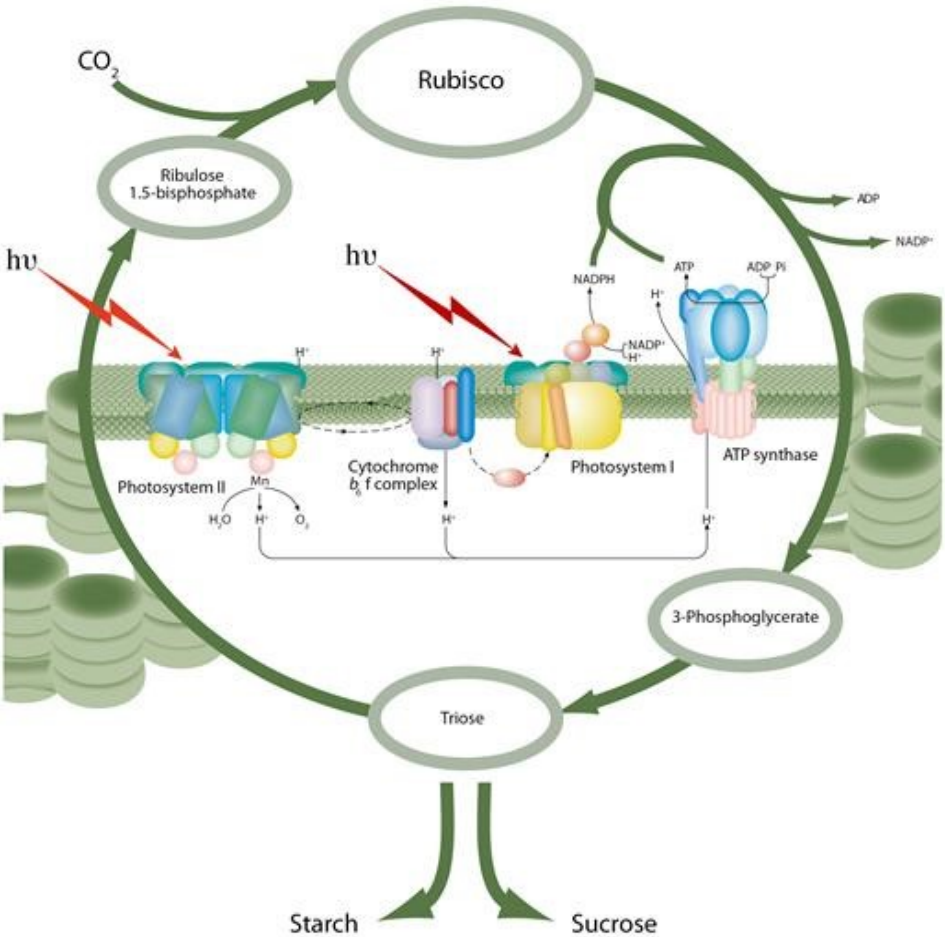
Advances in Photosynthesis and Respiration

Volume 34

Photosynthesis

Plastid Biology, Energy Conversion and Carbon Assimilation



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Springer

Photosynthesis

Plastid Biology, Energy Conversion and Carbon Assimilation

Advances in Photosynthesis and Respiration

VOLUME 34

The scope of our series reflects the concept that photosynthesis and respiration are intertwined with respect to both the protein complexes involved and to the entire bioenergetic machinery of all life. *Advances in Photosynthesis and Respiration* is a book series that provides a comprehensive and state-of-the-art account of research in photosynthesis and respiration. Photosynthesis is the process by which higher plants, algae, and certain species of bacteria transform and store solar energy in the form of energy-rich organic molecules. These compounds are in turn used as the energy source for all growth and reproduction in these and almost all other organisms. As such, virtually all life on the planet ultimately depends on photosynthetic energy conversion. Respiration, which occurs in mitochondrial and bacterial membranes, utilizes energy present in organic molecules to fuel a wide range of metabolic reactions critical for cell growth and development. In addition, many photosynthetic organisms engage in energetically wasteful photorespiration that begins in the chloroplast with an oxygenation reaction catalyzed by the same enzyme responsible for capturing carbon dioxide in photosynthesis. This series of books spans topics from physics to agronomy and medicine, from femtosecond processes to season-long production to evolutionary time scales, from the photophysics of reaction centers, through the electrochemistry of intermediate electron transfer, to the physiology of whole organisms, and from X-ray crystallography of proteins to the morphology of organelles and intact organisms. The goal of the series is to offer beginning researchers, advanced undergraduate students, graduate students, and even research specialists, a comprehensive, up-to-date picture of the remarkable advances across the full scope of research on photosynthesis, respiration and related processes.

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Photosynthesis

Plastid Biology, Energy Conversion and Carbon Assimilation

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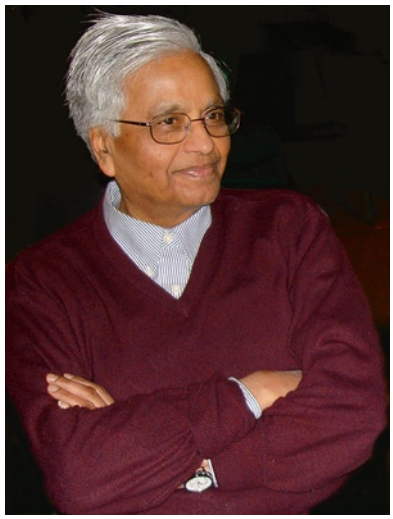
Dedication



Govindjee's passion for photosynthesis has been inspirational for students and researchers for over 50 years. At the start of his 80th year this volume of the Advances in Photosynthesis and Respiration series is dedicated to Govindjee to especially acknowledge not only his unique discoveries in Photosystem II, but also his commitment to educating students and researchers throughout the World (Photo taken in 2009 by Ram Singh).

Govindjee has provided unparalleled educational resources to the field of photosynthesis through his three *Scientific American* articles (1965; 1974; 1990; translated in Russian and in Japanese), and outstanding editorship of authoritative books on Photosynthesis (*Bioenergetics of Photosynthesis*, 1975; *Photosynthesis*, two volumes, 1982 (translated in Russian, 1987); *Light Emission by Plants and Bacteria*, 1986; *Chlorophyll a Fluorescence: A Signature of Photosynthesis*, 2004; reprinted in 2010; *Photosynthesis in Silico: from Molecules to Ecosystems*, 2009; and *Abiotic Stress in Plants*, 2010). Under his leadership, as the chief editor of *Photosynthesis Research*, he made this publication a World-class journal; it took a quantum jump (five-fold) in the number of pages published per year. He then founded the unique 'Historical Corner' of *Photosynthesis Research*, and by his shear persuasion attracted all the leaders in the field to write on their discoveries. This culminated in a unique book 'Discoveries in Photosynthesis'. Govindjee is also the founding Editor of the '*Advances in Photosynthesis and Respiration*' Series; under his leadership more than 30 highly-acclaimed authoritative volumes on all aspects of photosynthesis: from molecules to the whole plant have been published; they are available in all leading libraries and photosynthesis research laboratories of the World. In addition, Govindjee's 1969 book on '*Photosynthesis*' remains a classic as it was used in the past to teach photosynthesis to thousands of students around the World. Lastly, together with Larry Orr, Govindjee has produced the highly cited and heavily used web site '*Photosynthesis and the World Wide Web*' for education. We refer the readers to Chapter 31 (by George C. Papageorgiou), Chapter 32 (by Julian J. Eaton-Rye) and Chapter 33 (by Robert M. Clegg) on Govindjee's research during 1956–1969, 1970–1999, and 2000–2010, respectively; also see the Preface of this book and Govindjee's Biographical Sketch that follows. These achievements have been made possible by Govindjee's personal enthusiasm and dedicated service to photosynthesis research and education. His unparalleled commitment and knowledge have

made him a true advocate, and ambassador, of photosynthetic research and education around the World.

Biographical Sketch

Govindjee, born in 1932, obtained his BSc (Chemistry, Biology) and MSc (Botany) in 1952 and 1954, from the University of Allahabad, India. He was a graduate student of Robert Emerson and of Eugene Rabinowitch, receiving his Ph.D. (Biophysics), in 1960, from the University of Illinois at Urbana-Champaign (UIUC). He has focused mainly on "*Photosystem II*" (PS II, the Water:Plastoquinone Oxidoreductase) throughout his career; research on PS II has included discoveries on excitation energy transfer, light emission, primary photochemistry and electron transfer. His early research included the discovery of a short-wavelength form of chlorophyII (Chl) *a* functioning in the Chl *b*-containing system, now called PS II (in 1960, with Eugene Rabinowitch); and the two-light effect (Emerson Enhancement) in NADP-reduction in chloroplasts (1962–1964, with Rajni Govindjee and George Hoch). In collaboration with his ~25 graduate students and postdoctoral associates, he has worked on the origins of the different spectral fluorescing forms of Chl *a* and the temperature dependence of excitation energy transfer down to 4 K (1963–1970); established basic relationships between Chl *a* fluorescence and photosynthetic reactions (1968–1988); discovered a unique role of bicarbonate on the acceptor side of PS II, particularly in protonation events involving the Q_B binding region (1970–1998); formulated the theory of thermoluminescence in plants (1983, with Don C. De Vault); made the first picosecond measurement on the primary photochemistry of PS II (1989–1997, with Michael Seibert and Michael Wasielewski); and pioneered the use of the lifetime of Chl *a* fluorescence in understanding photoprotection against excess light (with Adam Gilmore). His current focus, however, is on the "*History of Photosynthesis Research*" and in "*Photosynthesis Education*" in addition to his dedicated research on the application of FLIM (Fluorescence Lifetime Imaging Microscopy) to photosynthetic systems to understand photoprotection in plants and algae (with Robert Clegg of UIUC) and on fluorescence spectroscopy of cyanobacterial cells during fluorescence induction (with Ondrej Prasil in The Czech Republic).

Govindjee has served the UIUC as an Assistant Professor, Associate Professor and Professor (1961–1999). Since 1999, he has been Professor Emeritus of Biochemistry, Biophysics and Plant Biology at the UIUC. His honors include: Fellow and Life Member of the National Academy of Sciences, India (1978); President of the American Society of Photobiology (1980–1981); Fulbright Senior Lecturer (1996–1997); Honorary President of the 13th International Photosynthesis Congress (Montréal, 2004); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology (2006); recipient of the ISPR (International Society of Photosynthesis Research) Communication Award (2007); and the LAS (Liberal Arts and Sciences) Lifetime Achievement Award of the University of Illinois at Urbana-Champaign (2008). He is also listed among the Eminent Indian Botanists, Past and Present (2010).

From the Co-Series Editor

Advances in Photosynthesis and Respiration

Volume 34: Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation

I am delighted to announce the publication, in the *Advances in Photosynthesis and Respiration* (AIPH) Series, of *Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation*. Julian Eaton-Rye and Baishnab Tripathy (coeditors of Vol. 34) conceived a comprehensive look at photosynthesis and I (TDS) joined them in the late stages to put together this volume. Julian and Baishnab had in mind a volume to be dedicated to Govindjee for his extraordinary contributions to both discoveries and education about photosynthesis. (See the Dedication page, his Biographical Sketch, the Preface of this book, and Chapters 31, 32 and 33 in this volume.) Volume 31 of the series marked a turning point, with Govindjee inviting a co-series editor beginning with that volume. Govindjee's invitation to me represents his strong desire to keep the series comprehensive and reflects his view that, while photosynthesis begins with the absorption of a photon, a full understanding of photosynthesis can take investigators on many journeys, some of which can lead to intricacies of carbon metabolism and sugar synthesis while others undertake studies of the role of photosynthesis in ecology and even its very significant role in global change. I can now tell the story of Govindjee's incredible efforts on behalf of this series from the inside. Editors of the previous 30 volumes will recount how Govindjee can be a task-master, making sure that books are produced in a timely manner and making sure the quality is upheld at every step of the process. Above all else, Govindjee cares about photosynthesis. The coeditors of Volume 34 are pleased to reflect on the history of Govindjee's contributions to photosynthesis.

Our Books: Thirty-Three Volumes

Below is listed information on all the past 33 volumes. We are pleased to note that Springer is now producing complete table of contents of these books and electronic copies of individual chapters of these books (http://www.springer.com/series/5599); their web sites include free downloadable front matter as well as indexes. All the available and anticipated web sites of books in this series *Advances in Photosynthesis and Respiration* are listed, within square brackets, at the end of each entry. This volume (34) will be the last with the familiar white cover. A green cover better suited to the increasing web presence will be used for volume 35, which will be published early 2012.

- **Volume 33 (2012): Functional Genomics and Evolution of Photosynthetic Systems**, edited by Robert L. Burnap and Willem F.J. Vermaas, from USA; Fifteen chapters, 428 pp, http://www.springer.com/life+sciences/book/978-94-007-1532-5 [http://www.springerlink.com/content/978-90-481-1532-5/]
- **Volume 32 (2011): C4 Photosynthesis and Related CO_2 Concentrating Mechanisms**, edited by Agepati S. Raghavendra and Rowan Sage, from India and Canada. Nineteen chapters, 425 pp, Hardcover, ISBN 978-90-481-9406-3 [http://www.springerlink.com/content/978-90-481-9406-3/]
- **Volume 31 (2010): The Chloroplast: Basics and Applications**, edited by Constantin Rebeiz (USA), Christoph Benning (USA), Hans J. Bohnert (USA), Henry Daniell (USA), J. Kenneth Hoober (USA), Hartmut K. Lichtenthaler (Germany), Archie R. Portis (USA), and Baishnab C. Tripathy (India). Twenty-five chapters, 451 pp, Hardcover, ISBN: 978-90-481-8530-6 [http://www.springerlink.com/content/978-90-481-8530-6/]
- **Volume 30 (2009): Lipids in Photosynthesis: Essential and Regulatory Functions**, edited by Hajime Wada and Norio Murata, both from Japan. Twenty chapters, 506 pp, Hardcover, ISBN:

978-90-481-2862-4; e-book, ISBN: 978-90-481-2863-1 [http://www.springerlink.com/content/978-90-481-2862-4/]

- **Volume 29 (2009): Photosynthesis in Silico: Understanding Complexity from Molecules**, edited by Agu Laisk, Ladislav Nedbal, and Govindjee, from Estonia, The Czech Republic, and USA. Twenty chapters, 525 pp, Hardcover, ISBN: 978-1-4020-9236-7 [http://www.springerlink.com/content/978-1-4020-9236-7/]
- **Volume 28 (2009): The Purple Phototrophic Bacteria**, edited by C. Neil Hunter, Fevzi Daldal, Marion C. Thurnauer and J. Thomas Beatty, from UK, USA and Canada. Forty-eight chapters, 1053 pp, Hardcover, ISBN: 978-1-4020-8814-8 [http://www.springerlink.com/content/978-1-4020-8814-8/]
- **Volume 27 (2008): Sulfur Metabolism in Phototrophic Organisms**, edited by Christiane Dahl, Rüdiger Hell, David Knaff and Thomas Leustek, from Germany and USA. Twenty-four chapters, 551 pp, Hardcover, ISBN: 978-4020-6862-1 [http://www.springerlink.com/content/978-1-4020-6862-1/]
- **Volume 26 (2008): Biophysical Techniques in Photosynthesis**, Volume II, edited by Thijs Aartsma and Jörg Matysik, both from The Netherlands. Twenty-four chapters, 548 pp, Hardcover, ISBN: 978-1-4020-8249-8 [http://www.springerlink.com/content/978-1-4020-8249-8/]
- **Volume 25 (2006): Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications**, edited by Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger, and Hugo Scheer, from Germany and Australia. Thirty-seven chapters, 603 pp, Hardcover, ISBN: 978-1-40204515-8 [http://www.springerlink.com/content/978-1-4020-4515-8/]
- **Volume 24 (2006): Photosystem I: The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase**, edited by John H. Golbeck, from USA. Forty chapters, 716 pp, Hardcover, ISBN: 978-1-40204255-3 [http://www.springerlink.com/content/978-1-4020-4255-3/]
- **Volume 23 (2006): The Structure and Function of Plastids**, edited by Robert R. Wise and J. Kenneth Hoober, from USA. Twenty-seven chapters, 575 pp, Softcover, ISBN: 978-1-4020- 6570–6; Hardcover, ISBN: 978-1-4020-4060-3 [http://www.springerlink.com/content/978-1-4020-4060-3/]
- **Volume 22 (2005): Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase**, edited by Thomas J. Wydrzynski and Kimiyuki Satoh, from Australia and Japan. Thirty-four chapters, 786 pp, Hardcover, ISBN: 978-1-4020-4249-2 [http://www.springerlink.com/content/978-1-4020-4249-2/]
- **Volume 21 (2005): Photoprotection, Photoinhibition, Gene Regulation, and Environment**, edited by Barbara Demmig-Adams, William W. Adams III and Autar K. Mattoo, from USA. Twenty-one chapters, 380 pp, Hardcover, ISBN: 978-14020-3564-7 [http://www.springerlink.com/content/978-1-4020-3564-7/]
- **Volume 20 (2006): Discoveries in Photosynthesis**, edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen, from USA, Canada and UK. One hundred and eleven chapters, 1304 pp, Hardcover, ISBN: 978-1-4020-3323-0 [http://www.springerlink.com/content/978-1-4020-3323-0/]
- **Volume 19 (2004): Chlorophyll *a* Fluorescence: A Signature of Photosynthesis**, edited by George C. Papageorgiou and Govindjee, from Greece and USA. Thirty-one chapters, 820 pp, Hardcover, ISBN: 978-1-4020-3217-2 [http://www.springerlink.com/content/978-1-4020-3217-2/]
- **Volume 18 (2005): Plant Respiration: From Cell to Ecosystem**, edited by Hans Lambers and Miquel Ribas-Carbo, from Australia and Spain. Thirteen chapters, 250 pp, Hardcover, ISBN: 978-14020-3588-3 [http://www.springerlink.com/content/978-1-4020-3588-3/]
- **Volume 17 (2004): Plant Mitochondria: From Genome to Function**, edited by David Day, A. Harvey Millar and James Whelan, from Australia. Fourteen chapters, 325 pp, Hardcover, ISBN: 978-1-4020-2399-6 [http://www.springerlink.com/content/978-1-7923-2399-6/]
- **Volume 16 (2004): Respiration in Archaea and Bacteria: Diversity of Prokaryotic Respiratory Systems**, edited by Davide Zannoni, from Italy. Thirteen chapters, 310 pp, Hardcover, ISBN: 978-14020-2002-5 [http://www.springerlink.com/content/978-1-4020-2002-5/]
- **Volume 15 (2004): Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers**, edited by Davide Zannoni, from Italy. Thirteen chapters, 350 pp, Hardcover, ISBN: 978-1-4020-2001-8 [http://www.springerlink.com/content/978-0-7923-2001-8/]
- **Volume 14 (2004): Photosynthesis in Algae**, edited by Anthony W. Larkum, Susan Douglas and John A. Raven, from Australia, Canada and UK. Nineteen chapters, 500 pp, Hardcover, ISBN:

978-0-7923-6333-0 [http://www.springerlink.com/content/978-0-7923-6333-0/]

- **Volume 13 (2003): Light-Harvesting Antennas in Photosynthesis**, edited by Beverley R. Green and William W. Parson, from Canada and USA. Seventeen chapters, 544 pp, Hardcover, ISBN: 978-07923-6335-4 [http://www.springerlink.com/content/978-0-7923-6335-4/]
- **Volume 12 (2003): Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism**, edited by Christine H. Foyer and Graham Noctor, from UK and France. Sixteen chapters, 304 pp, Hardcover, ISBN: 978-07923-6336-1 [http://www.springerlink.com/content/978-0-7923-6336-1/]
- **Volume 11 (2001): Regulation of Photosynthesis**, edited by Eva-Mari Aro and Bertil Andersson, from Finland and Sweden. Thirty-two chapters, 640 pp, Hardcover, ISBN: 978-0-7923-6332-3 [http://www.springerlink.com/content/978-0-7923-6332-3/]
- **Volume 10 (2001): Photosynthesis: Photobiochemistry and Photobiophysics**, authored by Bacon Ke, from USA. Thirty-six chapters, 792 pp, Softcover, ISBN: 978-0-7923-6791-8; Hardcover: ISBN: 978-0-7923-6334-7 [http://www.springerlink.com/content/978-0-7923-6334-7/]
- **Volume 9 (2000): Photosynthesis: Physiology and Metabolism**, edited by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer, from UK, USA and Australia. Twenty-four chapters, 644 pp, Hardcover, ISBN: 978-07923-6143-5 [http://www.springerlink.com/content/978-0-7923-6143-5/]
- **Volume 8 (1999): The Photochemistry of Carotenoids**, edited by Harry A. Frank, Andrew J. Young, George Britton and Richard J. Cogdell, from USA and UK. Twenty chapters, 420 pp, Hardcover, ISBN: 978-0-7923-5942-5 [http://www.springerlink.com/content/978-0-7923-5942-5/]
- **Volume 7 (1998): The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas***, edited by Jean David Rochaix, Michel Goldschmidt-Clermont and Sabeeha Merchant, from Switzerland and USA. Thirty-six chapters, 760 pp, Hardcover, ISBN: 978-0-7923-5174-0 [http://www.springerlink.com/content/978-0-7923-5174-0/]
- **Volume 6 (1998): Lipids in Photosynthesis: Structure, Function and Genetics**, edited by Paul-André Siegenthaler and Norio Murata, from Switzerland and Japan. Fifteen chapters, 332 pp, Hardcover, ISBN: 978-0-7923-5173-3 [http://www.springerlink.com/content/978-0-7923-5173-3/]
- **Volume 5 (1997): Photosynthesis and the Environment**, edited by Neil R. Baker, from UK. Twenty chapters, 508 pp, Hardcover, ISBN: 978-07923-4316-5 [http://www.springerlink.com/content/978-0-7923-4316-5/]
- **Volume 4 (1996): Oxygenic Photosynthesis: The Light Reactions**, edited by Donald R. Ort, and Charles F. Yocum, from USA. Thirty-four chapters, 696 pp, Softcover: ISBN: 978-0-7923- 3684–6; Hardcover, ISBN: 978-0-7923-3683-9 [http://www.springerlink.com/content/978-0-7923-3683-9/]
- **Volume 3 (1996): Biophysical Techniques in Photosynthesis**, edited by Jan Amesz and Arnold J. Hoff, from The Netherlands. Twenty-four chapters, 426 pp, Hardcover, ISBN: 978-0-7923-3642-6 [http://www.springerlink.com/content/978-0-7923-3642-6/]
- **Volume 2 (1995): Anoxygenic Photosynthetic Bacteria**, edited by Robert E. Blankenship, Michael T. Madigan and Carl E. Bauer, from USA. Sixty-two chapters, 1331 pp, Hardcover, ISBN: 978-0-7923-3682-8 [http://www.springerlink.com/content/978-0-7923-3681-5/]
- **Volume 1 (1994): The Molecular Biology of Cyanobacteria**, edited by Donald R. Bryant, from USA. Twenty-eight chapters, 916 pp, Hardcover, ISBN: 978-0-7923-3222-0 [http://www.springerlink.com/content/978-0-7923-3222-0/]

Further information on these books and ordering instructions can be found at http://www.springer.com/series/5599. Contents of volumes 1–29 can also be found at http://www.life.illinois.edu/govindjee/g/References.html.

Special 25% discounts are available to members of the International Society of Photosynthesis Research, ISPR http://www.photosynthesisresearch.org/: See http://www.springer.com/ispr

This Book: Volume 34

"*Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation*" was conceived as a comprehensive treatment touching on most of the processes important for photosynthesis. Most of the chapters provide a broad coverage that, it is hoped, will be accessible to advanced undergraduates, graduate students, and researchers looking to broaden their knowledge of photosynthesis. For biologists, biochemists, and biophysicists, this volume will provide quick background understanding for the breadth of issues in

photosynthesis that are important in research and instructional settings. This volume will be of interest to advanced undergraduates in plant biology, and plant biochemistry and to graduate students and instructors wanting a single reference volume on the latest understanding of the critical components of photosynthesis.

Solar energy is the source of almost all life on this Earth. An important factor in increasing the production of biomass, bioenergy and biofuels, which is needed to solve the global energy crisis, is the overall efficiency of photosynthesis. To manipulate overall efficiency we must understand the basic reactions of photosynthesis. This volume (*Photosynthesis: Plastic Biology, Energy Conversion and Carbon Assimilation*) in the *Advances in Photosynthesis and Respiration* series provides a comprehensive view of the current understanding of photosynthesis; it is divided into several sections to help the readers see the broad categories of photosynthetic processes. The first twenty-nine chapters in this volume cover the topics central to our current understanding of *Photosynthesis*.

A look back can be very informative and help make sense of the present. A historical perspective comes out in many of the chapters. The term "historical" can have different meaning to different people. A history of photosynthesis research (Chapter 30) caps the chapters on various aspects of photosynthesis in the volume; it traces the development of concepts in photosynthesis.

A strong theme in many of the chapters is a different type of history, the course of evolution that led to the current photosynthetic organisms that exist today. A strong evolutionary thread runs through various chapters; evolutionary insights aid in understanding why photosynthesis today has so many of its special properties. In this volume, readers will find discussions of the origins of photosynthesis as well as the future of photosynthesis, including the prospects for engineering artificial photosynthesis. Readers will learn about bacteria that use just parts of the photosynthetic system found in plants and how duplication and specialization in a small number of progenitor pigments, and proteins, gave rise to the vast array of pigments and proteins that are found in photosynthetic systems today. As Theodosius Dobzhansky wrote in 1973, "*Nothing in biology makes sense except in light of evolution*." Photosynthesis is a rich area for the study of evolution. This volume has a number of chapters that tell fascinating stories tracing the evolution of photosynthetic processes including the photosystems (Chapter 1), plastids (Chapter 2), carbon metabolism (Chapter 26), and sucrose metabolism (Chapter 27). Evolutionary perspectives can be found in many of the other chapters as well.

This book is appropriately dedicated to Govindjee, the founding series editor, on the occasion of a new board of advisors and a new co-editor (officially starting from Volume 31). Govindjee's contributions are enormous, and are included in the last three chapters (Chapters 31, 32 and 33) of this book. In my opinion, Govindjee is, perhaps, the best-known photosynthesis researcher alive today.

Authors

The current book contains 33 chapters written by 76 international authors from 20 different countries (Australia, Canada, China, Finland, France, Germany, Greece, Hungary, India, Israel, Italy, Japan, New Zealand, The Netherlands, Russia, Spain, Sweden, Switzerland, United Kingdom, and the United States of America). The series editors give a special thank you to each and every author for their valuable contribution to the successful production of this unique book:

Iwona **A**damska (Germany; Chapter 14); Elizabeth A. Ainsworth (USA; Chapter 29); Suleyman I. Allakhverdiev (Russia; Chapter 12); Yagut Allahverdiyeva (Finland; Chapter 13); Naohiro Aoki (Australia and Japan; Chapter 28); Eva-Mari Aro (Finland; Chapter 13); Neil R. **B**aker (UK; Chapter 23); Olivier Bastien (France; Chapter 9); Roberto Bassi (Italy; Chapter 5); Carl J. Bernacchi (USA; Chapter 29); Basanti Biswal (India; Chapter 10); Udaya C. Biswal (India; Chapter 10); Robert E. Blankenship (USA; Chapter 1); Maryse A. Block (France; Chapter 9); Robert **C**arpentier (Canada; Chapter 12); Wah Soon Chow (Australia; Chapter 24); Robert MacDonald Clegg (USA; Chapter 33); Gabriel Cornic (France; Chapter 23); Roberta Croce (The Netherlands; Chapter 6); Antoine **D**anon (France; Chapter 15); Ildikó Domonkos (Hungary; Chapter 11); Julian J. **E**aton-Rye (New Zealand; Chapters 20, 32); Johannes Engelken (Germany and Spain; Chapter 14); Denis **F**alconet (France; Chapter 2); Wayne D. Frasch (USA; Chapter 22); Hideya

Fukuzawa (Japan; Chapter 25); Robert T. Furbank (Australia; Chapter 28); Zoltán **G**ombos (Chapter 11; Hungary); Mounia **H**eddad (Switzerland; Chapter 14); Jane F. Hill (USA; Chapter 30); Tatsuro Hirose (Japan; Chapter 28); Martin F. Hohmann-Marriott (New Zealand; Chapter 1); Kenneth J. Hoober (USA; Chapter 16); Tassilo Hornung (USA; Chapter 22); Norman P. A. Hüner (Canada; Chapter 19); Robert **I**shmukhametov (USA; Chapter 22); Alexandra G. Ivanov (Canada; Chapter 19); Lixia **J**in-Day (USA; Chapter 22); Juliette Jouhet (France; Chapter 9); Jacques Joyard (France; Chapter 9); Toivo **K**allas (USA; Chapter 21); Aaron Kaplan (Israel; Chapter 25); Vyacheslav V. Kilmov (Russia; Chapter 12); Vladimir D. Kreslavski (Russia; Chapter 12); Andrew D.B. **L**eakey (USA; Chapter 29); Hartmut K. Lichtenthaler (Chapter 4; Germany); Steve P. Long (USA; Chapter 29); Dmitry A. Los (Russia; Chapter 12); John E. Lunn (Germany; Chapter 27); Elspeth **M**acRae (New Zealand; Chapter 27); Eric Marechal (France; Chapter 9); James Martin (USA; Chapter 22); Mamoru Mimuro (Japan; Chapter 12); Iain L. McConnell (USA; Chapter 20); Prasanna Mohanty (Russia and India; Chapter 12); Pranab K. Mohapatra (India; Chapter 10); Tomas Morosinotto (Italy; Chapter 5); Teruo **O**gawa (China; Chapter 25); Donald R. Ort (USA; Chapter 29); Ulrike Oster (Germany; Chapter 8); Gunnar Öquist (Sweden; Chapter 19); George C. **P**apageorgiou (Greece; Chapters 18, 31); Gopal K. Pattanayak (USA; Chapter 3); Mukesh K. **R**avel (India; Chapter 10); Gernot Renger (Germany; Chapter 17); Wolfhart Rüdiger (Germany; Chapter 8); Prafullachandra Vishnu **S**ane (India; Chapter 19); Thomas D. Sharkey (USA; Chapter 26); David Spetzler (USA; Chapter 22); Balázs Szalontai (Hungary; Chapter 11); Baishnab C. **T**ripathy (India; Chapter 3); Jack J. S. **v**an Rensen (The Netherlands; Chapter 20); Klaas J. van Wijk (USA; Chapter 7); Sean E. **W**eise (USA; Chapter 26); Justin **Y**ork (USA; Chapter 22); Xinguang **Z**hu (China; Chapter 29).

Future Advances in Photosynthesis and Respiration and Other Related Books

The readers of the current series are encouraged to watch for the publication of the following books (not necessarily arranged in the order of future appearance):

- The Bioenergetic Processes of Cyanobacteria: From Evolutionary Singularity to Ecological Diversity (Editors: Günter A. Peschek, Christian Obinger, and Gernot Renger) http://www.springer.com/life+sciences/book/978-94-007-0352-0
- Chloroplast Biogenesis: During Leaf Development and Senescence (Editors: Basanti Biswal, Karin Krupinska and Udaya Chand Biswal)
- The Structural Basis of Biological Energy Generation (Editor: Martin Hohmann-Marriott)
- Genomics of Chloroplasts and Mitochondria (Editors: Ralph Bock and Volker Knoop)
- Photosynthesis in Bryophytes and Early Land Plants (Editors: David T. Hanson and Steven K. Rice)

In addition to the above contracted books, the following topics are under consideration:

- Algae, Cyanobacteria, Biofuel and Bioenergy
- Artificial Photosynthesis
- ATP Synthase and Proton Translocation
- Bacterial Respiration II
- Biohydrogen Production
- Carotenoids II
- Cyanobacteria II
- The Cytochromes
- Ecophysiology
- Evolution of Photosynthesis
- Excitation Energy Transfer in Photosynthesis
- The FACE Experiments
- Global Aspects of Photosynthesis
- Green Bacteria and Heliobacteria
- Interactions between Photosynthesis and other Metabolic Processes
- Limits of Photosynthesis: Where do we go from here
- Photosynthesis, Biomass and Bioenergy
- Photosynthesis under Abiotic and Biotic Stress
- Plant Canopies and Photosynthesis
- Plant Respiration II

If you have any interest in editing/co-editing any of the above listed books, or being an author, please send an E-mail to Govindjee at gov@illinois.edu, and/or to me (tsharkey@msu.edu). Suggestions for additional topics are also welcome

In view of the interdisciplinary character of research in photosynthesis and respiration, it is our earnest hope that this series of books will be used in educating students and researchers not only in Plant Sciences, Molecular and Cell Biology, Integrative Biology, Biotechnology, Agricultural Sciences, Microbiology, Biochemistry, Chemical Biology, Biological Physics, and Biophysics, but

also in Agricultural Engineering, Bioengineering, Chemistry, and Physics.

We take this opportunity to thank and congratulate Julian Eaton-Rye and Baishnap Tripathy for their outstanding editorial work; they have done a fantastic job not only in editing, but also in organizing this book for Springer, and for their highly professional dealing with the typesetting process. We have thanked all the 76 authors of this book (see the list above): without their authoritative chapters, there would be no such volume. We give special thanks to A. Lakshmi Praba, Project Manager, SPi Content Solutions – SPi Global, India for directing the typesetting of this book; her expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Ineke Ravesloot and André Tournois (of Springer) thanks for their friendly working relation with us that led to the production of this book. Thanks are also due to Govindjee, the Series Founding Editor. Some of the text above was originally written by him and adapted by me. Govindjee has been involved and keenly interested at every stage to bring this book to fruition.

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Part VIII: The Career of Govindjee

Preface

During 1945–1956, Govindjee's Ph.D. mentor, Eugene Rabinowitch, wrote a comprehensive three-volume treatise on photosynthesis; however, today the amount of knowledge makes such a project out of the reach of a single author. Perhaps Govindjee recognized this when he produced his own excellent multi-author two-volume *Photosynthesis* in 1982. However, the Advances in Photosynthesis and Respiration series, under Govindjee's editorial leadership, has provided an ongoing authoritative presentation of photosynthesis and related research over the past 17 years. This book, volume 34 in the series, differs from many of its predecessors in that its scope includes topics in many branches of photosynthesis. This is no accident as the book is dedicated to Govindjee and reflects his far reaching interests and many of the chapters provide excellent introductions to different branches of the subject that are suitable for senior students or for researchers wishing to introduce themselves to aspects other than their own research specialty. The selection of topics also reflects the research interests of Govindjee. These include chapters on photosynthetic water splitting by photosystem II, fluorescence emission by the photosynthetic apparatus, thermoluminescence and the regulation of photosystem II electron transport by bicarbonate.

The material in this book is divided into *eight parts*. The *first part* is a single chapter that introduces the photosynthetic world in all its diversity. The chapter provides a unique perspective on the many ways nature has harvested light to drive photosynthesis and the variety of photosynthetic reaction centers served by these antenna both in prokaryotic and eukaryotic photosynthetic organisms. The *second part* covers nine topics in plastid biochemistry and physiology. Following from the material covered in the first introductory chapter, section two opens with a chapter on the origin, evolution and division of plastids: this is followed by four chapters that introduce chlorophyll and carotenoid biosynthesis, the assembly of pigments into light-harvesting pigment-protein complexes and the diversity of the chlorophyll-binding proteins of plants and cyanobacteria. Section two continues with chapters on the application of proteomics to photosynthesis research, the intricacies of cell signaling between the plastid and the nucleus and the role of the plastid envelope membrane in glycerolipid biosynthesis. Section two concludes with a chapter on leaf senescence and the interconversion of chloroplasts into gerontoplasts.

In *Part III* the subject matter turns to the response of the photosynthetic apparatus to different environmental stress factors. Individual chapters cover the susceptibility and adaptations to low-temperature stress, high temperatures, too much light and environmentally-induced oxidative stress. In *Part IV* the emphasis is on energy conversion and it is in these nine chapters that the topics most closely aligned with the research career of Govindjee are covered. The first of these chapters focuses on light absorption by chlorophyll and the unique biological functions of the accessory chlorophylls *b* and *c*. The next chapter focuses on the apparatus and mechanism of Photosystem II and presents a current understanding of water splitting in oxygenic photosynthesis. Govindjee's many seminal contributions to our understanding of Photosystem II began with establishing basic relationships between chlorophyll *a* fluorescence and photosynthetic reactions: these relationships are explored in the subsequent chapter that addresses fluorescence emission by the photosynthetic apparatus. Together with Don DeVault, Govindjee also formulated the theory of thermoluminescence and this is the next topic in this section of the book. Govindjee also discovered the role of bicarbonate in Photosystem II where it acts as a ligand to the non-heme iron of the photosystem; this subject has a long history going back to early experiments by Otto Warburg and this story is told in a chapter exploring the regulation of Photosystem II electron transport by bicarbonate. The remaining chapters of this section review the pivotal role of the cytochrome b_6f complex in photosynthetic electron transport, the mechanism of energy transduction by the F_1F_0 ATP synthase, electron transport in leaves and finally the currently available technologies for developing artificial photosynthetic systems for the planet's future energy needs.

The *fifth part* of this book turns to the topics of carbon assimilation as well as sucrose synthesis and transport. The uptake of CO_2 by cyanobacteria and microalgae is covered and the diversity of carbon fixing mechanisms together with the operation and control of the Calvin-Benson-Bassham cycle are discussed in a chapter devoted to autotrophic carbon fixation. This section concludes with a chapter providing a unique evolutionary perspective on photosynthetic sucrose biosynthesis and a chapter detailing sucrose transport in higher plants. The *sixth and the seventh parts* each contain a single chapter. The first looks at photosynthesis in the context of climate change and considers photosynthesis in a CO_2 rich atmosphere. The second provides a detailed history of the early pioneers of photosynthesis research. The book concludes with the last section (*Part VIII*) detailing Govindjee's extensive research, teaching and service contributions to the photosynthesis community.

We thank all the contributors to this volume. We have endeavored to edit these chapters to the best of our abilities and we apologize where we have missed something. We hope our readers enjoy learning more about photosynthesis from reading the contributions included here. We thank Govindjee for his inspiration to so many in the field of photosynthesis to do good science, to remember those whose contributions our own work has built on and to take up his example of excellence in teaching to our undergraduate and postgraduate students.

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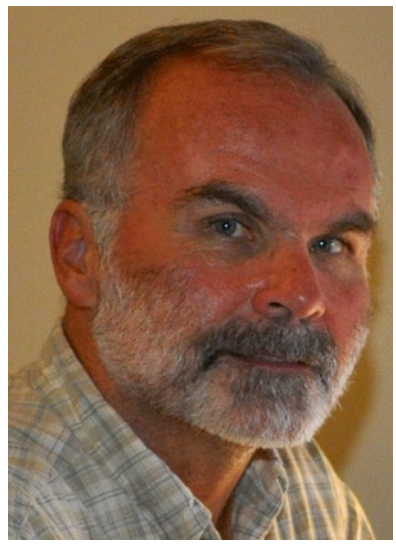
The Editors

Julian J. Eaton-Rye is currently in the Department of Biochemistry at the University of Otago in New Zealand. He completed a BSc Honors degree in Botany from the University of Manchester, UK in 1981 and obtained his Ph.D. (Plant Physiology) in 1987 at the University of Illinois at Urbana-Champaign under the supervision of Govindjee. His Ph.D. thesis research addressed the role of the bicarbonate cofactor in Photosystem II and showed that the reduction of the secondary plastoquinone electron acceptor and the turnover of the two-electron gate were impaired in bicarbonate-depleted membranes consistent with bicarbonate acting as a ligand to the non-heme iron of Photosystem II and participating in the protonation reactions associated with plastoquinone reduction on the acceptor side of the photosystem. He then obtained a Japan Society for the Promotion of Science postdoctoral fellowship (through the Royal Society, UK) and worked with Professor Norio Murata at the National Institute for Basic Biology in Okazaki, Japan (1987–1989). In Professor Murata's laboratory he studied the binding of the manganese-stabilizing protein (MSP or more usually nowadays, PsbO) to isolated Photosystem II complexes and demonstrated the requirement of an N-terminal domain of MSP for the binding of this protein to Photosystem II. In 1989 he moved to the Center for Early Event in Photosynthesis at Arizona State University to take up a postdoctoral position in the laboratory of Professor Wim Vermaas. In Professor Vermaas' laboratory he began working with the cyanobacterial model organism *Synechocystis* sp. PCC 6803, and produced the first site-directed mutations in the chlorophyll *a*-binding core antenna protein CP47 targeting putative chlorophyll ligands and the large hydrophilic loop of this protein that interacts with MSP. In 1993, he moved to Brookhaven National Laboratory as a Senior Research Fellow in the laboratory of Geoffrey Hind and demonstrated, using isoelectric focusing and kinase activity assays, that the then candidate Light-Harvesting Complex II protein kinase was in fact a *substrate* of a then as yet unidentified thylakoid-membrane-associated kinase. Since his appointment to a lectureship in the Department of Biochemistry at the University of Otago in 1994 his research group has studied the role of either the lumenal "extrinsic proteins" of Photosystem II or low-molecular-weight membrane-spanning "auxiliary" Photosystem II subunits found at the monomer-monomer interface of the Photosystem II supercomplex. His laboratory

is also actively studying the role of three additional Photosystem II lipoproteins (Psb27, CyanoP and CyanoQ) that are present in isolated Photosystem II complexes from *Synechocystis* sp. PCC 6803 but are absent from the current atomic resolution structures from the thermophilic cyanobacteria *Thermosynechococcus elongatus* and *T. vulcanus*. This work has produced the first X-ray-derived crystal structure of CyanoQ and NMR solution structures of Psb27 and CyanoP. With respect to other professional activities Julian currently serves on the Executive Committee of the International Society of Photosynthesis and the International Scientific Committee of the Triennial International Symposium on Phototrophic Prokaryotes. He is also a consulting editor for the Advances in Photosynthesis and Respiration book series and an Associate Editor of the New Zealand Journal of Botany. He served as the President of the New Zealand Society for Plant Biologists (2006–2008) and will serve as the President of the New Zealand Institute of Chemistry in 2012.

Baishnab C. Tripathy was born in Kutilo, Cuttack, located on the east eastern coast of India. After 11 years of primary, middle and high school education in a rural setting, he joined Ravenshaw College, Cuttack in 1967, and subsequently Buxi Jagabandhu Bidyadhar (BJB) College, Bhubaneswar in 1969, where he received a BSc (Major: Botany) in 1971. Then he obtained an MSc (Botany) in 1973 form Utkal University, Orissa, India. In 1981, he was awarded a Ph.D. degree, form Jawaharlal Nehru University (JNU), New Delhi, India, for his work on the 'Primary production and role of metal ions on the primary processes of photosynthesis' under the supervision of Professor Prasanna Mohanty, who had obtained his Ph.D. under Govindjee in 1972. During 1981–1983, he was a post-doctoral research associate, with Professors Elizabeth Gross and John S. Rieske, at the Ohio State University, Columbus, Ohio, USA, where he worked on '*The role of cations on chlorophyll a fluorescence and on electron transport from plastocyanin to cytochrome c through complex III of mitochondria*'. From December 1983 till March 1987, he worked with Professor Constantin A Rebeiz, at the University of Illinois at Urbana-Champaign, Illinois, USA on '*Chloroplast biogenesis and biochemistry of chlorophylls*'. In 1987, he joined as an Assistant Professor of Photobiology in the School of Life Sciences, JNU, where he was promoted to an Associate Professor and finally as a Professor in 2002. At JNU, Baishnab teaches and does research in plant physiology and photobiology. His research work has significantly contributed to our knowledge of plant tetrapyrroles and chloroplast biogenesis. His work on plant senescence in relation to chlorophyll metabolism has demonstrated the presence of different sets of enzymes i.e., kinetin-sensitive and kinetin-insensitive that are responsible for the degradation of chlorophyll and protochlorophyllide, respectively. His demonstration of the role of the meristematic layer in the intra- and inter-cellular signaling system in relation to chloroplast development of wheat and rice plants grown under red or red plus blue light, are novel and interesting. At the National Aeronautics and Space Administration (NASA), USA, he has published research papers on photosynthetic responses of plants growing in space. In addition to research articles, he has contributed several chapters to books and has edited books on photosynthesis. He has mentored a large number of Ph.D., MPhil and MSc students. Baishnab is on the editorial board of "*Horticulture, Environment and Biotechnology*". He is a member of the Board of Directors of the Rebeiz Foundation for Basic Research, is a fellow of the National Academy of Sciences, India, of the Indian National Science Academy and of the National Academy of Agricultural Sciences, India.



Thomas D. (Tom) Sharkey obtained his Bachelor's degree in Biology in 1974 from Lyman Briggs College, a residential science college at Michigan State University, East Lansing, Michigan. After 2 years as a research technician, Tom entered a Ph.D. program in the federally funded Plant Research Laboratory at Michigan State University under the mentorship of Professor Klaus Raschke and graduated in 1980 after 3 years and 3 months. Post-doctoral research was carried out with Professor Graham Farquhar at the Australian National University, in Canberra, where he co-authored a landmark review on photosynthesis and stomatal conductance that continues to receive much attention even today. For 5 years, he worked at the Desert Research institute, followed by 20 years as a professor of botany at the University of Wisconsin in Madison. In 2008, Tom became Professor and Chair of the Department of Biochemistry and Molecular Biology at Michigan State University. Tom's research interests center on the exchange of gases between plants and the atmosphere. The biochemistry and biophysics underlying carbon dioxide uptake and isoprene emission from plants form the two major research topics in his laboratory. Among his contributions are measurement of the carbon dioxide concentration inside leaves, and exhaustive study of short-term feedback effects on carbon metabolism, as well as a significant contribution to the elucidation of the pathway by which leaf starch breaks down at night. In the isoprene research field, Tom is recognized as the leading advocate for thermotolerance of photosynthesis as the explanation for why plants emit isoprene. In addition, his laboratory has cloned many of the genes that underlie isoprene synthesis, and he has published many papers on the biochemical regulation of isoprene synthesis. Tom has co-edited two books: T.D. Sharkey, E.A. Holland and H.A. Mooney (eds.) *Trace Gas Emissions from Plants*, Academic, San Diego, CA, 1991; and R.C. Leegood, T.D. Sharkey, and S, von Caemmerer (eds.) Physiology and Metabolism, Advances in Photosynthesis (and Respiration), Volume 9 of this Series, Kluwer (now Springer), Dordrecht, 2000. Tom is a "highly cited researcher" according to the Thomson Reuters Institute for Scientific Information.

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Part I

Introduction

Chapter 1

The Photosynthetic World

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DOI 10.1007/978-94-007-1579-0_1,

Summary

Photosynthesis, the conversion of sunlight into energy that is available to sustain cellular metabolism, is accomplished by a diverse group of organisms. The present photosynthetic diversity has been shaped over billions of years through the interactions of the genetic makeup and metabolic capabilities of each organism with its environment. Some photosynthetic bacteria found today can live in anaerobic conditions as must have been the case with the first photosynthetic organisms found on the primordial Earth. The oxygen of our present atmosphere was generated by ancient cyanobacteria. Cyanobacteria were incorporated into non-photosynthetic organisms in a process called endosymbiosis, giving rise to all the photosynthetic eukaryotes. Competition for light led to the development of a multitude of pigments that together span the entire solar spectrum. These pigments are arranged in special light-harvesting antenna systems that have evolved to efficiently channel excited-state energy to the reaction centers. In reaction centers, electrons are stripped away from donor molecules. This charge separation event, and successive electron transfer reactions, are catalyzed by multisubunit membrane-embedded protein complexes that are connected together via mobile electron carriers. The electron transfer reactions are ultimately used to convert inorganic carbon into organic carbon compounds. Most ecosystems rely on consuming photosynthesis-derived organic molecules and photosynthesis-derived oxygen to sustain life.

I. Introduction

Few places exist on Earth where there is an energy source to sustain life. In the oceans at depths where there is no sunlight some organisms are able to undergo autotrophic growth utilizing chemical gradients (Chivian et al., 2008) and blackbody radiation (Beatty et al., 2005). However, the cornucopia of life we encounter today exists because life mastered a way to harness the ubiquitous, albeit extraterrestrial, source of energy provided by the sun. Organisms have evolved complex and diverse machineries that utilize solar energy to accomplish the remarkable feat of photosynthesis (Björn and Govindjee, 2009; Hohmann-Marriott and Blankenship, 2011). While this chapter attempts to convey the diversity of photosynthetic organisms, more comprehensive introductions to photosynthesis are also available (Lawlor, 2000; Blankenship, 2002; Falkowski and Raven, 2007).

II. Photosynthesis

Photosynthesis is one of the most significant achievements of life on Earth. The word photosynthesis (Greek: phos "light" and syntithenai "put together") describes the complex process that converts light energy into stable chemical energy that is the fuel of most ecosystems (see Gest (2002) for history and definition of the term "photosynthesis").

Photosynthesis is an extremely ancient invention. We do not know when the first photosynthetic machinery evolved or what it looked like. We know, however, that the first photosynthetic organisms arose billions of years ago in an environment quite different from most places on Earth today. Photosynthesis originated in an anaerobic atmosphere that lasted for up to two billion years. Photosynthetic organisms were responsible for the transformation of this environment into the aerobic atmosphere we now take for granted. This evolutionary benchmark was the advent of oxygen-producing photosynthesis, which might have occurred as long ago as 2.4 billion years (Bekker et al., 2004). The accumulation of oxygen in the

Abbreviations: BChl – Bacteriochlorophyll; C3 – The pathway where CO_2 is fixed to ribulose 1,5 bisphosphate to produce a three-carbon sugar; C4 – The pathway where CO_2 is fixed to phosphoenolpyruvate to produce oxaloacetate; CAM – Crassulacean acid metabolism; Chl – Chlorophyll; FAP – Filamentous anoxygenic phototrophs; FeS – Iron-sulfur; FMO – Fenna-Matthews-Olson; LHCI – Light-harvesting complex I of oxygenic phototrophs; LHCII – Light-harvesting complex II of oxygenic phototrophs; LHI – Light-harvesting complex I of purple bacteria and filamentous anoxygenic phototrophs; LHII – Light-harvesting complex II of purple bacteria and filamentous anoxygenic phototrophs; PBP – Phycobilisome proteins of cyanobacteria and red algae; Pcb – *Prochlorococcus* chlorophyll-binding protein; PS I – Photosytem I; PS II – Photosystem II; Q – Quinone

atmosphere created a new frontier, a dangerous place, where oxygen and reactive molecular species met with destructive consequences. The photosynthetic organisms encountered today are the descendants of those that survived in the old atmosphere and those that ventured into the new (Fig. 1.1). Only the successes of trial and error live today to tell us about the evolutionary processes that shaped them. These surviving groups of photosynthetic organisms are able to flourish in diverse environments where some require anaerobic conditions (green sulfur bacteria, heliobacteria) and others are tolerant of aerobic conditions (filamentous anoxygenic phototrophs, purple bacteria), and the majority excels in aerobic conditions (cyanobacteria and eukaryotic phototrophs).

A. Living Together

1. Symbiosis

Being able to harvest sunlight is a sought-after ability, and many non-photosynthetic organisms have found that living with a photosynthetic partner is advantageous. These partnerships can be transient as found in symbiotic relationships or permanent as found in endosymbiosis (see below). Judged by its long evolutionary success, the symbiotic relationship that is recognized as a lichen is an extremely successful partnership. The two partners are a fungus (mostly belonging to the Ascomycetes, and rarely the Basidomycetes and Zygomycetes) as the mycobiont, and a photobiont. The photosynthetic partner can be a unicellular cyanobacterium (e.g., strains belonging to the genera *Chroococcus* and *Gloeocapsa*), or members of genera giving rise to heterocyst-forming cyanobacteria (e.g., strains belonging to the genera *Nostoc*, *Stigonema*, *Scytonema*, *Calothrix*) (Rai et al., 2000; Rasmussen and Nilsson, 2002).

Green alga of the genus *Trebouxia* (Trebouxiophyceae) are the most common eukaryotic photobiont, but lichenized algae also belong to the genera *Trentepohlia* (Ulvophyceae), *Stichococcus* (Trebouxiophyceae) and *Heterococcus* (Xanthophyceae) (Rambold et al., 1998). Green algae are often not the only photobiont and a nitrogen-fixing cyanobacterium is also present resulting in tripartite lichens. Over time, mycobiont and photobiont have become dependent on each other. While most photosynthetic symbionts can grow independently, the fungal partner is usually dependent on finding a suitable photosynthetic symbiont.

The ability to fix nitrogen make *Nostoc* species widely distributed symbionts. Ferns of the genus *Azolla* incorporate some *Nostoc* species inside special cavities. An important agricultural grain, rice, profits from this relationship since *Azolla* grows within rice fields (Sinha and Häder, 1996) and so provides a nitrogen source. Some symbiotic *Nostoc* species live in the gamete cavity of some Bryophytes, the roots of all cycads (Gymnospermata), and the stem glands of Gunnera (Angiospermata) (Usher et al., 2007).

Symbiotic photosynthetic organisms also contribute to the formation of coral reefs as in the polyps of cnidarians (Golden-Brown algae of the genus *Symbiodinium*) (Baird et al., 2008) and in the cavities of ascidians (cyanobacteria of the genus *Acaryochloris* and *Prochloron*) (Lewin and Cheng, 1975; Kott et al., 1984; Miyashita et al., 1996).

2. Endosymbiosis

Endosymbiosis, the permanent symbiotic relationship of two organisms by the incorporation of one into another, was first suggested by Schimper (1883) and Meyer (1883) and Mereschkowsky (1905, annotated translation by Martin and Kowallik, 1999), but it took the arrival of molecular biology to build this suggestion into an accepted theory (Margulis, 1970).

a. Primary Endosymbiosis

Several lines of evidence suggest that a single endosymbiotic event gave rise to the plastids of all eukaryotic phototrophs (*Glaucophyta*, *Rhodophyta*, *Chlorophyta*) (McFadden and Van Dooren, 2004) (Table 1.1), although independent endosymbiotic origins of chloroplasts cannot be excluded (Larkum et al., 2007). In either case, there is now overwhelming evidence that the three eukaryotic groups arose through the incorporation of a cyanobacterium-like organism into a eukaryotic organism. By some estimates this event occurred ~1,600 million years ago (Yoon et al., 2004). Part of the cyanobacterial legacy is the presence of a plastid genome, the retention within the organelle of transcription and translation

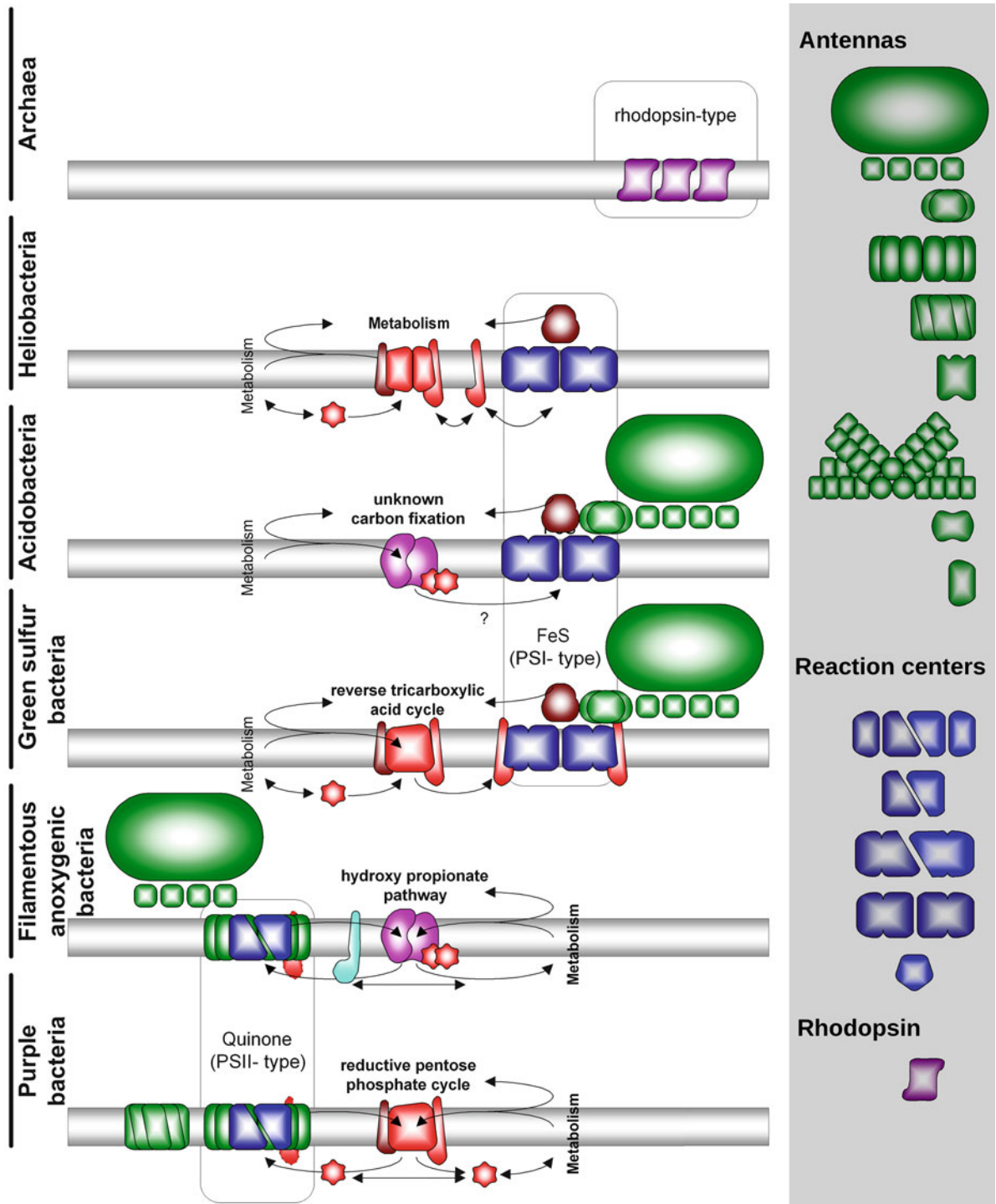

Fig. 1.1. Energy conversion of photosynthetic organisms. For description of complexes see the key included in this Figure.

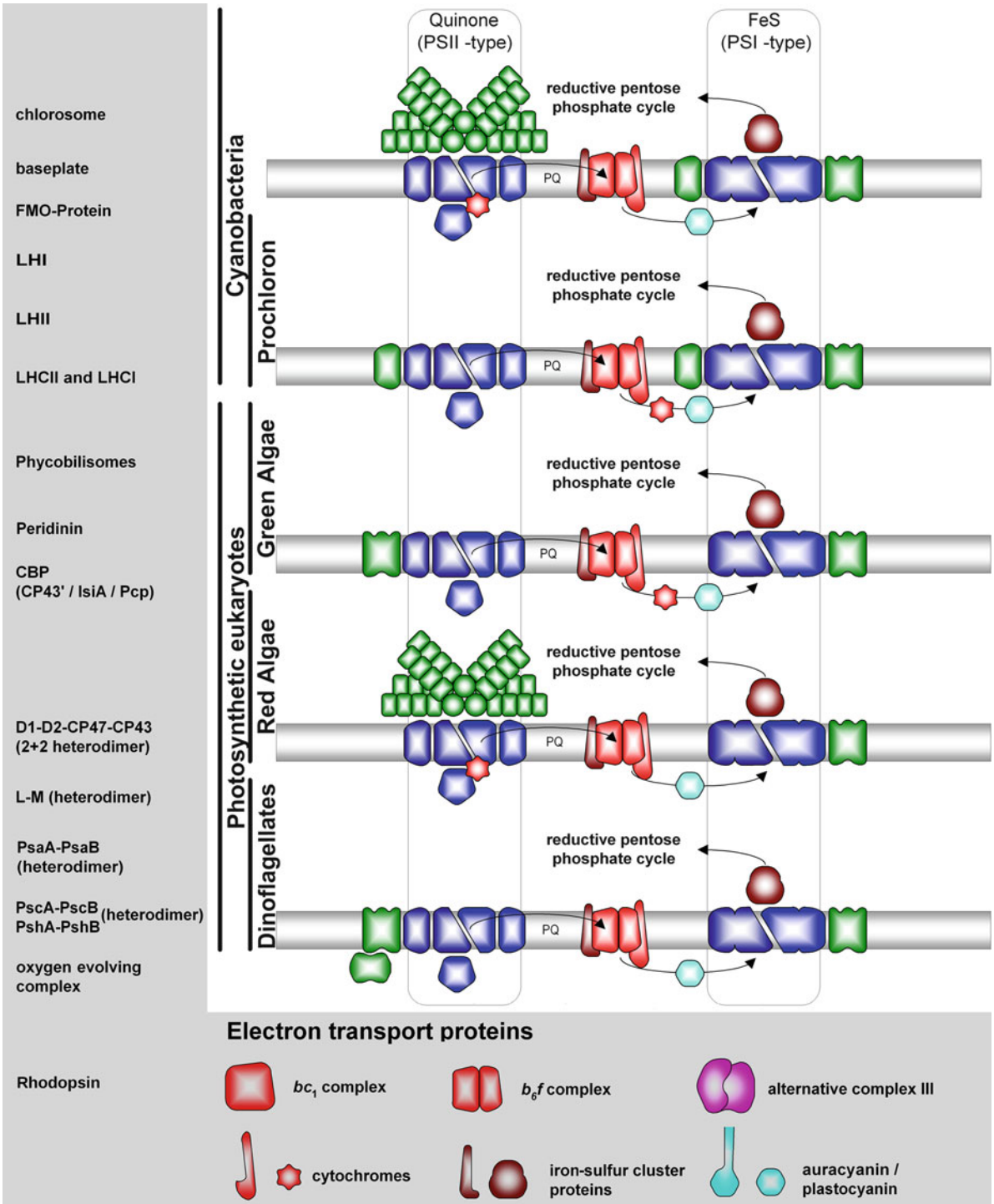

Fig. 1.1. (continued)

Table 1.1. Characteristics of primary and secondary eukaryotic endosymbionts

		Chl			Pb	Mb	Nm
Primary	Secondary	*a*	*b*	*c*	PBP[a]	Mem[b]	Nm[c]
Chlorophyta		X	X			2	
	Chloroarachniophyta	X	X			4	X
	Euglenophytes	X	X			3	
Rhodophyta		X			X	2	
	Cryptophytes	X		X	X	4	X
	Stramenophytes	X		X		4	
	Haptophytes	X		X		4	
	Dinoflagellates	X		X		3	
	Apicomplexan					4	
Glaucophyta		X			X	2	

[a]PBP: phycobiliproteins
[b]Mem: number of membranes surrounding the chloroplast
[c]Nm: presence of nucleomorph

machinery, and, in primary endosymbiosis, the presence of two membranes that separate the chloroplast stroma from the cytoplasm of the eukaryotic cell.

Symbiotic partners complement each other's metabolic needs. The exchange of metabolites has been established through the development of export and uptake machinery. Communication between the symbionts can be accomplished by chemicals or by exchange of genetic information. All plastids have transferred most of the genetic information to the nucleus, which gives the nucleus control over the plastid photosynthetic machinery and metabolism (Allen, 2003). In *Arabidopsis thaliana*, 18% of nuclear genes are potentially derived from an ancestral plastid genome (Martin et al., 2002). The transferred genes are transcribed in the nucleus and translated in the cytoplasm of the cell and then transported into the plastid. Several different import pathways can be distinguished (Inaba and Schnell, 2008). These pathways involve protein target sequences with distinct structural and chemical characteristics.

b. Secondary Endosymbiosis

Electron microscopy studies have revealed that some photosynthetic organisms possess chloroplasts that are surrounded by more than two membranes (Table 1.1). This finding can be explained by a secondary endosymbiotic event, in which an organism incorporates a second organism that was derived through a primary endosymbiotic event. Such secondary endosymbiosis occurred by incorporating a green alga-like organism, resulting in Euglenophytes and Chloroarachniophytes, while incorporation of red alga-like organism resulted in Cryptophytes, Stramenopiles, and Haptophytes. Phyogenetic analysis of the plastid genomes supports this model (Bhattacharya and Medlin, 1995); however, even before the genetic information became available the distribution of pigments also indicated the aforementioned relationships. Additional evidence for secondary endosymbiotic events comes from the presence of genetic material found within the four membranes that enclose the plastid in Chloroarachnophytes and Cryptophytes. These organisms have a rudimentary nucleus called a nucleomorph (Archibald, 2007) that is positioned between two membranes on each side. Moreover, it has been proposed that dinoflagellates might be the result of a tertiary or maybe even quaternary symbiotic event (Bhattacharya et al., 2004). The identification of a large number of nuclear genes that appear to be derived from an ancestral green alga in diatoms (Moustafa et al., 2009) may point to a more complex sequence of endosymbiotic events in Stramenopiles and other groups with multiple endosymbiotic events.

c. Transient Endosymbiosis

A "transient endosymbiotic" relationship was discovered in *Elysia chlorotica* (West, 1979; Pierce et al., 1996). This sea slug ingests green algae, extracts the chloroplasts away from the rest of the algal cell, and manages to preserve a structurally intact chloroplast that is incorporated into

the cytoplasm of the epithelial gut cells. These chloroplasts can stay functional in photosynthesis including oxygen evolution for at least 9 months and apparently sustain an otherwise food-deprived snail (Green et al., 2000; Rumpho et al., 2000; Rumpho et al., 2006).

3. Photosynthesis Lost

Surprisingly, some human diseases can be treated with chemicals that are known to interfere with chloroplast function (Jomaa et al., 1999). *Plasmodium falciparum* that causes malaria and *Toxoplasma gondii* that causes toxoplasmosis are organisms that belong to the Apicomplexa clade. Apicomplexans contain plastid remnants (McFadden and Ross, 1999) that appear to be derived from a secondary endosymbiotic event that involved an organism with a chloroplast similar to that found in red algae. There is also the suggestion that trypanosomes once possessed a chloroplast (Hannaert et al., 2003).

These animal parasites are not the only organisms that lost photosynthetic function. Several photosynthetic organisms rely on a photosynthetic host, while their own photosynthetic apparatus declined in functionality. These plant parasites include dodder (*Cuscuta polygonorum*, Convulaceae), and mistletoe (*Phoradendron macrophyllum*, Viscacea).

Another important instance where photosynthetic capacity was lost might have occurred in the endosymbiont that is common to all eukaryotic life, the mitochondrion. Phylogenetic analysis groups mitochondria with α-proteobacteria, which include besides non-photosynthetic organisms also the photosynthetic purple bacteria (Cavalier-Smith, 2006).

B. Photoconverter

At the center of biological light energy conversion are pigmented protein complexes. Two types of photoconverters can be distinguished by the pigments they use, and the type of energy gradient they generate. Retinal-based photoconverters generate an energy gradient by translocating protons across a membrane, while reaction centers containing chlorophyll (Chl) use light energy to transfer electrons from a donor to an acceptor.

1. Retinal-Based Photoconverters

Retinal-based photoconverters can be found in Archaea, bacteria, and eukaryotes and have functions in energy generation and sensing of light in the environment (Nagel et al., 2005). Retinal-based photoconverters are integral membrane proteins with 7 transmembrane alpha helices. These transmembrane helices form a channel with a covalently bound retinal as a cofactor. Light isomerizes all-trans retinal to 13-cis retinal. This conformational change also induces a conformational change in the protein that leads to the translocation of protons through the protein. Aspargine and arginine residues act as proton acceptors and donors of retinal. One amino acid is protonated by the cis retinal while another amino acid reprotonates the all trans retinal.

The retinal-based photoconverter of Archaea is called bacteriorhodopsin. Under low oxygen tension, Archaea like *Halobacterium salinarum* sp. NRC-1 can form paracrystalline arrays that contain thousands of bacteriorhodopsins.

For a long time it was thought that retinal-based photoconverters were limited to Archaea and eukaryotes. This changed when retinal-based photoconverters were found in the genomes of uncultivated marine γ-proteobacteria (Béjà et al., 2000). The suggestion that these proteorhodopsins can contribute to cellular energy generation has been experimentally confirmed (Gómez-Consarnau et al., 2007). Interestingly, proteorhodopsins have also recently been identified in Archaea (Frigaard et al., 2006), with lateral gene transfer a likely mechanism to explain this distribution.

Photoconverters that mediate retinal-based proton translocation can be distinguished from the Chl-based reaction centers that catalyze the extraction of electrons. They are almost certainly independent evolutionary solutions to the problem of energy acquisition.

2. Reaction Centers

The defining function of the reaction center is charge separation and stabilization of the resulting products. Upon excitation of a special set of Chls (used here to include bacteriochlorophylls (Bchls)) within the reaction center, charge separation occurs: next an electron is "stripped away" from the special set of Chls (the primary donor) and transferred to a primary electron acceptor,

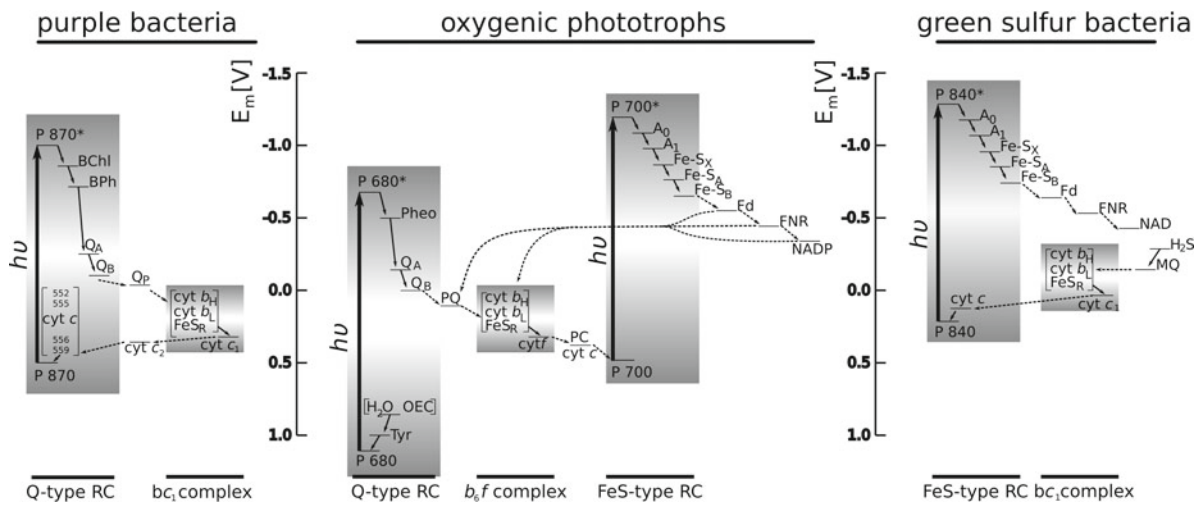

Fig. 1.2. Overview of reaction center types and photosynthetic electron transport chains of purple bacteria, oxygenic phototrophs and green sulfur bacteria. The redox midpoint potential (E_m) at physiological conditions for the cofactors is indicated. Protein complexes are shown as shaded blocks. Reaction centers are classified as Q-type or FeS-type, depending on whether quinones or a series of iron sulfur clusters are the final electron acceptors (see text). Absorption of a photon with energy of $h\nu$ (h, Planck's constant; ν, wavelength frequency) by reaction center pigments causes the transition from ground state (P) to the excited state (P*). The peak absorption wavelength is indicated for each type of reaction center. The cytochrome (cyt) bc_1 and b_6f complexes, together with their Rieske-type iron-sulfur clusters (FeS_R), both receive electrons from reaction centers (see dotted arrows) and are incorporated into the schemes to indicate linear and cyclic electron transport. Different species of cyt are indicated by lowercase letters in italics: the subscripts L and H refer to low potential and high potential forms of cyt *b*, respectively. In the purple bacterial reaction center four different forms of cyt *c* are indicated by their absorption maxima. Bacteriochlorophyll (BChl) and bacteriopheophytin (BPh) are early electron acceptors in purple bacteria, and pheophytin (Pheo) is the first electron acceptor from P680* in oxygenic phototrophs. Q_A and Q_B are primary and secondary quinone electron acceptors, respectively. In purple bacteria, the pool of membrane-associated quinone acceptors is referred to as Q_P and in oxygenic phototrophs this is designated as the plastoquinone (PQ) pool. In the green sulphur bacteria a membrane-associated pool of menaquinone (MQ) is present that is oxidized by the cyt bc_1 complex. In oxygenic phototrophs the oxygen-evolving center (OEC) catalyzes water oxidation and donates electrons via a conserved tyrosine (Tyr) to the P680 reaction center: while electrons are transferred between the cyt b_6f complex and the P700 reaction center by either a *c*-type cyt or plastocyanin (PC). In the FeS-type reaction centers of oxygenic phototrophs and green sulphur bacteria the primary electron acceptors are A_0, a (bacterio)chlorophyll, and A_1, an electron-accepting quinone species. Fe-S_X, Fe-S_A and Fe-S_B are reaction-center-associated iron sulfur clusters that transfer electrons to NADP or NAD via ferredoxin (Fd) and a ferredoxin-NAD(P) oxidoreductase (FNR).

and then on to a series of electron acceptors (Fig. 1.2). To avoid recombination of separated charges, the spatial orientation and distances, as well as energetic gradients of donors and acceptors are arranged to stabilize the charge efficiently away from the electron donating Chl(s). In addition to the efficient removal of the generated electron from the donating set of Chls, charge separation is also stabilized by a fast reduction of these Chls.

At the heart of each reaction center is a dimeric protein that serves as a scaffold for the components involved in excitation, electron and proton transfer. The reaction centers of green sulfur bacteria and heliobacteria consist of two identical subunits. This homodimeric composition probably represents the evolutionarily most ancient reaction center configuration, which is not to imply that heliobacterial and green sulfur bacterial reaction centers are identical with the ancient form (Hohmann-Marriott and Blankenship, 2008). The heterodimeric reaction centers found in all other organisms are likely to be derived forms that correspond to specialization in function. Each dimer subunit contains Chls that participate in charge separation and electron acceptors. Reaction centers can be grouped into two classes depending on whether the first stable electron acceptor is a quinone (Q-type) or iron-sulfur (FeS-type) cluster (Blankenship, 1992) (Fig. 1.2).

a. Iron-Sulfur-Type Reaction Centers

Each core subunit of FeS-type reaction centers consists of 11 transmembrane helices. The last five membrane-spanning helices contain the electron donors and acceptors that participate in charge separation and charge stabilization. The first 6 membrane-spanning helices of FeS-type reaction centers can be considered a core antenna domain and house carotenoids and Chls. The FeS-type reaction centers of green sulfur and heliobacteria are homodimeric, while the FeS-type reaction centers of cyanobacteria and photosynthetic eukaryotes are heterodimers.

The components of the electron transport chain in FeS-type reaction centers in cyanobacteria and eukaryotes have been identified (Golbeck, 2006). Electrons, which originated from excited state reaction center Chls, reduce a Chl called A_0, followed by a quinone called A_1 (Kjaer et al., 1998) and then a series of iron sulfur clusters (F_X, F_A and F_B). Although green sulfur bacterial and heliobacterial reaction centers also possess the same cofactors, there remains uncertainty about the function of the menaquinones in these reaction centers, as electrons can reach the iron sulfur cluster in reaction centers that lack quinones (Hager-Braun et al., 1997; Kleinherenbrink et al., 1993).

Because of the dimeric nature of FeS-type reaction centers electron transport may occur in two branches. In homodimeric FeS-type reaction centers both branches are used equally. In the heterodimeric FeS-type reaction centers of cyanobacteria and eukaryotes, there appears to be a preference towards one electron transport branch. There is only a small preference in eukaryotes (Guergova-Kuras et al., 2001; Fairclough et al., 2003; Muhiuddin et al., 2001) and a more significant preference in cyanobacteria (Xu et al., 2003a, b).

The oxidized reaction center Chl *a* in heliobacteria and green sulfur bacteria is reduced by cytochromes. The standard electron donor to the FeS-type reaction center of plants is the copper protein plastocyanin. In addition to plastocyanin, cytochromes can also act as electron donor to the FeS-type reaction center in cyanobacteria (Briggs et al., 1990) and algae (Merchant and Bogorad, 1987).

b. Quinone-Type Reaction Centers

The Q-type reaction centers of purple bacteria and filamentous anoxygenic phototrophic bacteria consist of two subunits (L and M), each containing 5 transmembrane alpha helices, that form a heterodimer. The electron carriers involved in charge separation and stabilization are contained within this heterodimer. The Q-type reaction centers of cyanobacteria and their eukaryotic relatives also possess a heterodimeric reaction center, composed of two 5-helix subunits (D1 and D2), which house electron carriers involved in charge separation and its stabilization. In addition to these 5-helix complexes, two additional 6-helix subunits (called CP43 and CP47, see Eaton-Rye and Putnam-Evans (2005)) that act as a core antenna are an essential part of the oxygenic reaction center.

In heterodimeric Q-type reaction centers, electrons only use one of the pheophytin molecules as electron acceptor. From there, electrons are transferred to a tightly bound quinone in one part of the dimer (Q_A) followed by an exchangeable second quinone (Q_B) in the other part of the dimer. The second quinone can leave the reaction center when it is reduced twice. Reduced Q_B leaves the reaction center and becomes a part of a membrane-bound pool of quinones that can be oxidized by quinol oxidoreductases.

The oxidized reaction center of anoxygenic purple bacteria is reduced by a cytochrome, while the copper containing auracyanin reduces the reaction centers of filamentous anoxygenic phototrophic bacteria. In cyanobacteria and eukaryotes, electrons are generated by the oxygen-evolving complex that is part of the reaction center complex.

c. Evolution of Reaction Centers

Although sequence homology between FeS-type and Q-type reaction centers does not clearly indicate a common origin, structural data strongly suggest that all the reaction centers are derived from a single common ancestor (Sadekar et al., 2006; Cardona et al., 2011). Common to all reaction centers is a dimeric core. As mentioned earlier, the core can be homodimeric (green sulfur bacteria and heliobacteria) or heterodimeric (purple bacteria, filamentous anoxygenic bacteria, and the

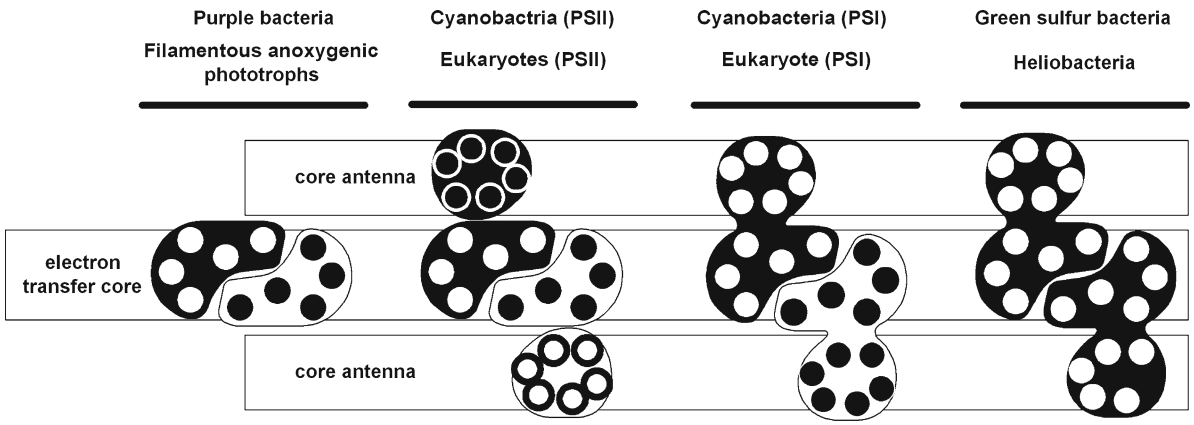

Fig. 1.3. Schematic diagram indicating the transmembrane helical composition of photosynthetic reaction centers. Purple bacteria and filamentous anoxygenic phototrophs possess a heterodimeric (L, M) Quinone-type (Q-type) reaction center. The Q-type reaction center (PS II) of cyanobacteria and photosynthetic eukaryotes consists of a heterodimeric electron transfer core (D1, D2) and two core antenna subunits (CP47, CP43). The iron-sulfur-type (FeS-type) reaction center (PS I) of cyanobacteria and higher plants is heterodimeric. Heliobacteria, acidobacteria and green sulfur bacteria possess a homodimeric FeS-type reaction center. The core antenna of heliobacteria, green sulfur bacteria, and PS I is structurally related to the separately encoded core antenna of PS II of oxygenic eukaryotes and cyanobacteria. Transmembrane helices are indicated by circles. Transmembrane helices encoded by separate genes are shown by different black and white patterns and are indicated by circles. Modified from Hohmann-Marriott and Blankenship (2008).

cyanobacterial lineage). The reaction center proteins of FeS-type reaction centers consist of 11 transmembrane helices. Functionally, the 6 N-terminal membrane helices bind many Chls and act as a core antenna, while the 5 C-terminal helices contain the electron transport components of FeS-type reaction centers (Fig. 1.3).

Complexes with a pair of subunits with 5 membrane helices also house the electron transport components in the Q-type reaction center proteins of purple bacteria and filamentous anaerobic bacteria as well as those of the cyanobacterial lineage. However, a functional Q-type reaction center of the cyanobacterial lineage also contains the CP43 and CP47 proteins that each have 6 membrane helices, bind Chl and act as antenna complexes just like the 6 N-terminal helices of FeS-type reaction centers. There is little doubt that the 6-membrane helix core antenna of FeS-type and Q-type reaction centers are related.

It is unclear, however, whether the 11-membrane helix configuration or the 5 + 6 membrane helix configuration is the more ancient form. The current reaction center composition could be caused by a splitting of an 11-membrane helix protein into a 5-helix electron transport core and a 6-helix antenna core that was lost in purple bacteria and filamentous anoxygenic phototrophs. Alternatively, a previously evolved electron transport core and antenna core might have been fused. Functionally, a split reaction center protein might be a necessity to enable the efficient diffusion of quinones, while a fused electron transport core and antenna core might simplify transcriptional control, and assembly of reaction centers.

d. Oxygenic Photosynthesis Paradox

The presence of both a FeS-type and a Q-type reaction center in cyanobacteria and photosynthetic eukaryotes seems at first paradoxical. However, two models have been put forward to account for this distribution.

The first model, the "selective loss model" (Olson, 1970; Olson and Pierson, 1987a, b) suggests that a common ancestor of all photosynthetic organisms contained both types of reaction centers. According to this model, the presence of FeS-type reaction centers in heliobacteria and green sulfur bacteria and the presence of Q-type reaction centers in purple bacteria and filamentous anoxygenic phototrophs, is caused by the evolutionary loss of the respective reaction center.

The second model, the "fusion model" (Mathis, 1990; Blankenship, 1992) suggests that a single ur-reaction center differentiated into the two reaction center types in different organisms. The occurrence of the two reaction center types in cyanobacteria and their relatives is due to the transfer of genetic material of organisms with the complementary reaction centers.

A third model is a hybrid of the previous two models (Allen, 2005). This model suggests that an organism with a homodimeric reaction center encoded by a single gene created a second copy of this gene within its genome that then developed independently. Depending on the physiological state of the organism, either one or the other copy of the gene was expressed, and over time the copies developed into FeS-type and Q-type reaction centers, which were subsequently selectively lost in all photosynthetic organism groups, with the exception of the cyanobacterial lineage (Allen and Martin, 2007).

C. Antenna Systems

Antenna systems provide an efficient way to harvest light. The evolutionary pressure to harvest light is probably the reason for the many different antenna systems that have evolved. All antenna systems follow the same basic blueprint: light energy is absorbed by pigments, which are part of a pigment collective. Within the collective, energy can be transferred from one pigment to another. The direction of energy transfer is dependent on the orientation of the pigments and the energy at which the respective pigments absorb. The net energy transfer usually follows a gradient established by pigments, from higher absorbed light energy to lower absorbed light energy with the reaction center being the ultimate recipient.

The pigments together with the first 6 transmembrane helices in FeS-type reaction centers can be considered as the core antenna. In fact, this is the only antenna system available to heliobacteria. This core antenna is also part of every Photosystem II (PS II) reaction center as CP43 and CP47. A CP43-like protein gave rise to a whole class of antenna systems that can be found in cyanobacteria that are collectively known as accessory Chl-binding proteins (Chen et al., 2008). Members of this antenna protein family, first described as IsiA proteins, are expressed, when cells experience stress conditions, forming a ring around PS I (Bibby et al., 2001; Boekema et al., 2001). Other members of this family, first described as Prochlorophyte Chl-binding (Pcb) proteins, constitute the antenna systems of *Prochlorophytes* (Bullerjahn and Post, 1993).

A large and diverse family of integral membrane light-harvesting complexes are the LHCs (Green, 2001). Depending on their association with Photosystem I (PS I) or PS II they are called LHCI and LHCII, but alternative nomenclature is also employed (Koziol et al., 2007). LHCs are found in green algae, some red algae and their respective endosymbiotic offspring. These protein complexes consist of three transmembrane helices and can contain carotenoids and Chl *a*, Chl *b* or Chl *c*. It has been proposed that the LHC family derived from the one-membrane-helix small cab-like proteins (SCPs) (Funk and Vermaas, 1999) also known as high-light-induced proteins (HLIPs) (Dolganov et al., 1995), which have been fused several times and so gave rise to LHC and Pcb proteins (Garczarek et al., 2003) (see Chapters 6 and 14). HLIP proteins are found in eukaryotes and cyanobacteria (Heddad and Adamska, 2002). A suggested function in cyanobacteria is the regulation of Chl biosynthesis (Xu et al., 2002).

A third group of membrane-embedded antenna complexes consists of the ring-forming LH1 and LH2 complexes of purple bacteria (Cogdell et al., 2004) and filamentous anoxygenic phototrophs (Blankenship and Matsuura, 2003). The first, LH1, forms a ring around the reaction center made up of 16 identical subunits. There is evidence that one member of this LHI ring may be a different protein that would enable the diffusion of quinone from and to the reaction center (Barz et al., 1995). The second light-harvesting system, LH2, forms smaller rings consisting of 8 or 9 identical subunits that are in the membrane adjacent to the LH1 reaction center ring. LH1 and LH2 are related, each consists of a heterodimer with a bound BChl *a* and a carotenoid.

Several groups of organisms developed antenna systems that are not embedded in membranes. The peridinin-Chl binding antenna system of dinoflagellates is located in the thylakoid lumen (Larkum, 1996), The phycobilisomes of cyanobacteria (MacColl, 1998; Zhao and Quin, 2006) and

of red algae form rod-shaped elements that are oriented towards the cytoplasm and stroma, respectively. Cryptomonads, which are thought to have arisen through a secondary endosymbiotic event, possess phycobiliprotein fragments that act as an antenna system and are located in the lumen (Wedemayer et al., 1996).

In contrast to all antenna systems mentioned so far, the bulk of antenna pigments in green sulfur bacteria, acidobacteria and filamentous anoxygenic phototrophs are not bound to proteins (Blankenship et al., 1995). Chlorosomes, the main antenna systems of these organism groups, are located within the cytoplasm adjacent to the cytoplasmic membrane. Chlorosomes contain hundreds of thousands of BChls *c, d* or *e* that form extended energetically coupled aggregates (Montaño et al., 2003). In addition to these aggregates, chlorosomes also possess a unique array of pigmented proteins that are collectively called the baseplate. In green sulfur bacteria, the baseplate is interfaced to the P840 reaction center via the Fenna-Matthews-Olson-protein (FMO-protein) (Fenna and Matthews, 1975), while in filamentous anoxygenic phototrophs the baseplate is coupled to the P866 reaction center via the membrane-internal LH808-866 antenna system, which is related to the LH1 antenna complexes of purple bacteria (Blankenship and Matsuura, 2003).

D. Quinol Oxidoreductases

Q-type reaction centers generate reduced quinones. A special class of proteins converts redox energy, which is stored in these quinones, into a proton gradient. When the quinones are reduced by the reaction centers, they pick up protons from one side of the membrane that they release when they are oxidized on the other side of the membrane. The quinones are oxidized by quinone oxidoreductases, which include the cytochrome bc_1 complex (Cyt bc_1 complex) in green sulfur bacteria, and purple bacteria (Crofts, 2004), the cytochrome b_6f complex (Cyt b_6f complex) in cyanobacteria and photosynthetic eukaryotes (Allen, 2004), and another cytochrome-containing complex in filamentous anoxygenic phototrophs (Yanyushin et al., 2005) and acidobacteria. Heliobacteria possess a quinone oxidoreductase with a bipartite Cyt *b* complex similar to the Cyt b_6f complex (see Chapter 21).

E. ATP Generation

The combined reactions of reaction centers and quinol oxidoreductases result in a transmembrane proton gradient. The conversion of energy stored in this proton gradient into an energy carrier that can be used to drive biochemical reactions is of central importance. Organisms throughout the tree of life use a single protein system for this task: The ATP synthase (see Chapter 22).

The ATP synthase is a membrane-spanning multisubunit protein complex. The membrane embedded part, called CF_o, consists of identical subunits. On the cytoplasmic side of the membrane, this proton channel interfaces with the CF_1 part of the ATPase. A multisubunit protein complex called "stator" is located at the periphery of the CF_1–CF_o complex. When protons pass from one side of the membrane to the other, CF_o and CF_1 rotate against each other. The conformational change induced by this rotation is used to catalyze the synthesis of ATP from ADP and inorganic phosphate (Senior et al., 2002; Adachi et al., 2007).

The energy that each transmembrane proton transfer can generate is dependent on the concentration difference of protons on both sides of the membrane, but because protons are charged, also on the electric potential difference of both membrane sides. That such energy transduction can be used by organisms was first postulated by Mitchell (1961, 1970) and is known as the chemiosmotic hypothesis of energy generation.

F. Carbon Fixation

Currently, six reaction sequences are known to lead to the incorporation of inorganic carbon (Thauer, 2007) (see Chapter 26). Green sulfur bacteria use a reverse or (reductive) tricarboxylic acid (TCA) cycle (Evans et al., 1966; Buchanan and Arnon, 1990). The reverse TCA is indeed a reversal of the TCA cycle concerning the intermediates; however, some enzymes distinct from the TCA cycle are required. Purple bacteria (Stoppani et al., 1955), cyanobacteria (Tabita, 1988) and photosynthetic eukaryotes utilize the reductive pentose phosphate cycle, also known as the Calvin-Benson-Bassham cycle (Calvin and Benson, 1948). The Calvin-Benson-Bassham cycle will be referred to as the Calvin-Benson

cycle throughout this book. In this cycle CO_2 is incorporated through a reaction with ribulose-1-5-bisphosphate catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Plants capable of C4 photosynthesis (Hatch, 1987) and Crassulacean acid metabolism (CAM) photosynthesis (Heyne, 1815; Cushman, 2001; Black and Osmond, 2003) perform a prefixation of CO_2 that is catalyzed by the enzyme phosphoenol pyruvate carboxylase. The resulting malate is then decarboxylated, and incorporated into organic molecules by the reductive pentose phosphate cycle. The prefixation can be separated from the fixation by compartments (C4) or compartments and time (CAM).

The first step of two additional carbon fixation cycles rely on the carboxylation of acetyl-CoA. Filamentous anoxygenic bacteria use the 3-hydroxypropionate/malonyl-CoA pathway (Eisenreich et al., 1993; Strauss and Fuchs, 1993). Berg et al. (2007) have discovered an additional acetyl-CoA-based carbon fixation cycle (3-hydroxypropionate/4-hydroxybutryate-CoA) in Archaea. The initial steps in carbon fixation are identical with carbon fixation found in filamentous anoxygenic bacteria, but further acceptor regeneration proceeds via succinyl-CoA and 4-hydroxybutryate-CoA.

While the previously described carbon fixations proceed via a cycle, in which the acceptor is regenerated, the reductive acetyl-CoA pathway is non-cyclic, generating acetyl-CoA as the fixation product. This CO_2 fixation pathway is found in organisms belonging to the genus *Clostridium* (Ragsdale, 1991; Roberts et al., 1994).

III. The Photosynthetic World

This section describes photosynthetic organisms and organism groups (Fig. 1.1). It is not meant to be a complete phylogeny of photosynthetic life, but rather is an attempt to illustrate the photosynthetic machinery and provide details on model organisms for the respective groups.

A. Archaea

Some Archaea (Table 1.2) possess retinal-based photoconverters that generate a proton gradient. This is in contrast to the Chl-based reaction centers of bacteria, which generate energy-rich reduced molecules through a charge separation event. The archaeal photoconverter, bacteriorhodopsin, and the eubacterial reaction centers belong to entirely different protein families. Some Archaea can fix carbon using the reductive TCA cycle (*Thermoproteus*) and reductive acetyl-CoA pathway (Methanogenic Archaea, *Archaeoglobus*) and the 3-hydroxypropionate/4-hydroxybutryate-CoA pathway (*Metallosphaera*, *Sulfolobus*, *Acidianus*, *Nitrosopumilus*, *Crenarchaeum*, *Archaeoglobus)* (Berg et al., 2007; Thauer, 2007).

Table 1.2. Characteristics of Archaea

Electron source	None – generates proton gradient
Reaction center	Bacteriorhodopsin (in some species) Retinal (~570 nm)
Antenna	None
Quinol oxidoreductase	Some contain a cytochrome bc_1 complex
Carbon fixation	Reductive TCA cycle (*Thermoproteus*) Reductive Acetyl-CoA cycle (Methanogenic Archaea, e.g., *Archaeoglobus*) 3-hydroxypropionate/4-hydroxybutryate-CoA (*Metallosphaera*, *Sulfolobus*, *Acidianus*, *Nitrosopumilus*, *Crenarchaeum*, *Archaeoglobus*)

Halobacterium salinarum sp. NRC-1 is the most investigated photosynthetic Archaea. Under low oxygen tensions this organism produces large amounts of bacteriorhodopsin that is incorporated into the cytoplasmic membrane. These "purple membranes" give photoheterotrophically growing halobacteria a vivid purple color.

B. Anoxygenic Photosynthesis

1. Heliobacteria

Heliobacteria are the only photosynthetic bacteria that are gram-positive and able to generate spores (Ormerod et al., 1996). The photosynthetic machinery is also unique (Oh-Oka, 2007) (Table 1.3). The homodimeric FeS-type reaction center contains BChl *g* and the reaction center core antenna is the only antenna system present. Heliobacteria are strictly anaerobic and appear not to be able to use reaction-center-derived electrons for the fixation of CO_2. *Heliobacterium chlorum* was the first photosynthetic heliobacterium

Table 1.3. Characteristics of heliobacteria

Electron source	Organic molecules – no photoautotrophic growth known
Reaction center	FeS-type – homodimeric (BChl *g*) PshA (11 TMH[a]) P800[b]
Donor	Cytochrome
Acceptor	BChl *g* (A_o) – Menaquinone (A_1) – F_X – F_A/F_B – Ferredoxin
Antenna	None, one core antenna that is part of reaction center
Quinol oxidoreductase	Cytochrome bc_1-like complex (with a bipartite cytochrome *b* complex similar to that in cytochrome b_6f *complexes*)
Carbon fixation	None

[a]TMH: transmembrane helices
[b]Absorption maximum of reaction center pigments

Table 1.4. Characteristics of acidobacteria

Electron source	Uncertain
Reaction center	FeS-type – homodimeric (BChl *a*) PscA (11 TMH[a]) P840[b]
Donor	Cytochrome
Acceptor	BChl *a* (A_o) – Menaquinone (A_1) – F_X – F_A/F_B – Ferredoxin
Antenna	Chlorosome (BChl *c*,*d*,*e* aggregates) Baseplate (BChl *a*) CsmA Fenna-Matthews-Olson (or FMO) protein (BChl *a*)
Quinol oxidoreductase	Cytochrome bc_1 complex
Carbon fixation	Uncertain (cannot grow on bicarbonate)

[a]TMH: transmembrane helices
[b]Absorption maximum of reaction center pigments

discovered (Gest and Favinger, 1983; Gest, 1994). The genome of *Heliobacterium modesticaldum* has been sequenced (Sattley et al., 2008).

2. Acidobacteria

Bryant et al. (2007) have discovered a novel phylum with Chl-based reaction centers. The initial characterization of the first member of this group, the acidobacterium *Candidatus* Chloracidobacterium thermophilum revealed many features of the photosynthetic machinery that are similar to that found in green sulfur bacteria (Table 1.4). The genome of this organism includes genes for an FeS-type reaction center, FMO-protein and baseplate protein and enzymes for the synthesis

Table 1.5. Characteristics of green sulfur bacteria

Electron source	Sulfides, sulfur, hydrogen
Reaction center	FeS-type – homodimeric (BChl *a*) PscA (11 TMH[a]) P840[b]
Donor	Cytochrome
Acceptor	BChl *a* (A_o) – Menaquinone (A_1) – F_X – F_A/F_B – Ferredoxin
Antenna	Chlorosome (BChl *c*,*d*,*e* aggregates) Baseplate (BChl *a*) CsmA Fenna-Matthews-Olson (or FMO) protein (BChl *a*)
Quinol oxidoreductase	Cytochrome bc_1 complex
Carbon fixation	Reverse tricarboxylic acid cycle

[a]TMH: transmembrane helices
[b]Absorption maximum of reaction center pigments

of BChl *a* and methylated BChl *c*. The accumulation of BChl and the formation of chlorosomes have also been demonstrated. However, judged by its machinery to respire oxygen, *Candidatus* Chloracidobacterium thermophilum seems to prefer a more aerobic life style than green sulfur bacteria, and seems more similar to filamentous anoxygenic phototrophs (Bryant et al., 2007).

3. Green Sulfur Bacteria

Green sulfur bacteria comprise a phylogenetic group of photosynthetic Gram-negative bacteria (Overmann and Tuschak, 1997). Green sulfur bacteria are obligate photoautolithotrophs that derive electrons required for CO_2 fixation from sulfur (or hydrogen) components and possess a very efficient antenna system. This combination of features enables green sulfur bacteria to flourish in light-limited, anoxic, water layers of lakes and littoral sediments.

The photosynthetic apparatus of green sulfur bacteria contains a homodimeric reaction center of the FeS-type (Hauska et al., 2001) (Table 1.5). Closely associated with the reaction center is the BChl *a*-containing FMO protein (Fenna and Matthews, 1975; Olson, 2004). The main light-harvesting system, however, consists of BChl *c*, *d* or *e* aggregates. These aggregates have hundreds of thousands of BChls located in a vesicle called a chlorosome, which is surrounded by a unilayer membrane towards the cytoplasmic side (Blankenship and Matsuura, 2003). The chlorosomes are energetically coupled to the FMO-reaction center

complex via a BChl *a*-containing baseplate protein array, which delineates the chlorosome towards the cytoplasmic membrane. The baseplate proteins of green sulfur bacteria and filamentous anoxygenic phototrophs are homologous structures. Green sulfur bacteria are unique in using the reverse tricarboxylic acid cycle for carbon fixation (Buchanan and Arnon, 1990).

Chlorobium tepidum is the most prominent green sulfur laboratory strain. True to its name, *Chlorobium tepidum* prefers a warm growth temperature of 42°C. The main antenna pigment is BChl *c*. The organism forms rod-shaped cells of 1.0–2.5 µm length. *Chlorobium tepidum* is amenable to genetic manipulation and the first member of this group with a sequenced genome (Eisen et al., 2002).

4. Purple Bacteria

Purple bacteria (Hunter et al., 2008) comprise a group of photosynthetic organisms that can grow in aerobic and anaerobic conditions. Many species prefer anaerobic conditions for photoautotrophic growth. However, some others are also able to grow photoheterotrophically in aerobic conditions. Many species of this group are able to fix nitrogen. Some species can use sulfide and sulfur components to grow photosynthetically. The photosynthetic machinery of purple bacteria consists of a heterodimeric Q-type reaction center and two membrane-embedded light-harvesting systems (Table 1.6). Reaction centers and antenna complexes are housed in the invaginations of the cytoplasmic membrane known as intracytoplasmic membranes.

Blastochloris (Rhodopseudomonas) viridis is an unusual purple bacterium that uses BChl *b* as a reaction center pigment and therefore uses extremely far-red light (960 nm). The reaction center of *B. viridis* was the first reaction center to be crystallized (Deisenhofer et al., 1984). Recently, *Roseobacter denitrificans*, an oceanic purple bacterium, has been identified, which might play a major role in carbon/nitrogen cycling in the ocean. A genome project for this organism has been completed (Swingley et al., 2007) and has revealed that this organism is not capable of photoautotrophic growth.

The genomes of *Rhodopseudomonas palustris* and the non-sulfur oxidizing *Rhodobacter capsulatus* (also known as *Rhodopseudomonas capsulatus*) (Haselkorn et al., 2001) were the first in the purple bacteria group to be sequenced.

Table 1.6. Characteristics of purple bacteria

Electron source	Organic molecules, sulfides
Reaction center	Q-type – heterodimeric PufL (5 TMH[a]) + PufM (5 TMH) (BChl *a*) most species (P870[b]) (BChl *b*) some species e.g., *Blastochloris viridis* (P960[b])
Donor	Cytochrome
Acceptor	Bacteriopheophytin *a or b* – Ubiquinone/Menaquinone (Q_A) – Ubiquinone/Menaquinone (Q_B)
Antenna	LH1 LH2
Quinol oxidoreductase	Cytochrome bc_1 complex
Carbon fixation	Reductive pentose phosphate pathway

[a] TMH: transmembrane helices
[b] Absorption maximum of reaction center pigments

5. Filamentous Anoxygenic Phototrophs

The old nomenclature (green non-sulfur bacteria) was abandoned when it became clear that some members of this group are able to obtain electrons from sulfide and sulfur components. Filamentous anoxygenic phototrophs possess a Q-type reaction center and a ring of membrane-embedded LH proteins that is homologous to that found in purple bacteria (Table 1.7). The main light-harvesting system is, however, the chlorosome that consists of BChl aggregates very similar to the chlorosomes found in green sulfur bacteria. These similarities extend to an array of baseplate proteins that couple the chlorosome to the membrane-embedded LH antenna system and reaction center. Filamentous anoxygenic phototrophs use the 3-hydroxypropionate pathway for carbon fixation. Filamentous anoxygenic phototrophic bacteria have a very flexible metabolism that includes photoautotrophic or chemoorganotrophic growth in anaerobic conditions, and photoheterotrophic growth in aerobic conditions.

Chloroflexus aurantiacus is the most studied organism of this group with a sequenced genome. The name is a consequence of the golden streaky appearance of cell cultures caused by the filaments.

Table 1.7. Characteristics of filamentous anoxygenic phototrophs (formerly: Green non sulfur bacteria)

Electron source	Organic molecules, sulfides
Reaction center	Q-type – heterodimeric (BChl *a*) PufL (5 TMH[a]) + PufM (5 TMH) P870[b]
Donor	Cytochrome
Acceptor	Bacteriopheophytin *a* – Menaquinone (Q_A) – Menaquinone (Q_B)
Antenna	Chlorosome (BChl *c*) in some species Baseplate (BChl *a*) CsmA in some species LHI 808-866 antenna (BChl *a*)
Quinol oxidoreductase	Cytochrome complex (alternative complex III)
Carbon fixation	3-hydroxypropionate pathway

[a]TMH: transmembrane helices
[b]Absorption maximum of the reaction center pigments

The pigmentation is most dominant in cells grown under anaerobic conditions. In nature, *Chloroflexus aurantiacus* is often found in mats as a layer under a layer of cyanobacteria. *Chloroflexus aurantiacus* is a thermophile with an optimal growth temperature of 55°C. Some species, e.g., *Roseoflexis castenholzii*, lack chlorosomes but appear to have a similar membrane-bound antenna, reaction center and electron transport chain.

C. Oxygenic Photosynthesis in Bacteria

Oxygenic photosynthesis requires the collaboration of PS II, a Q-type reaction center with an added oxygen-evolving complex and PS I, an FeS-type reaction center (Table 1.8). Electrons are generated by PS II by oxidation of water. PS II then reduces plastoquinone, which is oxidized by the cytochrome b_6f complex. The cytochrome b_6f complex in turn reduces the soluble carriers cytochrome c_6 or plastocyanin, which are electron donors to PS I. Morphologically significant are paired internal membranes called thylakoids. Each membrane pair encloses a space called the lumen, into which protons are deposited and which contains soluble electron carriers (cytochromes and plastocyanin). Oxygenic photosynthesis first appeared in cyanobacteria-like organisms. Through endosymbiotic events these organisms became the ancestors of modern Glaucophyta, Rhodophyta and Chlorophyta (see the following section).

Table 1.8. Characteristics of oxygenic phototrophs

Electron source	Water
Reaction center II	Q-type – heterodimer (Chl *a*) PsbA or D1 (5 TMH[a]), and PsbC or CP43 (6 TMH) *and* PsbD or D2 (5 TMH) and PsbB or CP47 (6 TMH) P680[b]
Donor	Oxygen-evolving center
Acceptor	Pheophytin *a* – Plastoquinone (Q_A) – Plastoquinone (Q_B)
Quinone oxidoreductase	Cytochrome b_6f complex
Reaction center I	FeS-type – heterodimer (Chl *a*) PsaA (11 TMH) PsaB (11 TMH) P700[b]
Donor	Plastocyanin, cytochrome c_6
Acceptor	Chl *a* (A_0) – Phylloquinnone (A_1) – F_X – F_A/F_B – Ferredoxin
Carbon fixation	Reductive pentose phosphate pathway (C3 pathway) Phosphoenol pyruvate pathway (C4 pathway) Crassulacean acid metabolism (CAM pathway)

[a]TMH: transmembrane helices
[b]Absorption maximum of reaction center pigments

Table 1.9. Characteristics of cyanobacteria

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	CBP[a] (Chl *a*) Phycobilisomes (Allophycocyanin, Phycocyanin, Phycoerythrin)

[a]CBP: chlorophyll-binding protein

1. Cyanobacteria

Cyanobacteria are a very diverse group of gram-negative bacteria that carry out oxygenic photosynthesis (Table 1.9). Microfossil evidence and geological signatures for oxygen date the possibility of cyanobacterial oxygenic photosynthesis to at least 2,500 million years ago (Knoll, 2003). Present day cyanobacteria are found in sea and fresh water. Cyanobacteria can be unicellular or filamentous, and some species can convert atmospheric nitrogen into nitrates and organic nitrogen components (Whitton and Potts, 2000).

The main light-harvesting system in this group are phycobilsomes that are usually found associated with PS II (Mullineaux, 2008) and arranged in spherical arrays within the cell cytoplasm

(Gantt, 1996). Most cyanobacteria possess thylakoids that harbor the photosynthetic electron transport chain (Mullineaux, 2005).

Phylogenetic analysis of the numerous sequenced cyanobacterial genomes indicates that *Gloeobacter violaceus* branches early from other cyanobacteria (Swingley et al., 2008). Because of this fact and the absence of internal thylakoid membranes, *Gloeobacter violaceus* may be a primitive cyanobacterium, having retained many of the common features of all cyanobacteria.

All *Synechocystis* and *Synechococcus* species are unicellular. *Synechocystis* sp. PCC 6803 is the most prominent cyanobacterial laboratory organism. *Synechocystis* sp. PCC 6803 was the first photosynthetic organism to be sequenced (Kaneko et al., 1996). It has a small genome size and can be genetically manipulated through homologous recombination. *Synechcocystis* sp. PCC 6803 can grow heterotophically on glucose with a doubling time of 8–12 h. *Synechococccus* sp. PCC 7942 (also known as *Synechococcus elongatus* and as *Anacystis nidulans* R2) is a model system for carbon fixation, nutrient acquisition and the investigation of circadian rhythms (Iwasaki and Kondo, 2004; Mackey and Golden, 2007). *Thermosynechococcus elongatus* is a fast-growing thermophilic organism. The heat-stable reaction centers were the first to be crystallized and revealed the atomic structure of PS II (Zouni et al., 2001) and PS I (Jordan et al., 2001). The closely related thermophilic strain *Thermosynechococcus vulcanus* has also been used and crystals of PS II isolated from this cyanobacterium have yielded the highest resolution structure of PS II that is currently available (Umena et al., 2011).

Nostoc punctiforme PCC 73102 (Meeks et al., 2001) and *Anabaena* (also known as *Nostoc*) sp. PCC 7120 (Kaneko et al., 2001) are filamentous cyanobacteria. *Nostoc* species are model organisms for nitrogen fixation and the formation of heterocysts, specialized cells that are formed by some cyanobacteria as a nitrogen fixation compartment. Because nitrogen fixation and oxygen are incompatible, due to the oxygen-sensitivity of nitrogenases, heterocysts do not have PS II activity (Fay, 1992).

2. Prochlorophytes

Phylogenetic analysis assigns Prochlorophytes to the cyanobacterial clade (Lewin, 2002). However, the prochlorophyte genera *Prochloron* (Lewin, 1976), *Prochlorothrix* (Burger-Wiersma et al., 1986), and *Prochlorococcus* (Chisholm et al., 1988) may not constitute a monophyletic group, and they appear scattered within different cyanobacterial groups based on 16 s RNA analysis (Urbach et al., 1992).

Table 1.10. Characteristics of prochlorophytes

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	CBP[a]/Pcb (Chl *a* + *b*) Phycoerythrin (present in genome, limited expression)

[a] CBP: chlorophyll-binding protein

An unusual feature that connects these three genera is the presence of Chl *b* in addition to Chl *a* in their light-harvesting systems (Table 1.10). Initial speculation that Chl *b* indicated that a Prochlorophyte-like organism was the ancestor to photosynthetic eukaryotes has been weakened when it became clear that the light-harvesting systems of both groups of organisms have different origins (La Roche et al., 1996). The Chl *a*- and Chl *b*-containing Pcb proteins of Prochlorophytes are members of the CP43 core-antenna family of light-harvesting proteins. The antenna of *Prochloron* contains the Chl *c*-like pigment (Mg-2,4-divinyl pheoporphyrin a_5 monomethyl ester or divinyl protochlorophyllide) as well as Chls *a* and *b*, while *Prochlorococcus* Pcb binds divinyl Chls *a* and *b* (Green and Durnford, 1996).

Prochlorocccus marinus is a unicellular marine organism that due to its small size had escaped detection for a long time. The genome of the *Prochlorococcus* strains SS120 (low-light-adapted ecotype), strain MED4 (high-light-adapted ecotype) and MIT9313 (low-light-adapted ecotype) have been sequenced. In addition to phycobilisomes and Pcbs, some *Prochlorococcus* strains also contain phycoerythrin. While in the high light ecotype the expression of phycoerythrin has been demonstrated (Hess et al., 1996), the low light ecotypes appear to completely lack phycoerythrin (Hess et al., 2001).

3. Acaryochlorophytes

Acaryochloris marina is a unicellular organism that has been isolated from a colonial ascidian (*Lissoclinum patella*). *Acaryochloris marina* is not a Prochlorophyte, but has some unique features at

Table 1.11. Characteristics of Acaryochlorophytes

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	CBP[a]/Pcb (Chl *d*+*a*) Allophycocyanin+phycocyanin

[a] CBP: chlorophyll-binding protein

the level of light harvesting in common with this group (Table 1.11). Similar to *Prochlorococcus*, *Arayochloris marinus* uses Pcb proteins as the main light-harvesting system, while phycobilins are of only negligible function. *Acarychloris marina* uses Chl *d* as the main pigment in its external light-harvesting system, with only small amounts of Chl *a* being present. Whether the reaction center contains Chl *a* at all remains unclear (Chen et al., 2005; Tomo et al., 2007; Schliep et al., 2010). The genome of this organism has been sequenced (Swingley et al., 2008).

D. Oxygenic Photosynthesis in Eukaryotes

It appears that there may have been a single endosymbiotic event (see Section II.A.2.) as the basis of all eukaryotic photosynthesis (Yoon et al., 2004; Blair et al., 2005). Due to this endosymbiotic event involving a cyanobacterial-like organism the resulting organisms and derived groups have electron transport pathways that are typical of cyanobacteria (see Section III.C.1.). However, while some groups maintained the light-harvesting system that they inherited from their cyanobacterial ancestors, other groups evolved a variety of unique light-harvesting systems (Larkum et al., 2003; Green, 2003; Green et al., 2003).

1. Glaucophyta

The clade Glaucophyta is composed of unicellular algae. The cells are pigmented with Chl *a* as well as *β*-carotene, zeaxanthin (Chapman, 1966), *β*-cryptoxanthin (Schmidt et al., 1979), allophycocyanin and phycocyanin (Table 1.12). Glaucophyta contain a plastid, called a cyanelle (Evrard et al., 1990), which is surrounded by two membranes indicating a primary endosymbiosis involving a cyanobacterium (McFadden, 2001). Another indication for this event is the presence of unstacked thylakoids, phycobilliproteins, and the existence of a peptidoglycan layer (Pfanzagl et al., 1996). This unique feature is thought to derive from the peptidoglycan layer of the incorporated cyanobacterium-like organism and distiguishes the Glaucophytes from the photosynthetic endosymbionts in Rhodophyta and Chlorophyta.

Table 1.12. Characteristics of Glaucophyta

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	IsiA (Chl *a*) Phycobilisomes

Cyanophora paradoxa is so far the only member of the Glaucophyta that can be easily cultivated. Due to its unique features and phylogenetic position, this example of the Glaucophyta is of interest to answer questions relating to the evolution of the photosynthetic machinery, the import of proteins into the plastid and endosymbiosis (Steiner and Löffelhardt, 2002).

2. Red Alga Line (Rhodophyta)

The red algal line originates from a primary endosymbiotic event that is the basis for Rhodophytes, but also includes groups that originated by secondary endosymbiosis. Secondary endosymbiotic events gave rise to Cryptophytes, Stramenopiles (that include brown algae and golden-brown algae) diatoms (that include yellow-green algae) and dinoflagellates (Li et al., 2006). The Apicomplexa are also the result of a secondary endosymbiotic event involving a red alga-like organism. Currently known members of the Apicomplexan clade, however, have lost photosynthetic capability, while they still maintain a rudimentary plastid (Waller and McFadden, 2005).

a. Rhodophytes (Red Algae)

Red algae occur in unicellular or filamentous forms. The multicelluar forms can reach the functional complexity of thalli. Red algae contain Chl *a*, phycobilliproteins as well as α- and β-carotene (Table 1.13). The thylakoids are unstacked.

The small (1.5–2.0 μm) thermo-acidophilic red alga *Cyanidioschyzon merolae* is unicellular; it contains only one chloroplast and one mitochondrion per cell. This organism has been investigated as a model for chloroplast and mitochondrion division (Miyagishima et al., 2003). The small nuclear genome (~16 Mb) and the chloroplast and mitochondrial genomes have been sequenced.

Table 1.13. Characteristics of Rhodophytes (red algae)

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	LHCI Phycobilins (*c*-phycoerythrin, allo-phycocyanin, *r*-phycocyanin)

Table 1.14. Characteristics of Cryptophytes

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	Phycobiliproteins, no allophycocyanin Chl *a*/*c*-binding proteins (CAC, LHC family)

Transformation and gene replacement through homologous recombination have also been reported (Minoda et al., 2004).

The thallus-forming *Porphyra* species (*P. purpurea and P. yezoensis*) have been investigated extensively; further, chloroplast and mitochondrial genomes of *Porphyra purpurea* have been sequenced (Reith and Munholland, 1995; Burger et al., 1999). Interestingly, *Porphyra yezoensis* is cultivated for food, known as "*Nori*".

Gracilaria (Gracilariaceae) species have been cultivated; extracts of their branched thalli are used for making agar. The chloroplast genome of *Gracilaria tenuistipitala* has also been sequenced (Hagopian et al., 2004).

b. Cryptophytes

The Cryptophytes are derived through a secondary endosymbiotic event: Cryptophytes possess 4 membranes that separate the cell cytoplasm from the chloroplast cytoplasm. The nucleomorph of this group (Gillot and Gibbs, 1980) is thought to represent the nucleus remnant of the primary symbiont. Cryptophytes are able to synthesize α-carotene and diatoxanthin (Stransky and Hager, 1970). The light-harvesting complex is of the LHC-type and contains Chl *a* and Chl *c* (Table 1.14). The location of the phycobiliproteins (phycoerythrin and phycocyanin) in the thylakoid lumen (Gantt et al., 1971) is unique to this group.

The cryptomonad *Guillardia theta* is the first Cryptophyte that has been sequenced (including the nucleomorph genome) (Douglas et al., 2001).

Table 1.15 Characteristics of Haptophytes

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	Phycobiliproteins Fucoxanthin–Chl *a*/*c* proteins (FCP, LHC family)

c. Haptophytes

Haptophytes are a group of unicellular organisms that were derived by secondary endosymbiosis of a red alga-like organism. The chloroplast is surrounded by 4 membranes, and contains phycobiliproteins, Chl *a*, Chl *c* and fucoxanthin-Chl-binding proteins that belong to the LHC family (Table 1.15). Haptophytes are able to synthesize different fucoxanthins as well as β-carotene.

Emiliana huxleyi is a spherical unicellular organism with ~4-μm diameter that is admired for its exquisitely ornamental calcium carbonate structures. The *Emiliana huxleyi* chloroplast and mitochondrion (Sanchez Puerta et al., 2004; Puerta et al., 2005) and nuclear genome (http://genome.jgi-psf.org/Emihu1/Emihu1.home.html) have been sequenced.

d. Dinoflagellates

Most dinoflagellates are the result of a secondary or tertiary endosymbiotic event involving either a red alga or a red alga containing a protist (Hackett et al., 2004; Yoon et al., 2005). As a result, the chloroplasts from these organisms are surrounded by three membranes. In some dinoflagellates, the red-alga-derived chloroplast was substituted with the plastid from a green alga in a secondary endosymbiotic event. However, in some dinoflagellates, their original red-alga-derived chloroplast seems to have been replaced with the chloroplast of cryptomonads, heterokonts and Haptophytes in a tertiary endosymbiotic event (Archibald and Keeling, 2002).

Dinoflagellates are marine organisms and most species are non photosynthetic. The majority of both photosynthetic and non-photosynthetic organisms are also predators of other algae. Some dinoflagellates cause "red tides" that can be deadly for marine life.

The most distinguishing feature of the dinoflagellates is the carotenoid peridinin (Table 1.16), which is only found in this group of organisms.

Table 1.16. Characteristics of Dinoflagellates

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	Intrinsic peridinin – Chl *a* proteins (iPCP, LHC family) Soluble peridinin – Chl *a/c* proteins (sPCP)

Peridinin is a major light-harvesting pigment that is incorporated into intrinsic peridinin-Chl proteins that belong to the LHC family, and a seemingly unrelated soluble peridinin-Chl protein (Hiller et al., 1991). The structure of the soluble peridinin-Chl protein, which is located in the lumen of thylakoids, has been determined to atomic resolution (Hofmann et al., 1996). In addition to peridinin, dinoflagellates can synthesize β-carotene, diadinoxanthin, Chl *a* and Chl *c*.

The genomes of dinoflagellate plastids have a unique organization. A large number of 2-3-kb plasmids, called "minicircles", contain the genetic information that is contained within a single large plasmid in the plastids of all other known eukaryotes (Zhang et al., 1999; Howe et al., 2008). The best-studied dinoflagellate genera are *Ceratium* and *Peridinium.*

e. Stramenopiles

The Stramenopiles include photosynthetic eukaryotes belonging to the brown algae, yellow-green algae, golden-brown algae, and the diatom group. All these groups probably share a common ancestor that resulted from the secondary endosymbiosis involving a red-alga-like partner (Archibald and Keeling, 2002) and this common ancestry is reflected in their having similar features. All members of the Stramenopiles possess a chloroplast that is surrounded by 4 membranes. The main light-harvesting system is the fucoxanthin-Chl-protein member of the LHC-antenna family (Table 1.17).

Brown algae (Phaeophytes) comprise a group of multicellular photosynthetic organisms with mainly marine habitat. Brown algae possess specialized photosynthetic tissues organized in blades and some species possess specialized gas filled bladders that keep the blades afloat. *Macrocystis pyrifera*, the giant kelp, can grow up to 60 m in size and is harvested for the alginates that are contained in its cell wall. The brown alga

Table 1.17. Characteristics of Stramenopiles that include brown algae (Phaeophytes), gold-brown algae (Chrysophytes), diatoms (Bacillariophytes), and yellow-green algae (Xanthophytes)

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	Fucoxanthin (most)–Chl *a/c* proteins (FCP, LHC family)

Table 1.18. Characteristics of oxygenic eukaryotes, green algae (Prasinophytes, Chlorophytes), mosses (Bryophytes), ferns (Pterophytes), and seed plants (Spermatophytes) and green alga-derived organisms (Chloroarachniophytes and Euglenaphytes)

Electron transport	See electron transport of oxygenic phototrophs (Table 1.8)
Antenna	(LHCII) Lhcb1 – 6 (Chl *a*,*b*) (LHCI) Lhca1 – 4 (Chl *a*)

Ectocarpus siliculosus is a small brown alga that offers genetic tools (Peters et al., 2004).

Yellow-green algae (Xanthophytes) are mostly fresh water algae of coccoid or filamentous organization. Yellow-green algae are the only members of the Stramenopiles that do not synthesize fucoxanthin. Yellow-green algal species belong to the genera *Tribonema*, *Vaucheria and Botrydium*.

Golden-brown algae (Chrysophytes) are mostly unicellular organisms that can form silica structures. Golden-brown algal species include *Ochromonas danica*, *Ripidodendron splendidum and Dinobryon balticum.*

Diatoms (Bacillariophytes) comprise a group of unicellular or colonial algae that might contribute 20% of the marine productivity (Field et al., 1998). Many organisms of this group form very delicate silicate shells. The symmetry of these shells was used to classify diatoms into centric (radially symmetric) and pinnate (bilateral symmetric) forms. The genomes of both the centric diatom *Thalassiosira pseudonana* (Armbrust et al., 2004) and the pennate genetically transformable diatom *Phaeodactylum tricornutum* have been sequenced (Bowler et al., 2008).

3. Green Alga Line (Chlorophyta)

The green algal line (Table 1.18) includes eukaryotes with a chloroplast that is derived from a cyanobacterium-like organism. The evolutionary steps that were required to transform a unicellular alga into a plant are thought to be preserved, at least in part, in the organism groups that share a

common ancestor with plants. The group that shares the earliest common ancestor with plants is the Prasinophytes, followed by Chlorophytes (green alga), Bryophytes (mosses), and Pterophytes (ferns and horse tails). The green algal line also includes groups (Chloroarachinophytes and Euglenophytes) that are the result of a secondary endosymbiotic event.

a. Prasinophytes

Ostreococcus tauri (Prasinophyceae) is unusually small (1 μm) and has a very compact genome size (~12 Mb). Typical for this group *Ostreococcus tauri* contains Prasinophyte-specific light-harvesting proteins, which bind Chl *a*, Chl *b*, and a Chl *c*-like pigment (Mg-2,4-divinyl-pheoporphyrin a_5 monomethyl ester) (Six et al., 2005). There is great interest in Prasinophyte*s*, because this is the group that split early from the lineage that led to higher plants. Nucleus (Derelle et al., 2006), mitochondrion and chloroplast (Robbens et al., 2007) genomes of *Ostreococcus tauri* have been completely sequenced.

b. *Chlorophytes*

The most studied green alga is *Chlamydomonas reinhardtii* (Chlorophyceae) (Rochaix et al., 1998), which also has completely sequenced nuclear (Merchant et al., 2007) mitochondrial and chloroplast genomes. This alga can grow heterotrophically with acetate as carbon source and has a fast doubling time, as short as 6 h. Transformation of the nucleus and the single chloroplast, tetrad analysis and a great body of biochemical protocols are available (Harris, 1989). *Chlorella vulgaris* and *Chlorella pyrenoidosa* (Chlorophyceae) are similar to *Chlamydomonas reinhardtii*, but feature multiple, smaller chloroplasts. *Chlorella pyrenoidosa* was extensively used by Robert Emerson and Otto Warburg in their disagreement over the quantum requirement for oxygen evolution during some of the pioneering studies of oxygenic photosynthesis (reviewed by Govindjee, 1999). Another green alga, *Haematococcus pluvialis* (Chlorophyceae), is a model organism for the investigation of carotenoid biogenesis. *Volvox carteri* (Chlorophyceae) forms spheres that can contain several thousand cells and is a model for multicellularity and intercellular communication.

c. Bryophytes

The Bryophytes contain the liverworts, hornworts, and mosses. It is thought that this organism group maintained features characteristic for the transition of plants from water to land. Such features are the emergence of roots and vascular tissue that is needed for water transport, as well as the establishment of guard cells in hornworts and mosses. In Bryophytes, the haploid gametophyte is the dominating and photosynthetic life form. *Physcomitrella patens* and *Ceratodon purpureus* are two mosses that have become model organisms for this group and are used to explore plant development, cell polarity and secondary metabolism and also have the potential to be a valuable system for studying photosynthesis (Thornton et al., 2005). A sequencing project for *Physcomitrella patens* has been completed (Rensing et al., 2008) and the genetic manipulation of the nuclear genome has been demonstrated (Strepp et al., 1998). Limited genetic tools have been developed for *Ceratodon purpureus*, which unlike *Physcomitrella patens*, can grow heterotrophically on glucose (Thornton et al., 2005). *Marchantia polymorpha* is the most often used liverwort for photosynthetic studies.

d. Pterophytes

In ferns (Pterophytes) the diploid sporophyte is the dominant photosynthetic life form, which features many characteristics that are typical for land plants. Ferns possess vascular tissue and roots. The photosynthetic tissue is highly differentiated and surrounded by a layer of non-photosynthetic epidermal cells. Under the epidermis is a layer of tightly packed cells, the palisade layer, which is followed by a space that possesses less tightly packed photosynthetic cells. Through this spongy tissue, CO_2 can reach the inside of the leaf. To avoid water loss, access between leaf interior and the atmosphere is controlled by pore forming guard cells. These guard cells are located on the lower side of a leaf within the epidermis.

The investigation of photosynthesis in ferns has not focused on a few model organisms. However, the tropical homosporous fern *Ceratopteris richardii* has a model status (Stout et al., 2003) for the investigation of vascular plant development. A body of photosynthetic research exists on the aquatic fern *Azolla*

microphylla in connection with its symbiosis with *Nostoc* species (Sinha and Häder, 1996).

e. Spermatophytes

Seed plants (Spermatophytes) are of the greatest importance for the production of food and energy. Depending on the fertilization pattern, gymnosperms (Cycads, Ginkgo, Gnetophytes and conifers) and angiosperms (flowering plants) can be distinguished. In Spermatophytes, as in Pterophytes, the dominant photosynthetic life form is diploid. Higher plants possess the same leaf morphologies described for the Pterophytes and an even better developed root system.

The gymnosperm pine (e.g., *Pinus sylvestris*, *Pinus nigra*) and spruce *Picea abies* are of great importance for forestry and objects of extensive photosynthesis research. Pines and spruce, like most other conifers, have the photosynthetic tissue organized in needles.

Arabidopisis thaliana (Brassicaceae) has developed into the most prominent angiosperm model organism. In addition to photosynthesis research, *Arabidopsis thaliana* is a model organism for plant development, and general plant physiology. *Arabidopsis thaliana* was selected as the first angiosperm for a genome project. This plant features a small number of chromosomes (haploid 5), a compact genome (120 Mb), and a short generation cycle of 60–70 days.

Spinach (*Spinacia oleracea*, Chenopodiaceae), pea (*Pisum sativum*, Fabaceae) and tobacco (*Nicotiana tabacum*, Solanaceae) are angiosperms that are usually harvested to obtain large amounts of photosynthetic material. For these plants many protocols for chloroplast isolation and the isolation of different photosynthetic complexes have been developed. Virtually all "photosynthetic" proteins in these plants are characterized on a genetic and biochemical level. Pea was used to obtain the crystal structure of PS I (Ben-Shem et al., 2003). The genome of *Medicago truncatula* (Fabaceae), also called barrel clover, has been sequenced (http://www.medicago.org/genome/) and might complement or to some extent replace pea as a photosynthetic model system.

The cottonwood (*Populus trichocarpa*, Populacae) is a common, rapidly growing tree species. The importance for forestry and the availability of genetic tools contributed in the selection of cottonwood for the first complete tree genome project (Tuskan et al., 2006).

Grasses are some of the most important crop plants. Rice (*Oryza sativa*, Poacea) uses the reductive pentose phosphate pathway (Calvin-Benson cycle) for CO_2 fixation. Maize (*Zea mays*, Poaceae) is a C4 plant which uses an enzyme with higher affinity for CO_2 for a prefixation step, and then refixes the CO_2 using the reductive pentose phosphate pathway cycle (see Chapter 26). Maize is a model organism for this mode of carbon fixation (C4 carbon fixation) and the accompanying differentiation of photosynthetic tissue known as "Kranzanatomie." Maize and rice have sequenced genomes (http://www.maizesequence.org/index.html, http://rice.plantbiology.msu.edu/).

Kalanchoe species (Chlorarachniophytes) and the common ice plant *Mesembryanthemum crystallinum* (Aizoaceae) are model plants for the investigation of CAM photosynthesis. The halophytic ice plant has become a model system to investigate mechanisms of salinity resistance. The CAM plant with most agricultural interest and detailed photosynthetic and physiological work is the pineapple (*Ananas comosus*, Bromeliaciae).

f. Chlorarachniophytes

Chlorarachniophytes are unicellular amoebae that contain a chloroplast that is surrounded by four membranes indicative of a secondary endosymbiosis. The cells also contain a nucleomoroph, in the space framed by two membranes from the cytoplasm, and two membranes from the chloroplast stroma. This space is the equivalent of the cytoplasm of the primary endosymbiont. *Chlorarachnion globosum* is the most investigated organism in this group.

g. Euglenophytes

Euglenophytes are unicellular algae that arose through secondary endosymbiosis of a green alga-like organism. Most Euglenophytes live in fresh water. This organism group is able to synthesize astaxanthan, antheraxanthan, diadinoxanthan and neoxanthan. Many Euglenophytes live photoheterotrophically. *Euglena gracilis* is the most investigated organism in this group and has a sequenced genome. Interestingly, *Euglena gracilis* is able to live without a chloroplast, which

can be permanently removed (Krajcovic et al., 1989) by treatment with specific reagents and photobleaching.

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References

Adachi K, Oiwa K, Nishizaka T, Furuike S, Noji H, Itoh H, Yoshida M and Kinosita K Jr (2007) Coupling of rotation and catalysis in F1-ATPase revealed by single-molecule imaging and manipulation. Cell 130: 309–321.

Allen JF (2003) Why chloroplasts and mitochondria contain genomes? Comp Funct Genom 4: 31–36

Allen JF (2004) Cytochrome b_6f: structure for signalling and vectorial metabolism. Trends Plant Sci 9: 130–137

Allen JF (2005) A redox switch hypothesis for the origin of two light reactions in photosynthesis. FEBS Lett 579: 963–968

Allen JF and Martin W (2007) Evolutionary biology: out of thin air. Nature 445: 610–612

Archibald JM (2007) Nucleomorph genomes: Structure, function, origin and evolution. Bioessays 29: 392–402

Archibald JM and Keeling (2002). Recycled plastids: a green movement in eukaryotic evolution. Trends Genet 18: 577–584

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kröger N, Lau WW, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsar DS (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306: 79–86

Baird AH, Bhagooli R, Ralph PJ and Takahashi S (2008) Coral bleaching: the role of the host. Trends Ecol Evol 24: 16–20

Barz WP, Vermeglio A, Francia F, Venturoli G, Melandri BA and Oesterhelt D (1995) Role of the PufX protein in photosynthetic growth of *Rhodobacter sphaeroides*. 2. PufX is required for the efficient ubiquinone/ubiquinol exchange between the reaction center Q_B site and the cytochrome bc_1 complex. Biochemistry 34: 15248–15258

Beatty JT, Overmann J, Lince MT, Manske AK, Lang AS, Blankenship RE, Van Dover CL, Martinson TA, and Plumley FG (2005) An obligately photosynthetic bacterial anaerobe from a deepsea hydrothermal vent. Proc Natl Acad Sci USA 102: 9306–9310

Béjà O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN and DeLong EF (2000) Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. Science 289: 1902–1906

Bekker A, Holland HD, Wang PL, Rumble D 3rd, Stein HJ, Hannah JL, Coetzee LL and Beukes NJ (2004) Dating the rise of atmospheric oxygen. Nature 427: 117–120

Ben-Shem A, Frolow F and Nelson N (2003) Crystal structure of plant photosystem I. Nature: 426: 630–635

Berg IA, Kockelkorn D, Buckel W and Fuchs G (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Science 318: 1782–1786

Bhattacharya D and Medlin L (1995) The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions. J Phycol 31: 489–498

Bhattacharya D, Yoon HS and Hackett JD (2004) Photosynthetic eukaryotes unite: endosymbiosis connects the dots. Bioessays 26: 50–60

Bibby TS, Nield J and Barber J (2001) Iron deficiency induces the formation of an antenna ring around trimeric photosystem I in cyanobacteria. Nature 412: 743–745

Björn LO and Govindjee (2009) The evolution of photosynthesis and chloroplasts. Curr Sci 96: 1466–1474

Black CC and Osmond CB (2003) Crassulacean acid metabolism photosynthesis: working the night shift'. Photosynth Res 76: 329–341

Blair JE, Shah P and Hedges SB (2005) Evolutionary sequence analysis of complete eukaryote genomes. BMC Bioinformatics 6: 53–56

Blankenship RE (1992) Origin and early evolution of photosynthesis. Photosynth Res 51: 91–111

Blankenship RE (2002) Molecular Mechanisms of Photosynthesis, Blackwell, London

Blankenship RE and Matsuura K (2003) Antenna complexes from green photosynthetic bacteria. In: Green BR and Parson WW (eds) Light Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 195–217. Kluwer Academic Publishing, Dordrecht

Blankenship RE, Miller M and Olson JM (1995) Antenna complexes from green photosynthetic bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, Advances in

Photosynthesis, Vol 2, pp 399–435. Kluwer Academic Publishing, Dordrecht

Boekema EJ, Hifney A, Yakushevska AE, Piotrowski M, Keegstra W, Berry S, Michel K-P, Pistorius EK and Kruip J (2001) A giant chlorophyll-protein complex induced by iron deficiency in cyanobacteria. Nature 412: 745–748

Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K, Kuo A, Maheswari U, Martens C, Maumus F, Otillar RP, Rayko E, Salamov A, Vandepoele K, Beszteri B, Gruber A, Heijde M, Katinka M, Mock T, Valentin K, Verret F, Berges JA, Brownlee C, Cadoret JP, Chiovitti A, Choi CJ, Coesel S, De Martino A, Detter JC, Durkin C, Falciatore A, Fournet J, Haruta M, Huysman MJJf, Jenkins B, Jiroutova K, Jorgensen RE, Joubert Y, Kaplan A, Kroger N, Kroth PG, La Roche J, Lindquist E, Lommer M, Martin-Jezequel V, Lopez PJ, Lucas S, Mangogna M, McGinnis K, Medlin LK, Montsant A, Secq M-PO-L, Napoli C, Obornik M, Parker MS, Petit J-L, Porcel BM, Poulsen N, Robison M, Rychlewski L, Rynearson TA, Schmutz J, Shapiro H, Siaut M, Stanley M, Sussman MR, Taylor AR, Vardi A, von Dassow P, Vyverman W, Willis A, Wyrwicz LS, Rokhsar DS, Weissenbach J, Armbrust EV, Green BR, Van De Peer Y and Grigoriev IV (2008) The Phaeodactylum genome reveals the evolutionary history of diatom genomes. Nature 456: 239–244

Briggs LM, Pecoraro VL and McIntosh L (1990) Copper-induced expression, cloning, and regulatory studies of the plastocyanin gene from the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Mol Biol 15: 633–642

Bryant DA, Garcia Costas AM, Maresca JA, Gomez Maqueo Chew A, Klatt CG, Bateson MM, Tallon LJ, Hostetler J, Nelson WC, Heidelberg JF and Ward DM (2007) *Candidatus* Chloracidobacterium thermophilum: An aerobic phototrophic acidobacterium. Science 317: 523–526

Buchanan BB and Arnon DI (1990) A reverse Krebs cycle in photosynthesis: consensus at last. Photosynth Res 24: 47–53

Bullerjahn GS and Post AF (1993) The prochlorophytes: are they more than just chlorophyll *a/b*-containing cyanobacteria? Crit Rev Microbiol 19: 43–59

Burger G, Saint-Louis D, Gray MW and Lang BF (1999) Complete sequence of the mitochondrial DNA of the red alga *Porphyra purpurea*. Cyanobacterial introns and shared ancestry of red and green algae. Plant Cell 11: 1675–1694

Burger-Wiersma T, Veenhuis M, Korthals HJ, Van de Wiel CCM and Mur LR (1986) A new prokaryote containing chlorophylls *a* and *b*. Nature 320: 262–264

Calvin M and Benson AA (1948) The path of carbon in photosynthesis. Science 107: 476-480

Cardona T, Sedoud A, Cox N and Rutherford AW (2011) Charge separation in photosystem II: A comparative and evolutionary overview. Biochim Biophys Acta, doi: 10.1016/j.bbabio.2011.07.012

Cavalier-Smith T (2006) Origin of mitochondria by intracellular enslavement of a photosynthetic purple bacterium. Proc Royal Soc B 273: 1943–1952

Chapman DJ (1966) The pigments of the symbiotic algae (cyanomes) of *Cyanophora paradoxa* and *Glaucocystis ostochinearum* and two Rhodophyceae, *Porphyridium aerugineum* and *Asterocytis ramosa*. Arch Mikrobiol 55: 17–25

Chen M, Telfer A, Lin S, Pascal A, Larkum AW, Barber J and Blankenship RE (2005) The nature of the photosystem II reaction centre in the chlorophyll *d*-containing prokaryote, *Acaryochloris marina*. Photochem Photobiol Sci 4:1060–1064

Chen M, Zhang Y and Blankenship RE (2008) Nomenclature for membrane-bound light-harvesting complexes of cyanobacteria. Photosynth Res 95: 147–54

Chisholm SW, Olson RJ, Zettler ER, Goericke R, Waterbury JB and Welschmeyer NA (1988) A novel free-living Prochlorophyte abundant in the oceanic euphotic zone. Nature 334: 340–343

Chivian D, Brodie EL, Alm EJ, Culley DE, Dehal PS, Desantis TZ, Gihring TM, Lapidus A, Lin LH, Lowry SR, Moser DP, Richardson PM, Southam G, Wanger G, Pratt LM, Andersen GL, Hazen TC, Brockman FJ, Arkin AP and Onstott TC (2008) Environmental genomics reveals a single-species ecosystem deep within Earth. Science 322: 275–278

Cogdell RJ, Gardiner AT, Roszak AW, Law CJ, Southall J and Isaacs NW (2004) Rings, ellipses and horseshoes: how purple bacteria harvest solar energy. Photosynth Res 81: 207–214

Crofts AR (2004) Proton-coupled electron transfer at the Q_0-site of the bc_1 complex controls the rate of ubihydroquinone oxidation. Biochim Biophys Acta 1655: 77–92

Cushman JC (2001) Crassulacean acid metabolism. A plastic photosynthetic adaptation to arid environments. Plant Physiol 127: 1439–1448

Deisenhofer J, Epp O, Miki K, Huber R and Michel H (1984) X-ray structure analysis of a membrane protein complex. Electron density map at 3 Å resolution and a model of the chromophores of the photosynthetic reaction center from *Rhodopseudomonas viridis*. J Mol Biol 180: 385–398

Derelle E, Ferraz C, Rombauts S, Rouze P, Worden AZ, Robbens S, Partensky F, Degroeve S, Echeynie S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C, Panaud O, Piegu B, Ball SG, Ral J-P, Bouget F-Y, Piganeau G, De Baets B, Picard A, Delseny M, Demaille J, Van De Peer Y and Moreau H (2006) Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc Natl Acad Sci USA 103: 11647–11652

Dolganov NA, Bhaya D and Grossman AR (1995) Cyanobacterial protein with similarity to the chlorophyll *a/b*-binding proteins of higher plants: Evolution and regulation. Proc Natl Acad Sci USA 92: 636–640

Douglas S, Zauner S, Fraunholz M, Beaton M, Penny S, Deng LT, Wu X, Reith M, Cavalier-Smith T and Maier UG (2001) The highly reduced genome of an enslaved algal nucleus. Nature 410: 1091–1096

Eaton-Rye JJ and Putnam-Evans C (2005) The CP47 and CP43 core antenna components. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 45–70. Springer, Dordrecht

Eisen JA, Nelson KE, Paulsen IT, Heidelberg JF, Wu M, Dodson RJ, Deboy R, Gwinn ML, Nelson WC, Haft DH, Hickey EK, Peterson JD, Durkin AS, Kolonay JL, Yang F, Holt I, Umayam LA, Mason T, Brenner M, Shea TP, Parksey D, Nierman WC, Feldblyum TV, Hansen CL, Craven MB, Radune D, Vamathevan J, Khouri H, White O, Gruber TM, Ketchum KA, Venter JC, Tettelin H, Bryant DA and Fraser CM (2002) The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc Natl Acad Sci USA 99: 9509–9514

Eisenreich W, Strauss G, Werz U, Fuchs G and Bacher A (1993) Retrobiosynthetic analysis of carbon fixation in the phototrophic eubacterium *Chloroflexus aurantiacus*. Eur J Biochem 215: 619–32

Evans MC, Buchanan BB and Arnon DI (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. Proc Natl Acad Sci USA 55: 928–34

Evrard JL, Johnson C, Janssen I, Löffelhardt W, Weil JH and Kuntz M (1990) The cyanelle genome of *Cyanophora paradoxa*, unlike the chloroplast genome, codes for the ribosomal L3 protein. Nucleic Acids Res 18: 1115–1119

Fairclough WV, Forsyth A, Evans MCW, Rigby SEJ, Purton S and Heathcote P (2003) Bidirectional electron transfer in photosystem I: electron transfer on the PsaA side is not essential for phototrophic growth in *Chlamydomonas*. Biochim Biophys Acta 1606: 43–55

Falkowski PG and Raven JA (2007) Aquatic Photosynthesis, 2nd edition, Princeton University Press, Princeton

Fay P (1992) Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56: 340–364

Fenna RE and Matthews BW (1975) Chlorophyll arrangement in a bacteriochlorophyll protein from *Chlorobium limicola*. Nature 258: 573–577

Field CB, Behrenfeld MJ, Randerson JT and Falkowski PG (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281: 237–240

Frigaard N-U, Martinez A, Mincer TJ and DeLong EF (2006) Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. Nature: 439: 847–850

Funk C and Vermaas WFJ (1999) Expression of cyanobacterial genes coding for single-helix polypeptides resembling regions of light-harvesting proteins from higher plants. Biochemistry 38: 9397–9404

Gantt E (1996) Pigment protein complexes and the concept of the photosynthetic unit: chlorophyll complexes and phycobilisomes. Photosynth Res 48: 47–53

Gantt E, Edwards MR and Provasoli L (1971) Chloroplast structure of the Cryptophyceae. Evidence for phycobiliproteins within intrathylakoidal spaces. J Cell Biol 48: 280–290

Garczarek L, Poupon A and Partensky F (2003) Origin and evolution of transmembrane Chl-binding proteins: hydrophobic cluster analysis suggests a common one-helix ancestor for prokaryotic (Pcb) and eukaryotic (LHC) antenna protein superfamilies. FEMS Microbiol Lett 222: 59–68

Gest H (1994) Discovery of the heliobacteria. Photosynth Res 41: 17–21

Gest H (2002) History of the word photosynthesis and evolution of its definition. Photosynth Res 73: 7–10

Gest H and Favinger JL (1983) *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a "new" form of bacteriochlorophyll. Arch Microbiol 136: 11–16

Gillot MA and Gibbs SP (1980) The cryptomonad nucleomorph: its ultrastructure and evolutionary significance. Phycology 16: 558–568

Golbeck JH (ed) (2006) Photosystem I: The Light-Driven Plastocyanin:Ferredoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24. Springer, Dordrecht

Gómez-Consarnau L, Gonzalez JM, Coll-Lladó M, Gourdon P, Pascher T, Neutze R, Pedrós-Alió C and Pinhassi J (2007) Light stimulates growth of proteorhodopsin-containing marine Flavobacteria. Nature 445: 210–213

Govindjee (1999) On the requirement of minimum number of four versus eight quanta of light for the evolution of one molecule of oxygen in photosynthesis: A historical note. Photosynth Res 59: 249–254

Green BJ, Li W-Y, Manhart JR, Fox TC, Summer EJ, Kennedy RA, Pierce SK and Rumpho ME (2000) Mollusc-algal chloroplast endosymbiosis. Photosynthesis, thylakoid protein maintenance, and chloroplast gene expression continue for many months in the absence of the algal nucleus. Plant Physiol 124: 331–342

Green BR (2001) Was "molecular opportunism" a factor in the evolution of different photosynthetic light-harvesting pigment systems? Proc Natl Acad Sci USA 98: 2119–2121

Green BR (2003) The evolution of light-harvesting antennas. In: Green BR and Parson WW (eds) Light-harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 129–168. Kluwer Academic Publishers, Dordrecht

Green BR and Durnford D (1996) Chlorophyll-carotenoid proteins in oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 47: 685–714

Green BR, Anderson JM and Parson WW (2003) Photosynthetic membranes and their light harvesting antennas. In: Green BR and Parson WW (eds) Light Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 1–28. Kluwer Academic Publishers, Dordrecht

Guergova-Kuras M, Boudreaux B, Joliot A, Joliot P and Redding K (2001) Evidence for two active branches for electron transfer in photosystem I. Proc Natl Acad Sci USA 98: 4437–4442

Hackett JD, Anderson DM, Erdner DL and Bhattacharya D (2004) Dinoflagellates: A remarkable evolutionary experiment. Am J Bot 91: 1523–1534

Hager-Braun C, Jarosch U, Hauska G, Nitschke W and Riedel A (1997) EPR studies of the terminal electron acceptors of the green sulfur bacterial reaction center. Revisited. Photosynth Res 51: 127–36

Hagopian JC, Reis M, Kitajima JP, Bhattacharya D and De Oliveira MC (2004) Comparative analysis of the complete plastid genome sequence of the red alga *Gracilaria tenuistipitata* var. liui provides insights into the evolution of rhodoplasts and their relationship to other plastids. J Mol Evol 59: 464–477

Hannaert V, Saavedra E, Duffieux F, Szikora JP, Rigden DJ, Michels PA and Opperdoes FR (2003) Plant-like traits associated with metabolism of Trypanosoma parasites. Proc Natl Acad Sci USA 100: 1067–1071

Harris EH (ed) (1989) The *Chlamydomonas* Sourcebook. A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego

Haselkorn R, Lapidus A, Kogan Y, Vlcek C, Paces J, Paces V, Ulbrich P, Pecenkova T, Rebrekov D, Milgram A, Mazur M, Cox R, Kyrpides N, Ivanova N, Kapatral V, Los T, Lykidis A, Mikhailova N, Reznik G, Vasieva O and Fonstein M (2001) The *Rhodobacter capsulatus* genome. Photosynth Res 70: 43–45

Hatch MD (1987) C4 photosynthesis – a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895: 81–106

Hauska G, Schoedl T, Remigy H and Tsiotis G (2001) The reaction center of green sulfur bacteria. Biochim Biophys Acta 1507: 260–277

Heddad M and Adamska I (2002) The evolution of light stress proteins in photosynthetic organisms. Comp Funct Genomics 3: 504–510

Hess WR, Partensky F, Van Der Staay GWM, Garcia-Fernandez JM, Borner T and Vaulot D (1996) Coexistence of phycoerythrin and a chlorophyll *a/b* antenna in a marine prokaryote. Proc Natl Acad Sci USA 93: 11126–11130

Hess WR, Rocap G, Ting CS, Larimer F, Stilwagen S, Lamerdin J and Chisholm SW (2001) The photosynthetic apparatus of *Prochlorococcus*: Insights through comparative genomics. Photosynth Res 70: 53–71

Heyne B (1815) On the deoxidation of the leaves of *Coltyledon calycina*. Trans Linn Soc Lond 11: 213–215

Hiller RG, Anderson JM and Larkum AWD (1991) The chlorophyll-protein complexes of algae. In: Scheer H (ed) Chlorophylls, pp 529–547. CRC Press, Baton Rouge

Hofmann E, Wrench PM, Sharples FP, Hiller RG, Welte W and Diederichs K. (1996) Structural basis of light harvesting by carotenoids: peridinin-chlorophyll-protein from *Amphidinium carterae*. Science 272: 1788–1791

Hohmann-Marriott MF and Blankenship RE (2008) Anoxygenic type I photosystems and evolution of photosynthetic reaction centers. In: Fromme P (ed) Photosynthetic Protein Complexes: A Structural Approach, pp 295–324 Wiley-VCH, Hoboken

Hohmann-Marriott MF and Blankenship RE (2011) Evolution of photosynthesis. Annu Rev Plant Biol 62: 515–548

Howe CJ, Nisbet RER and Barbrook AC (2008) The remarkable chloroplast genome of dinoflagellates. J Exp Bot 59: 1035–1045

Hunter CN, Daldal F, Thurnauer MC and Beatty JT (eds) (2008) The Purple Phototrophic Bacteria, Advances in Photosynthesis and Respiration, Vol 28, Springer, Dordrecht

Inaba T and Schnell DJ (2008) Protein trafficking to plastids: one theme, many variations. Biochem J 413: 15–28

Iwasaki H and Kondo T (2004) Circadian timing mechanism in the prokaryotic clock system of cyanobacteria. J Biol Rhythms 19: 436–444

Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Türbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D and Beck E (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science 285: 1573–1576

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W and Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. Nature 411: 909–917

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A and Nakazaki N (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3: 109–136

Kaneko T, Nakamura Y, Wolk CP, Kuritz T, Sasamoto S, Watanabe A, Iriguchi M, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kohara M, Matsumoto M, Matsuno A, Muraki A, Nakazaki N, Shimpo S, Sugimoto M, Takazawa M, Yamada M, Yasuda M, and Tabata S (2001) Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res 8: 205–213

Kjaer B, Frigaard NU, Yang F, Zybailov B, Miller M, Golbeck JH and Scheller HV (1998) Menaquinone-7 in the reaction center complex of the green sulfur bacterium *Chlorobium vibrioforme* functions as the electron acceptor A_1. Biochemistry 37: 3237–3242

Kleinherenbrink FAM, Ikegami I, Hiraishi A, Otte SCM and Amesz J (1993) Extraction of menaquinone does not change electron transport considerably. Biochim Biophys Acta 1142: 69–73

Knoll AH (2003) The geological consequences of evolution. Geobiology 1: 3–14

Kott P, Parry DL and Cox GC. (1984) Prokaryotic symbionts with a range of ascidian hosts. Bull Mar Sci 34: 308–312

Koziol AG, Borza T, Ishida K-I, Keeling P, Lee RW and Durnford DG (2007) Tracing the evolution of the light-harvesting antennae in chlorophyll *a/b*-containing organisms. Plant Physiol 143: 1802–1816

Krajcovic J, Ebringer L, Polónyi J (1989) Quinolones and coumarins eliminate chloroplasts from *Euglena gracilis*. Antimicrob Agents Chemother 33: 1883–1889

Larkum T (1996) How dinoflagellates make light work with peridinin. Trends Plant Sci 1: 247–248

Larkum AWD, Douglas SE and Raven JA (2003) Photosynthesis in Algae, Advances in Photosynthesis and Respiration, Vol. 14, Kluwer Academic Publishers, Dordrecht

Larkum AWD, Lockhart PJ and Howe CJ (2007) Shopping for plastids. Trends Plant Sci 12: 189–195

La Roche J, Van der Staay GW, Partensky F, Ducret A, Aebersold R, Li R, Golden SS, Hiller RG, Wrench PM, Larkum AWD and Green BR (1996) Independent evolution of the prochlorophyte and green plant chlorophyll *a*/*b* light-harvesting proteins. Proc Natl Acad Sci USA 93: 15244–15248

Lawlor DW (2000), Photosynthesis, 3rd edition, Springer, New York

Lewin RA (1976) Prochlorophyta as a proposed new division of algae. Nature 261: 697–698

Lewin RA (2002) Prochlorophyta – a matter of class distinctions. Photosynth Res 73: 59–61

Lewin RA and Cheng L (1975) Associations of microscopic algae with didemnid ascidians. Phycologia 14: 149–152

Li S, Nosenko T, Hackett JD and Bhattacharya D (2006) Phylogenomic analysis identifies red algal genes of endosymbiotic origin in the Chromalveolates. Mol Biol Evol 23: 663–674

MacColl R (1998) Cyanobacterial phycobilisomes. J Struct Biol 124: 311–334

Mackey SR and Golden SS (2007) Winding up the cyanobacterial circadian clock. Trends Microbiol 15: 381–388

Margulis L (1970) Origin of Eukaryotic Cells. Yale University Press, New Haven

Martin W and Kowallik KV (1999) Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Europ J Phycol 34: 287–295

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M and Penny D (2002) *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid evolutionary analysis of phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci USA 99: 12246–12251

Mathis P (1990) Compared structure of plant and bacterial photosynthetic reaction centers. Evolutionary implications. Biochim Biophys Acta 1018: 163–167

McFadden GI (2001) Primary and secondary endosymbiosis and the origin of plastids. J Phycol 37: 951–959

McFadden GI and Roos DS (1999) Apicomplexan plastids as drug targets. Trends Microbiol 6: 328–333

McFadden GI and Van Dooren GG (2004) Evolution: red algal genome affirms a common origin of all plastids. Curr Biol 14: R514–R516

Meeks JC, Elhai J, Theil T, Potts M, Larimer F, Lameridin J, Predki P and Atlas R (2001) An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth Res 70: 85–106

Merchant S and Bogorad L (1987) Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552. EMBO J 6: 2531–2535

Merchant S, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L, Marshall WF, Qu L-H, Nelson DR, Sanderfoot AA, Spalding MH, Kapitonov VV, Ren Q, Ferris P, Lindquist E, Shapiro H, Grimwood J, Schmutz J, Lucas S, Chlamydomonas Community Annotation Team, JGI Annotation Team, Grigoriev IV, Rokhsar DS and Grossman AR (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318: 245–250

Mereschkowsky C (1905) Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biol Centralbl 25: 593–604

Meyer A (1883) Über Krystalloide der Trophoplasten and über die Chromoplasten der Angiospermen. Bot Zeit 39: 841–846, 857–864

Minoda A, Sakagami R, Yagisawa F, Kuroiwa T and Tanaka K (2004) Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, *Cyanidioschyzon merolae* 10D. Plant Cell Physiol 45: 667–671

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 191: 144–148

Mitchell P (1970) Aspects of the chemiosmotic hypothesis. Biochem J 116: 5P–6P

Miyagishima S-Y, Nishida K and Kuroiwa T (2003) An evolutionary puzzle: chloroplast and mitochondrial division rings. Trends Plant Sci: 8: 432–438

Miyashita H, Ikemoto H, Kurano N, Adachi K, Chihara M and Miyachi S (1996) Chlorophyll *d* as a major pigment. Nature 383: 402

Montaño GA, Bowen BP, LaBelle JT, Woodbury NW, Pizziconi VB and Blankenship RE (2003) Characterization of *Chlorobium tepidum* chlorosomes: A calculation of bacteriochlorophyll *c* per chlorosome and oligomer modeling. Biophys J 85: 2560–2565

Moustafa A, Beszteri B, Maier UG, Bowler C, Valentin K and Bhattacharya D (2009) Genomic footprints of a cryptic plastid endosymbiosis in diatoms. Science 324: 1724–1726

Muhiuddin IP, Heathcote P, Carter S, Purton S, Rigby SEJ and Evans MCW (2001) Evidence from timeww resolved studies of the $P700^+/A_1$ radical pair for photosynthetic

electron transfer on both the PsaA and PsaB branches of the Photosystem I reaction centre. FEBS Lett 503: 56–60

Mullineaux CW (2005) Function and evolution of grana. Trends Plant Sci 10: 521–525

Mullineaux CW (2008) Phycobilisome-reaction centre interaction in cyanobacteria. Photosynth Res 95: 175–182

Nagel G, Szellas T, Kateriya S, Adeishvili N, Hegemann P and Bamberg E (2005) Channel rhodopsins: directly light-gated cation channels. Biochem Soc Trans 33: 863–866

Oh-Oka H (2007) Type 1 reaction center of photosynthetic heliobacteria. Photochem Photobiol 83: 177–186

Olson JM (1970) Evolution of photosynthesis. Science 168: 438–446

Olson JM (2004) The FMO protein. Photosynth Res 80: 181–187

Olson JM and Pierson BK (1987a) Evolution of reaction centers in photosynthetic prokaryotes. Ann Rev Cytol 108: 209–248

Olson JM and Pierson BK (1987b) Origin and evolution of photosynthetic reaction centers. Orig Life 17: 419–430

Ormerod, JG, Kimble LK, Nesbakken T, Torgersen, YA, Woese CR and Madigan MT (1996) *Heliophilum fasciatum* gen. nov. sp. nov. and *Heliobacterium gestii* sp. nov.: endospore-forming heliobacteria from rice field soils. Arch Microbiol 165: 226–234

Overmann J and Tuschak C (1997) Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch Microbiol 167: 302–309

Peters, AF, Marie D, Scornet D, Kloareg B, Cock JM (2004) Proposal of *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) as a model organism for brown algal genetics and genomics. J Phycol 40: 1079–1088

Pfanzagl B, Allmaier G, Schmid ER, De Pedro MA, Löffelhardt W (1996) N-acetylputrescine as a characteristic constituent of cyanelle peptidoglycan in Glaucocystophyte algae. J Bacteriol 178: 6994–6997

Pierce SK, Biron RW and Rumpho ME (1996) Endosymbiotic chloroplasts in molluscan cells contain proteins synthesized after plastid capture. J Exp Biol 199: 2323–2330

Puerta MVS, Bachvaroff TR and Delwiche CF (2005) The complete plastid genome sequence of the haptophyte *Emiliania huxleyi*: A comparison to other plastid genomes. DNA Res 12: 151–156.

Ragsdale SW (1991) Enzymology of the acetyl-CoA pathway of autotrophic CO_2 fixation. Crit Rev Biochem Mol Biol 26: 261–300

Rai AN, Söderbäck E and Bergman B (2000) Cyanobacterium–plant symbioses. New Phytol 147: 449–481

Rambold, G, Friedl T and Beck A (1998) Photobionts in lichens: possible indicators of phylogenetic relationships? Bryologist 101: 392–397

Rasmussen U and Nilsson M (2002) Cyanobacterial diversity and specificity in plant symbiosis. In: Rai AN, Bergman B and Rasmussen U (eds) Cyanobacteria in Symbiosis, pp 313–328. Kluwer Academic Publishers, Dordrecht

Reith ME and Munholland J (1995) Complete nucleotide sequence of the *Porphyra purpurea* chloroplast genome. Plant Mol Biol 13: 333–335

Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud PF, Lindquist EA, Kamisugi Y, Tanahashi T, Sakakibara K, Fujita T, Oishi K, Shin-I T, Kuroki Y, Toyoda A, Suzuki Y, Hashimoto S, Yamaguchi K, Sugano S, Kohara Y, Fujiyama A, Anterola A, Aoki S, Ashton N, Barbazuk WB, Barker E, Bennetzen JL, Blankenship R, Cho SH, Dutcher SK, Estelle M, Fawcett JA, Gundlach H, Hanada K, Heyl A, Hicks KA, Hughes J, Lohr M, Mayer K, Melkozernov A, Murata T, Nelson DR, Pils B, Prigge M, Reiss B, Renner T, Rombauts S, Rushton PJ, Sanderfoot A, Schween G, Shiu SH, Stueber K, Theodoulou FL, Tu H, Van de Peer Y, Verrier PJ, Waters E, Wood A, Yang L, Cove D, Cuming AC, Hasebe M, Lucas S, Mishler BD, Reski R, Grigoriev IV, Quatrano RS and Boore JL. (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science 319: 64–69

Robbens S, Derelle E, Ferraz C, Wuyts J, Moreau H and Van De Peer Y (2007) The complete chloroplast and mitochondrial DNA sequence of *Ostreococcus tauri*: Organelle genomes of the smallest eukaryote are examples of compaction. Mol Biol Evol, 24: 956–968

Roberts DL, Zhao S, Doukov T and Ragsdale SW (1994) The reductive acetyl coenzyme A pathway: Sequence and heterologous expression of active methyltetrahydr ofolate:corrinoid/iron-sulfur protein methyltransferase from *Clostridium thermoaceticum*. J Bacteriol 176: 6127–6130

Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) (1998) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7. Kluwer Academic Publishers, Dordrecht

Rumpho ME, Summer EJ and Manhart JR (2000) Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis. Plant Physiol 123: 29–38

Rumpho ME, Dastoor FP, Manhart JR and Lee J (2006) The kleptoplast. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 451–473. Springer, Dordrecht

Sadekar S, Raymond J and Blankenship RE (2006) Conservation of distantly related membrane proteins: Photosynthetic reaction centers share a common structural core. Mol Biol Evol 23: 2001–2007

Sanchez Puerta MV, Bachvaroff TR and Delwiche CF (2004) The complete mitochondrial genome sequence of the haptophyte *Emiliania huxleyi* and its relation to heterokonts. DNA Res 11: 1–10

Sattley WM, Madigan MT, Swingley WD, Chen M, Cheung PC, Clocksin KM, Conrad AL, Dejesa LC, Honchak BM, Jung DO, Karbach LE, Kurdoglu A, Lahiri S,

Mastrian SD, Page LE, Taylor HL, Wang ZT, Raymond J, Blankenship RE and Touchman JW (2008) The genome of *Heliobacterium modesticaldum*, a phototrophic representative of the Firmicutes containing the simplest photosynthetic apparatus. J Bacteriol 190: 4687–4696

Schimper AFW (1883) Über die Entwicklung der Chlorophyllkörner and Farbkörper. Bot Zeit 41: 105–114, 121–131, 137–146, 153–162

Schliep M, Crossett B, Willows RD and Chen M (2010) ^{18}O labeling of chlorophyll *d* in *Acaryochloris marina* reveals that chlorophyll *a* and molecular oxygen are precursors. J Biol Chem 285: 28450–28456

Schmidt VB, Kies L and Weber A (1979) Die Pigmente von *Cyanophora paradoxa*, *Gloechaete wittrockiana* und *Glaucocystis nostochinearum*. Arch Protistenk 122: 164–170

Senior AE, Nadanaciva S and Weber J (2002) The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase. Biochim Biophys Acta 1553: 188–211

Sinha RP and Häder D-P (1996) Photobiology and ecophysiology of rice field cyanobacteria. Photochem Photobiol 64: 887–896

Six C, Worden AZ, Rodríguez F, Moreau H and Partensky F (2005) New insights into the nature and phylogeny of Prasinophyte antenna proteins: *Ostreococcus tauri*, a case study. Mol Biol Evol 22: 2217–2230

Steiner JM, and Löffelhardt W (2002) Protein import into cyanelles. Trends Plant Sci 7: 72–77

Stoppani AOM, Fuller RC and Calvin M (1955) Carbon dioxide fixation by *Rhodospeudomonas capsulatus*. J Bacteriol 69: 491–501

Stout SC, Clark GB, Archer-Evans S and Roux SJ (2003) Rapid and efficient suppression of gene expression in a single-cell model system, *Ceratopteris richardii*. Plant Physiol 131: 1165–1168

Stransky H and Hager A (1970) Das Carotinoidmuster und die Verbreitung des lichtinduzierten Xanthophyllcyclus in verschiedenen Algenklassen V. Einzelne Vertreter der Cryptophyceae, Euglenophyceae, Bacillariophyceae, Chrysophyceae und Phaeophyceae. Arch Microbiol 73: 77–89

Strauss G and Fuchs G (1993) Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle. Eur J Biochem 215: 633–643

Strepp R, Scholz S, Kruse S, Speth V and Reski R (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. Proc Natl Acad Sci USA 95: 4368–4373

Swingley WD, Sadekar S, Mastrian SD, Matthies HJ, Hao J, Ramos H, Acharya CR, Conrad AL, Taylor HL, Dejesa LC, Shah MK, O'huallachain ME, Lince MT, Blankenship RE, Beatty JT and Touchman JW (2007) The complete genome sequence of *Roseobacter denitrificans* reveals a mixotrophic rather than photosynthetic metabolism. J Bacteriol 89: 683–690

Swingley WD, Blankenship RE and Raymond J (2008) Integrating Markov clustering and molecular phylogenetics to reconstruct the cyanobacterial species tree from conserved protein families. Mol Biol Evol 25: 643–654

Tabita FR (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol Mol Biol Rev 52: 155–189

Thauer RK (2007) A fifth pathway of carbon fixation. Science 318: 1732–1733

Thornton LE, Keren N, Ohad I and Pakrasi HB (2005) *Physcomitrella patens* and *Ceratodon purpureus*, mosses as model organisms in photosynthesis studies. Photosynth Res 83: 87–96

Tomo T, Okubo T, Akimoto S, Yokono M, Miyashita H, Tsuchiya T, Noguchi T and Mimuro M (2007) Identification of the special pair of photosystem II in a chlorophyll *d*-dominated cyanobacterium. Proc Natl Acad Sci USA 104: 7283–7288

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Déjardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjärvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leplé JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouzé P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, Van de Peer Y and Rokhsar D (2006) The genome of black cottonwood *Populus trichocarpa* (Torr. & Gray). Science 313: 1596–1604

Umena Y, Kawakami K, Shen J-R and Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473: 55–60

Urbach E, Robertson DL and Chishholm SW (1992) Multiple evolutionary origins of Prochlorophytes within the cyanobacterial radiation. Nature 355: 267–270

Usher KM, Bergman B and Raven JA (2007) Exploring cyanobacterial mutualisms. Annu Rev Ecol Evol and Syst 38: 255–273

Waller RF and McFadden GI (2005) The apicoplast: A review of the derived plastid of apicomplexan parasites. Curr Issues Mol Biol 7: 57–80

Wedemayer GJ, Kidd DG and Glazer AN (1996) Cryptomonad biliproteins: Bilin types and locations. Photosynth Res 48: 163–170

West HH (1979) Chloroplast symbiosis and development of the ascoglossan opisthobranch *Elysia chlorotica*. PhD thesis, Northeastern University, Boston

Whitton BA and Potts M (eds) (2000) The Ecology of Cyanobacteria: Their Diversity in Time and Space. Kluwer Academic Press, Dordrecht

Xu H, Vavilin D, Funk C and Vermaas W (2002) Small CAB-like proteins regulating tetrapyrrole biosynthesis. Plant Mol Biol 49: 149–160

Xu W, Chitnis PR, Valieva A, Van der Est A, Pushkar YN, Krzystyniak M, Teutloff C, Zech SG, Bittl R, Stehlik D, Zybailov B, Shen G and Golbeck JH (2003a) Electron transfer in cyanobacterial photosystem I: I. Physiological and spectroscopic characterization of site-directed mutants in a putative electron transfer pathway from A_0 through A_1 to F_X. J Biol Chem 278: 27864–278675

Xu W, Chitnis PR, Valieva A, Van der Est A, Brettel K, Guergova-Kuras M, Pushkar Y N, Zech SG, Stehlik D, Shen G, Zybailov B and Golbeck JH (2003b) Electron transfer in cyanobacterial Photosystem I. II. Determination of forward electron transfer rates of site-directed mutants in a putative electron transfer pathway from A_0 through A_1 to F_X. J Biol Chem 27: 27876–27887

Yanyushin MF, del Rosario MC, Brune DC and Blankenship RE (2005) New class of bacterial membrane oxidoreductases. Biochemistry 44: 10037–10045

Yoon HS, Hackett JD, Ciniglia C, Pinto C and Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21: 809–818

Yoon HS, Hackett JD, Van Dolah FM, Nosenko T, Lidie KL and Bhattacharya D (2005) Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. Mol Biol Evol 22: 1299–1308

Zhang Z, Green BR and Cavalier-Smith T (1999) Single gene circles in dinoflagellate chloroplast genomes. Nature 400: 155–159

Zhao F and Qin S (2006) Evolutionary analysis of phycobiliproteins: Implications for their structural and functional relationships. J Mol Evol 63: 330–340

Zouni A, Witt HT, Kern J, Fromme P, Krauss N, Saenger W and Orth P (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. Nature 409: 739–743

Part II

Plastid Biochemistry and Physiology

Chapter 2

Origin, Evolution and Division of Plastids

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Summary

All living eukaryotic cells with mitochondria, and plastids if any, within their cytoplasm, are the result of two billion years of evolution. These organelles are the result of two distinct endosymbioses. The increase in oxygen in the atmosphere supports the origin for mitochondria about 2.2 billion years ago, an origin probably due to a single invasion of a host cell by an α-proteobacterium-like organism. Plastids originated between 1.6 and 0.6 billion years ago as a result of a symbiotic association between a cyanobacterium and a mitochondriate eukaryote. This endosymbiotic event generated the green, red and blue algal lineages, which subsequently spread their chloroplasts when the new photosynthetic eukaryotes were, in their turn, engulfed by nonphotosynthetic eukaryotes (between, 1.2 and 0.55 billion years ago) generating more algal divisions. These symbiotic events would have been in vain if the continuity of the newly acquired organelles had not been maintained. Since the first observations of chloroplast in the mid nineteenth century, progress made in microscopy techniques, during the first half of the twentieth century, demonstrated without ambiguity that this continuity is the result of division of pre-existing chloroplasts. Moreover, thanks to the completion of sequencing projects and the use of classical and reverse genetic approaches, it was then possible to show that the chloroplast division machinery is an evolutionary hybrid, which has retained the activity of several prokaryotically-derived proteins together with components that have evolved from proteins present in the eukaryotic ancestor.

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I. Introduction

It is very difficult to imagine how the Earth would have been without the presence of plastids. But, certainly Earth would have been very different from today's planet with no plant and animal life. There is evidence that chloroplasts were observed for the first time during the seventeenth century by Nehemiah Grew in England and Anthony van Leeuwenhoek in the Netherlands (for a more comprehensive overview on pioneer researchers on chloroplasts, see Gunning et al., 2006). In the mid nineteenth century two French microscopists, Jean Baptiste Arthur Gris (1857) and August Adolphe Lucien Trécul (1858), published drawings of chloroplasts termed vesicles. The term plastid was introduced later by Schimper (1883) defining a category of organelles of the plant cell. Schimper also classified the plastids into different groups (chloroplasts, leucoplasts and chromoplasts) and suggested that the latter arose from the transformation of the former. Concomitantly with the observations of chloroplasts, the concept of plastid continuity, stipulating that plastids do not arise de novo but from preexisting plastids was announced. This concept was proposed after extensive studies on the development of plastids, first observed in algae (Nägeli, 1846; Schmitz, 1883) and then extended to higher plants (Meyer, 1883; Schimper, 1883, 1885). But, undoubtedly, it was Constantin Mereschkowsky (1905), a Russian botanist, who published at the beginning of the twentieth century the more convincing demonstration of the endosymbiotic origin of chloroplasts. Mereschkowsky's demonstration was based upon three lines of evidence known at the time as: (1) the known principle of symbiosis, already stated to designate the coexistence of two distinct organisms; (2) the observations described above that chloroplasts divided and the subsequent notion of plastid continuity; and (3) the growing evidence, observed at the time, of a resemblance between cyanobacteria and plastids. After Mereschkowsky, the role of symbiosis in evolution was advocated by others (Portier, 1918; Wallin, 1927), but in the absence of more convincing arguments the idea was almost universally rejected for a long period (Lumière, 1919).

Meanwhile, studies on chloroplast structure progressed remarkably well during the next few decades due to the development of microscopy techniques. In 1935, the first photomicrographs of grana in chloroplasts observed by light microscopy were published (Doutreligne, 1935), and the structure of the grana described (Heitz, 1936). Five years later, the first observations of chloroplast, by electron microscopy, were reported (Kausche and Ruska, 1940; Menke, 1940). These were then followed by numerous observations revealing the internal structure of the different plastids (for a review: see Steahelin, 2003). The visualization of DNA within the chloroplast using electron microscopy was then reported (Ris and Plaut, 1962), followed 1 year later by a similar report concerning the observation of DNA within mitochondria (Nass and Nass, 1963). These reports, together with a detailed comparison between cyanobacteria and chloroplasts (Ris and Singh, 1961), led to the revival of the endosymbiotic theory; an elegant synthesis of this theory was published in 1967 by Lynn Margulis (Sagan, 1967). Following advances in microbiology, biochemistry and genetics, it was demonstrated, in the late 1960s and early 1970s, that chloroplasts (and mitochondria) had many other features consistent with an endosymbiotic origin (Kirk and Tilney-Bassett, 1978). Finally, with the completion of the *Arabidopsis thaliana* genome sequencing project, among the approximately 25,000 nuclear genes present in the genome, about 1,550 (Abdallah et al., 2000) to 4,500 (Martin et al., 2002) protein-coding genes were predicted to be of cyanobacterial origin. Thus, a large number of genes from the cyanobacterium-like predecessor of plastids were transferred to the nuclear genome in the course of eukaryotic genome evolution. Concomitantly with the rediscovery of the endosymbiotic theory, progress was made in understanding chloroplast division. Much of this relied on the observation of dividing chloroplasts and of ring structures at the division site. In the late 1980s and early 1990s, a genetic approach showed

Abbreviations: ARC – Accumulation and replication of chloroplasts; BDLP – Bacterial dynamin-like protein; CFP – Cyan fluorescent protein; DRP – Dynamin related protein; Fts – Filamentous temperature sensitive; LCA – Last common ancestor; LGT – Lateral gene transfer; LUCA – Last universal common ancestor; MORN – Membrane occupation and recognition nexus; PD ring – Plastid division ring; TEM – Transmission electron microscopy; YFP – Yellow fluorescent protein

that plastid division is under nuclear control. Finally, in 1995, sequence analyses led to the identification of the first gene encoding a plastid division protein (Osteryoung and Vierling, 1995).

Since the mechanisms involved in plastid division reflect the complex origin of this organelle, I have attempted, in this chapter, to provide an overview of chloroplast origin, evolution and division mechanisms. I have also tried to place the progress made in the understanding of these mechanisms in their historic and scientific context. A number of excellent reviews address some of the different aspects presented here and are listed in the text.

II. Plastid Origin

Geological evidence shows that the 'true consensus for life's existence' dates from more than 3.5 billion years ago (Ga) (Nisbet and Sleep, 2001; Schopf, 2006) and could coincide in time with the end of the late heavy bombardment occurring at the end of the earlier Archaean period (Forterre and Gribaldo, 2007) (Fig. 2.1). It is widely accepted that the last common ancestor (LCA, also named the last universal common ancestor or LUCA, or cenancestor) of all extant organisms must exist. However, the nature of the LCA, whether it was a discrete entity or a diverse community of

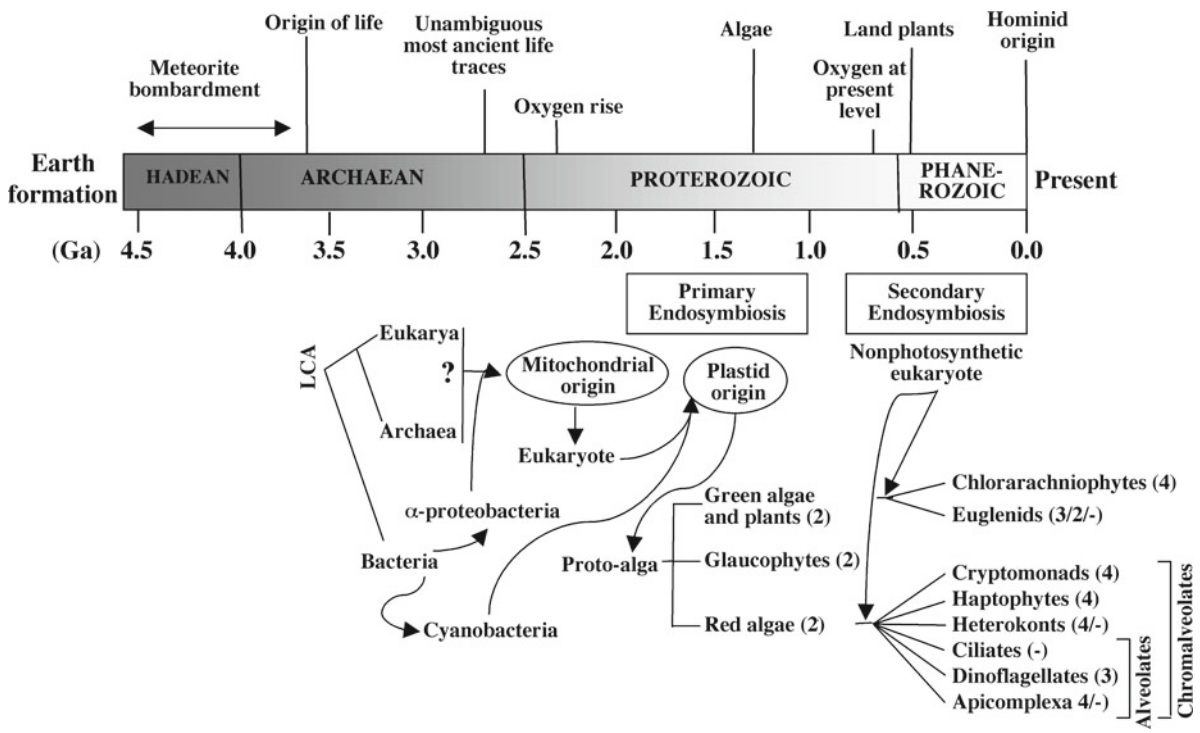

Fig. 2.1. Evolution of plastids in the geological time scale. Origin of Last Common Ancestor (LCA) dates from 3.5 Ga (billion years ago) and origin of cyanobacteria from 2.7 Ga. Mitochondrial origin, giving rise to all modern eukaryotes, occurs 2.2 Ga with a symbiotic event between a host cell and an α-proteobacterium ancestor, (the nature of the host, Archaea or Eukarya, is still controversial as indicated with a question mark). Primary endosymbiotic origin of red, green and glaucophyte plastids (all surrounded by two membranes) resulting from a symbiotic event between a cyanobacterium and a mitochondriate eukaryote is believed to have occurred between 1.6 and 0.6 Ga. Subsequent endosymbioses involving the uptake of red and green algae by nonphotosynthetic eukaryotes occurred between 1.2 and 0.55 Ga, and have given rise to a large and distinct number of algal groups with plastids surrounded by a variable number of membranes. The number of membranes surrounding the plastid is indicated between parentheses. For more details and references see text.

cells is still disputed (Woese, 1998; Forterre and Philippe, 1999; Doolittle, 2000; Poole and Penny, 2007a). Whatever the LCA was, the widely endorsed universal tree of life, first defined by sequences of small subunit ribosomal RNA, recognizes three major domains (Bacteria, Archaea and Eukarya) (Woese and Fox, 1977). During the late-Archaean period (3.0–2.5 Ga), the oxygen concentration became significant (but still very low) at 2.7 Ga as indicated by organic carbon isotopes studies (Forterre and Gribaldo, 2007) and then started to rise steadily from 2.4 Ga (Holland, 2006). During the last decade, studies on molecules contained in late-Archaean (2.7 Ga) rocks suggest not only the presence of cyanobacteria but also of eukaryotes (Brocks et al., 1999; Summons et al., 1999; Ventrual et al., 2007). However, recent results suggest that the evidence provided by molecular fossils for the advent of oxygenic photosynthesis in the Archaean is not valid (Rasmussen et al., 2008). Given so much contradictory evidence, the debate whether the oxygen-producing cyanobacteria appear before the rise in oxygen is far from over (Fischer, 2008). Nethertheless, the rise in oxygen known as "the great oxidation event" supports the aerobically driven origin of mitochondria about 2.2 Ga ago. This origin is probably due to a single invasion of an Archaea-type host or a protoeukaryote host (the nature of the host cell being still controversial: Esser and Martin, 2007; Poole and Penny, 2007b) by an α-proteobacterium-like ancestor with different possible scenarios (Gray et al., 1999; Poole and Penny, 2007a). All known modern eukaryotes are thus considered to have evolved from the mitochondrion-bearing ancestor.

Phylogenetic, structural and biochemical analyses support a single primary origin of plastids as a result of a single symbiotic association between a cyanobacterium and a mitochondriate eukaryote occurring between 1.6 and 0.6 Ga ago (Yoon et al., 2004; Cavalier-Smith, 2006). Over time, the foreign cell was reduced to a plastid bound by two membranes: the inner and outer membranes of the cyanobacterium (Reumann et al., 2005; Block et al., 2007). This endosymbiosis gave rise to a proto-alga, which based on photosynthetic pigments, generated three lineages: the green lineage (Viridiplantae, the green algae and land plants), the red lineage (Rhodophyta, the red algae) and the blue lineage (Glaucophyta, the glaucophytes) (Delwhich, 1999; Moreira et al., 2000; Martin et al., 2002; Palmer, 2003; Bhattacharya et al., 2004; Dyall et al., 2004; McFadden and Van Dooren, 2004). However, recent suggestions that endosymbionts in an amoeba (*Paulinella chromatophora*) represent an independent origin of plastids from those in plants and algae still raise the endless question of how many times plastids have evolved (Marin et al. 2005; Larkum et al., 2007). The next remarkable stage in the evolution process was the propagation of plastids by secondary endosymbioses, which occurred between 1.2 and 0.55 Ga, ago (Yoon et al., 2004; Cavalier-Smith, 2006), whereby the new photosynthetic eukaryotes were, in their turn, engulfed by nonphotosynthetic eukaryotes (Gibbs, 1978; Douglas, 1998; Archibald and Keeling, 2002; Bhattacharya et al., 2004). Two algal divisions (Chlorarachniophyta, and Euglenophyta) acquired green algal plastids and four algal divisions (Cryptophyta, Haptophyta, Heterokontophyta and Dinoflagellata) together with one parasitic phylum (Apicomplexa) acquired red algal plastids, thus explaining the diversity of the algal world. Interestingly, a nucleomorph, the remnant nucleus of the algal symbiont, is present between the second and third plastid membranes in the cryptophytes (Douglas et al., 1991) and the chlorarachniophytes (McFadden et al., 1995). For additional reviews on the evolution of plastids, the reader may refer to Keeling (2004), Sato (2006), Bhattacharya et al., (2007) and Reyes-Prieto et al., (2007). These symbiotic events were the release mechanisms for the phanerozoic explosive radiations of autotrophic organisms, making possible all vertebrate life on land, including humans.

III. Studies on Plastids and Plastid Division

Our knowledge on plastid and plastid division is directly related to the progress made in the comprehension of mechanisms for evolution and symbiosis; we summarize these concepts in Fig. 2.2. The first suggestion that all organisms may have had a common ancestor and diverged through random variation and natural selection was made in 1745 by the French mathematician and scientist Pierre-Louis Moreau de Maupertuis (1698–1759) in his work 'Vénus physique' (the

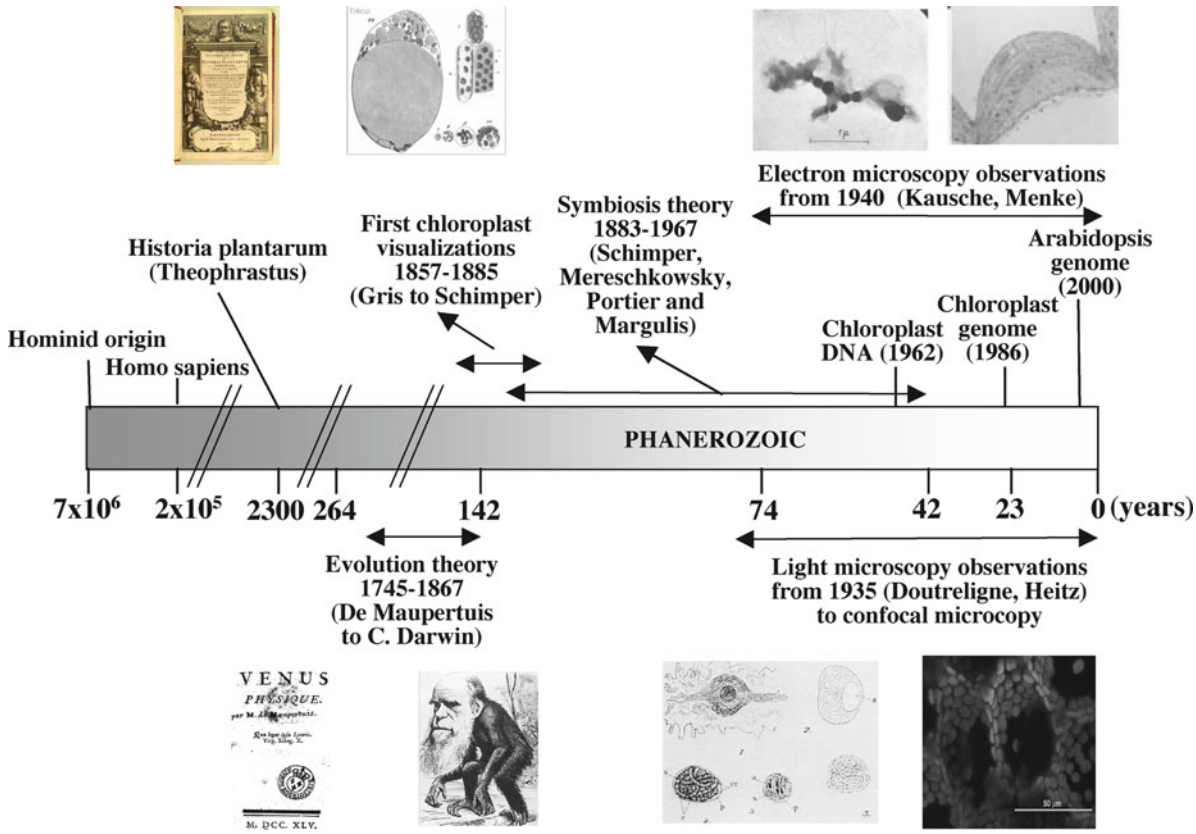

Fig. 2.2. Progress made in plastid comprehension on human time scale. Origin of man dates from seven million years and *Homo sapiens* 200,000 years ago. The first botanical encyclopedia is believed to have been written 2,300 years ago and a chloroplast was visualized for the first time in the mid nineteenth century. The evolution and symbiosis theories postulated at the end of the eighteenth century and the end of the nineteenth century, respectively together with technical progress made in microscopy observations in the twentieth and genome sequencing projects are probably the major milestones of human comprehension in plastid biology.

earthly Venus) when he wrote "… *les espèces que nous voyons aujourd'hui ne sont que la plus petite partie de ce qu'un destin aveugle avait produit…*" (… The species we see today are but the smallest part of what blind destiny has produced…"). Towards the end of the eighteenth century, philosophers and scientists suggested that living organisms could have evolved gradually along with changes in the Earth's crust. Among these authors we can mention Immanuel Kant in his '*Kritik der Urtheilskraft*' (Critique of Judgement) published in 1790, in which he states that the analogy of animal forms implies a common original type and thus a common parent, and Erasmus Darwin (Charles Darwin's grandfather) who hypothesized in 'Zoonomia or The Laws of Organic Life' (1792–1795) that all warm-blooded animals were descended from a single "living filament". Erasmus Darwin's views anticipated those of Lamarck, the first thinker to develop a coherent evolutionary theory in 1809 in his book '*Philosophie zoologique ou exposition des considérations relatives à l'histoire naturelle des animaux*' (meaning: zoological philosophy: an exposition with regard to the natural history of animals) in which he outlines his theory that is known as Lamarckism.

In 1859, near the end of his book 'The Origin of Species by Means of Natural Selection', Charles Darwin wrote: "….that probably all the organic beings which have ever lived on this Earth have descended from someone primordial form, into which life was first breathed". In this short sentence Darwin summarized 3.5 billion of years of organic evolution but without knowing the mechanisms underlying the evolution process.

We had to wait for another century in order to be able to decipher such mechanisms. At the time Charles Darwin wrote his essay, the first observations and drawings of the chloroplast within the cell were published (Gris, 1857; Trécul, 1858). A few decades later, Schimper (1883) suggested in a footnote (translated in Martin and Kowallik, 1999) that "If it can be conclusively confirmed that plastids do not arise de novo in egg cells, the relationship between plastids and the organisms within which they are contained would be somewhat reminiscent of a symbiosis. Green plants may in fact owe their origin to the unification of a colorless organism with one uniformly tinged with chlorophyll". One century later it is astonishing to realize that in such a statement, Schimper had already understood that plastids divide, and more amazingly that they come from another organism. It is only a century later, with the works of many scientists, among them Mereschkowsky, Portier and Margulis, and the progress in techniques (microscopy and molecular biology) that the role played by symbiosis in evolution was accepted (for a comprehensive review, see Sapp, 1994).

A. Observation of Chloroplast Division

The understanding of chloroplast division followed the same development as the one observed in symbiosis studies. Following the pioneering work of the nineteenth and early twentieth centuries, only a few reports concerning chloroplast division can be found before the beginning of the 1950s. Light microscopic observations of mature chloroplasts dividing by constriction have been made in land plants ranging from Bryophytes (Sharp, 1934) through the Angiosperms (Randolf, 1922; Kusunoki and Kawasaki, 1936; Dangeard, 1947). In 1941, Hollande and Hollande (1941) wrote '*les chloroplastes constitués sont des corpuscules différenciés, indivis, libres, répartis dans le périplasme au contact direct duquel ils se trouvent. Ils ont une vie propre et sont aptes à se diviser*' that translates as: 'chloroplasts are free individual corpuscles, which are differentiated and distributed within the periplasm. They have a proper life and are able to divide'. Yuasa (1949), when studying the structure of the chloroplast of *Selaginella savatieri*, a Pteridophyte, described the amoeboid type of chloroplast division, besides the three types of division, i.e., the ordinary, the longitudinal and lateral division types.

It was Strugger (1950) who initiated the revival of Schimper-Meyer's theory on the individuality and continuity of plastids by the publication of drawings showing both proplastids and chloroplast division (Fig. 2.3). Further, Fasse-Franzisket (1955) showed that in normal development of the light grown leaf of *Agapanthus umbellatus,* the plastids replicated several times. At about the same time, investigation of the behavior of plastids by means of electron microscopy revealed the mechanisms by which plastids divided. The mechanism by which fully developed chloroplasts divided has been reported to consist of an initial constriction around the entire chloroplast, giving rise to a dumbell-shaped chloroplast, which is followed by a pinching into two daughter chloroplasts. Constriction is accompanied by fusion of the inner and outer layers of the chloroplast membrane. Such a division was documented first in a brown alga belonging to the *Fucus* genus (von Wettstein, 1954) and then in two dicotyledonous plants (Mühlethaler, 1960) and in a red alga *Lomentaria baileyana* (Harv.) Farlow, a tubular member of the Rhodymeniales (Bouck, 1962). Division of proplastids in higher plants was shown to be similar to that in algal and plant chloroplasts, in that pinching is also accompanied by fusion of all the layers of the chloroplast membrane (Strugger, 1957). A second method of mature chloroplast division termed 'concentralization' was soon thereafter described; in the gametophyte of the fern *Matteucia struthiopteris* (L.) Todaro, the mature chloroplast is divided by the centripetal invagination of the inner limiting membrane (Gantt and Arnott, 1963). In 1964, the first cinematographic study on division of chloroplasts in growing cells of the green alga *Nitella axillaris* was published (Green, 1964) demonstrating that indeed the dumbell-shaped chloroplasts divide. A few years later microscopic observations of developing leaf cells in tobacco (Boasson and Laetsch, 1969) and spinach (Possingham and Saurer, 1969) established that there is a correlation between the increase in chloroplast number and the presence of dumbell-shaped chloroplasts. Soon thereafter cinematographic observations on chloroplast division in leaf cells of the moss *Mnium cuspidatum* were reported (Ueda et al., 1970) and it was shown that once the chloroplast division was initiated it can be completed outside the cell (Ridley and Leech, 1970; Kameya and Takahashi, 1971).

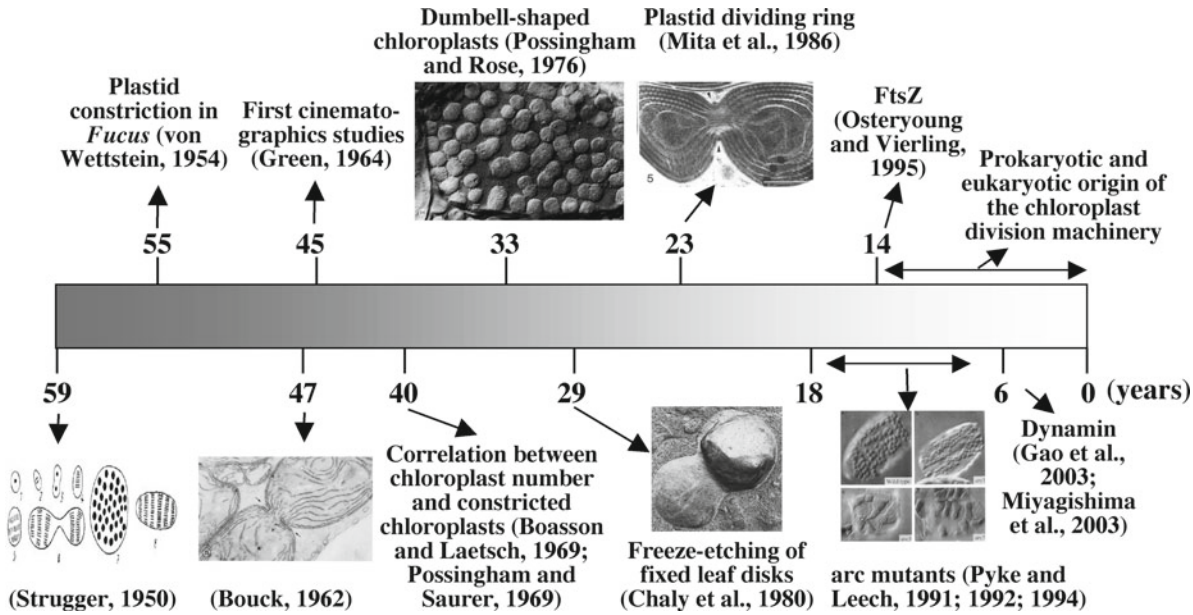

Fig. 2.3. Progress made in plastid division studies during the last half century. Major discoveries in plastid division started with the observation of plastid constriction, followed by the observation that the chloroplast number correlated with the number of constricted chloroplasts. Observation of dividing rings associated with plastids, *arc* mutant analysis and protein identification were the next important steps in our understanding of the chloroplast division machineries.

Ultrastructural studies established that plastids divide by a binary fission mechanism involving constriction of the envelope membranes (reviewed by Possingham and Lawrence, 1983; Leech and Pyke, 1988; Whatley, 1988) with the exceptions of the concentralization mechanism or partition (Grantt and Arnott, 1963; Modrusan and Wrischer, 1990; Miyake and Taniguchi, 1995), the accidental division induced by the cell plate (Staff and Parthasarathy, 1984) and the budding mode reported in a *Bryophyllum* spp. (Kulandaivelu and Gnanam, 1985).

B. The Plastid Dividing Ring

A number of specific electron dense structures were found associated with dividing plastids, at the narrowly constricted isthmus of the dumbell-shaped plastids, in the lumen between the two membranes of the envelope (Suzuki and Ueda 1975; Luck and Jordan, 1980; Chaly and Possingham, 1981; Leech et al., 1981). An electron dense annulus, termed the plastid division ring (PD ring), associated with the constriction and present at the cytosolic surface of the chloroplast, was first described in the red alga *Cyanidium caldarium* RK-1 (Mita et al., 1986). In *Avena sativum,* the PD ring was shown to be a double-ring structure lying on the stromal and cytosolic sides of the envelope (Hashimoto, 1986). The PD ring doublet has been detected in all Angiosperms investigated (Hashimoto, 1986, Kuroiwa, 1989; Oross and Possingham, 1989), red algae (Mita et al., 1986; Mita and Kuroiwa, 1988; Kuroiwa, 1989) and ferns (Duckett and Ligrone, 1993a) (Table 2.1). The situation is more complex in Bryophyta with the moss *Funaria hygrometrica* having only the outer ring (Tewinkel and Volkmann, 1987) while the liverwort *Odontoschisma denudatum* has the two rings (Duckett and Ligrone, 1993b). In Chlorophyta, *Trebouxia potteri* (Chida and Ueda, 1991) has only the outer ring, but *Nannochloris bacillaris* (Ogawa et al., 1995) has a pair of belts on both side of the chloroplast envelope. In the red alga *Cyanidioschyzon merolae,* a middle PD ring has been identified in the lumen between the inner and outer envelopes (Miyagishima et al., 1998), thus raising to three the number of PD rings in certain members of Rhodophyta. The middle PD ring has not been observed in other organisms yet. A single inner stromal ring was observed in cyanelles, the photosynthetic plastids of the glaucocystophyte *Cyanophora paradoxa* (Iino and

Table 2.1. PD ring numbers and sizes. Maximum sizes in nanometers (width × thickness) of the PD rings are given during the chloroplast contraction

Group		Land plants	Green algae	Red algae	Glaucocystophytes	Heterokonts
PD ring	Number	2	1 or 2	3	1	1
	Size (nm)	*A. sativa* (1) *S. oleracea* (2) *P. vulgaris* (2) *N. tabacum* (2) *T. aestivum* (2) *P. Zonale* (3) 15–40 × 5	*T. potteri* (4) 100 × 20 *N. bacillaris*[a](5) 60 × 14	*C. merolae*[b] (6) Outer 80–100 × 25 Middle 80–110 × 2 Inner 100–120 × 5	*C. Paradoxa*[c] (7) 50 × ?	*H. akashiwo* (8) 100 × ? *M. Splendens* (9) 200–240 × 5–10 *M. Rasilis* (9) 80–100 × ?

References: (1) Hashimoto (1986), (2) Oross and Possingham (1989), (3) Kuroiwa et al. (2002), (4) Chida and Ueda (1991), (5) Ogawa et al. (1995), (6) Kuroiwa (2000), Miyagishima et al. (2001b), (7) Iino and Hashimoto (2003), (8) Hashimoto (1997), (9) Weatherill et al. (2007)

Symbol used: ? not addressed

[a]In *Nannochloris bacillaris*, the belts become wider while their thickness remains constant during chloroplast division

[b]As the outer ring contracts, the ring grows thicker and maintains a constant volume. The middle and inner rings do not change in thickness, and their volumes decrease at a constant rate in proportion with contraction (Miyagishima et al., 1999)

[c]Ring does not seem to increase in thickness and width during the division of the inner envelope suggesting that components of the ring are lost during the constriction

Hashimoto, 2003). Observations of a PD ring in secondary plastids are scarce. The presence of a single ring at the periplastidial surface of the inner pair of four surrounding membranes has been documented in heterokont algae, *Mallomonas splendens* and *Mallomonas rasilis* (Synurophyceae) (Beech and Gilson, 2000; Weatherill et al., 2007), *Heterosigma akashiwo* (Raphidophyceae) (Hashimoto, 1997) and *Nannochloropsis oculata* (Hashimoto, 2005). In the Apicomplexan parasite, *Toxoplasma gondii*, no obvious division structures have been observed and the dividing plastids (termed apicoplasts) have been shown to associate with the centrosomes of the mitotic spindles (Striepen et al., 2000). However, Matsuzaki et al. (2001) have observed rings at the constriction between dividing apicoplasts suggesting the presence of a possible plastid-dividing ring. Whether the absence of detection of the inner stromal ring in secondary plastids is due to technical problems is not yet known. The almost universal presence of the PD ring in plastids arising by primary as well as by secondary endosymbiosis (Table 2.1) suggests that these rings, acquired after the endosymbiotic events, play a key role in plastid division (Hashimoto, 2003, 2005).

Beside the difference in PD ring numbers, the width and thickness of the rings differ between higher plants and algae (Table 2.1). To date, the widest ring has been observed on the outside surface of the heterokont *Mallomonas splendens* (Weatherill et al., 2007). The timing of the ring appearance during chloroplast division differs greatly between organisms. The outer and inner PD rings can be observed during early, middle and late stages in red algae and most, if not all, green algae, but they are only observed in the late stage of chloroplast division in land plants. For reviews and interpretations of the structures observed, see reviews by Kuroiwa (1989, 2000) and Kuroiwa et al. (1998). The identity of the components of the PD ring has been addressed by cytochemical and cyto-physiological analyses and TEM (transmission electron microscopy) observations. The first studies suggested that actin could be a component of the PD ring (Mita and Kuroiwa, 1988; Hashimoto, 1992) but it was finally demonstrated that actin is not involved in the plastid outer ring, but rather a 56 kDa protein that forms filaments 5 nm in diameter (Miyagishima et al., 2001b).

C. Genetic Approach to Study Plastid Division

The first evidence for nuclear control of plastid division and plastid DNA synthesis was suggested when a barley mutant, with plastids lacking ribosomes, was shown to have similar plastid number per cell, similar levels of DNA per plastid as in the wild-type plants (Scott et al., 1982). The presence of the double ring at the constricting neck of

these ribosome-deficient plastids, suggested that nuclear gene products are responsible for the formation of such structures (Hashimoto and Possingham, 1989). In order to identify the genes involved in chloroplast division, the strategy of isolating and analyzing mutants of chloroplast division in *Arabidopsis thaliana* was used. Ethyl methane sulfonate (EMS) mutagenized seedlings (Pyke and Leech, 1991), T-DNA mutagenized seedling populations, and plants mutagenized with Nae I deleted ac element, were screened for altered chloroplast number and size. Eleven independent *arc* (accumulation and replication of chloroplasts) mutants were identified and their main characteristics are summarized in Table 2.2. The relationship among five of the *arc* genes was addressed through a series of double mutant studies (Marrison et al., 1999). ARC1 down-regulates proplastid division but is on a separate pathway from ARC3, ARC5, ARC6 and ARC11. ARC6 initiates both proplastid and chloroplast division. ARC3 controls the rate of chloroplast expansion and ARC11 the central positioning of the final division plane in chloroplast division. Finally ARC5, acting at a late stage, facilitates separation of the two daughter chloroplasts.

IV. The Plastid Division Machineries

The definitive evidence for conservation of the division mechanism between plastids and bacteria came from the discovery of an *FtsZ* (filamentous temperature sensitive protein Z) homolog in the *Arabidopsis thaliana* nuclear genome (Osteryoung and Vierling, 1995). The identification in plants of other homologs of the bacterial division genes, and the localization of their products inside the chloroplast, suggested that the endosymbiont-derived machinery was likely to be involved in the constriction of the chloroplast inner envelope membrane. A second division machinery of the host-cell origin was first postulated to be present at the surface of the chloroplast (Miyagishma et al., 2001c). A couple of years later a dynamin was shown to be involved in chloroplast division, forming a ring at the chloroplast division site on the outer surface of the organelle (Gao et al., 2003; Miyagishma et al., 2003). Actually, ten proteins have been identified participating directly in plastid division, confirming that the plastid division machinery is an evolutionary hybrid, with proteins originating from both the symbiont and the host cell implicated in the endosymbiotic event (Table 2.3). For further information on plastid division mechanisms, see reviews by several authors (Miyagishma and Kuroiwa, 2006; Glynn et al., 2007; Maple and Moller, 2007a, c; Yang et al., 2008).

A. The Endosymbiont Derived Division Machinery

1. The Skeletal Proteins FtsZ and the Associated Protein ARC6

The division machinery must provide the force to split the mother cell into two daughter cells. In eukaryotic cells, the cytoskeleton plays an important role in such a process called cytokinesis. A medial ring of actin and myosin, together with other proteins, contracts to divide animal and fungal cells. Cytokinesis in plant cells occurs by cell plate formation, which is dependent upon the phragmoplast (or phycoplast in some algae), a complex assembly of microtubules and other cytoskeletal elements. Prokaryotes, which include Bacteria as well as Archaea, possess homologs of eukaryotic cytoskeletal proteins, and most of them use a tubulin homolog, FtsZ, to divide. FtsZ has been identified in a genetic screen of *Escherichia coli* cells unable to divide at the restrictive temperature and shown to form a contractile ring (Z-ring) at the division site (Lutkenhaus and Addinall, 1997). FtsZ is a highly conserved protein that is found in most of the major groups of Bacteria and Archaea (Vaughan et al., 2004; Margolin, 2005). However, FtsZ is absent in a few bacterial groups such as the chlamydiaceae family, which are obligate intracellular bacteria and may therefore use host mechanisms for division. FtsZ is also absent in the planctomycetes, a group of budding, peptidoglycan-less bacteria with unusual distinctive features such as compartmentalized cell organization and a membrane-bound nucleoid (Lindsay et al., 2001). The mycoplasmas, the wall-less mollicutes, have lost many cell division proteins, but contain the *FtsZ* gene, except for *Ureaplasma urealyticum*. In Archaea, *FtsZ* has been found in the genome of all euryarchaeota for which sequence data are available, but is absent in the crenarchaeota. The mechanism by which cell division occurs in the species lacking *FtsZ* is unknown.

Table 2.2. Phenotype of *Arabidopsis thaliana arc* mutants

Geno type	Eco type	Mesophyll cell size (μm^2)	Mean Chloroplast plan area (μm^2)	Chloroplasts/ cell	Chloroplasts/10^3 μm^2 mesophyll cell plan area	Chloroplast characteristics (observed in mesophyll cell, albeit specified)	References
WT	Ler	4,778–4,800	50	120	25	Spherical	(Pyke and Leech, 1992; Marrisson et al., 1999)
WT	Ws	5,087	52	83	16	Spherical	(Pyke et al., 1994; Rutherford, 1996)
WT	Col		50	100	23	Spherical	(Osteryoung et al., 1998)
arc1	Ler (ems)	3,388	26	108	32	Increase in chloroplast number of smaller size. Pales leaves	(Pyke and Leech, 1992, 1994)
arc2	Ler (ems)	4,339	111	40	9	Reduced chloroplast number	(Pyke and Leech, 1992)
arc3	Ler (ems)	3,582	202	18	5	Few very large chloroplasts	(Pyke and Leech, 1992)
arc5	Ler (ems)	3,500	290	13	4	Few very large chloroplasts, with central constriction	(Pyke and Leech, 1994; Robertson et al., 1996)
arc6	Ws (T-DNA)	3,700	1,080	2	0,4	In average only 2 giant chloroplasts. Reduced size of proplastids in both shoot and root meristems. Guard cells lack plastids.	(Pyke et al., 1994; Robertson et al., 1995; Pyke and Page, 1998)
arc7	Ws		40	80	26	Increase in chloroplast number. Pale leaves	(Rutherford, 1996; Pyke, 1999)
arc8	Ws		110	45	10		(Rutherford, 1996)
arc9	Ws		140	34	12		(Rutherford, 1996)
arc10[a]	Ws		170	38	6	Highly heterogeneous in size.	(Rutherford, 1996; Pyke, 1999)
arc11[b]	Ler (NaeI deleted ac)	4,200	110	29	7	Heterogeneous population of chloroplast. Normal ultrastructure	(Marrisson et al., 1999)
arc12	Col			2		Similar to *arc6*. Guard cells lack plastids	(Pyke, 1999)

WT wild-type plants

[a] The chloroplast division phenotype in arc10 is a consequence of the atFtsZ1 missense mutation that alters a glycine residue highly conserved among plant and cyanobacterial FtsZ proteins (Yoder et al., 2007)

[b] Arc11 contains a missense mutation in AtMinD changing an alanine at position 296 to glycine (Fujiwara et al., 2004)

Table 2.3. Components of the internal and external plastid division machineries

Machinery	Protein	Localization	Primary plastids[a]				Secondary plastids[a]	
Stromal side			Land plants	Green algae	Red algae	Glaucocystophytes	Heterokonts	Cryptomonads
	FtsZ1	Ring associated	+	+		+		
	FtsZ2	with inner	+	+		?		
	FtsZA	membrane			+		+	+
	FtsZB	(mainly in the stroma)[b]			+		–	?
	ARC6	Spans inner membrane	+	+	–	?	–	?
	MinD/ARC11	Polar loci	+	+	–	?	–	+
	MinE	Polar loci	+	+	–	?	–	+
	ARC3	Ring and Polar loci	+	+	–	?	–	?
	GC1 (AtSulA)	Associated with inner membrane	+	+	+	?	–	?
Cytosolic side	ARC5 (DRP5B)	Cytosolic patches (ARC5)	+	+	+	?	+	?
	PDV1	and ring associated	+	–	–	?	–	?
	PDV2	with outer membrane	+	–	–	?	–	?

Symbols used: (+) identified and (–) not identified in the complete genome sequences available. (?) not identified yet, but genome sequence is incomplete

[a]To date only a few algal genomes are completely sequenced, which include for organisms with primary plastids: *Ostreococcus tauri, Ostreococcus lucimarinus*, *Chlamydomonas reinhardtii* (green algae) and *Cyanidioshyzon merolae* (red algae); and for organisms with secondary plastids: *Thalassiosira pseudonana* as well as *Phaeodactylum tricornutum* (two diatoms, heterokonts) (Misumi et al., 2008). None of the proteins listed have been identified in the Apicomplexan parasites genomes. The list of complete eukaryotic genomes can be found on the following http sites: http://www.ncbi.nlm.nih.gov/genomes/ and http://genome.jgi-psf.org/euk_cur1.html. The list of all organelle and nucleomorph genomes is available on http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2759&type=4&name=Eukaryotae%20Organelles. The site http://chloroplast.cbio.psu.edu/index.html is dedicated to chloroplast genome analyses and for a comprehensive review, see Simpson and Stern (2002)

[b]In *Arabidopsis thaliana*, the localization of the FtsZ1 proteins is dependent on plant development (El-Kafafi et al., 2008)

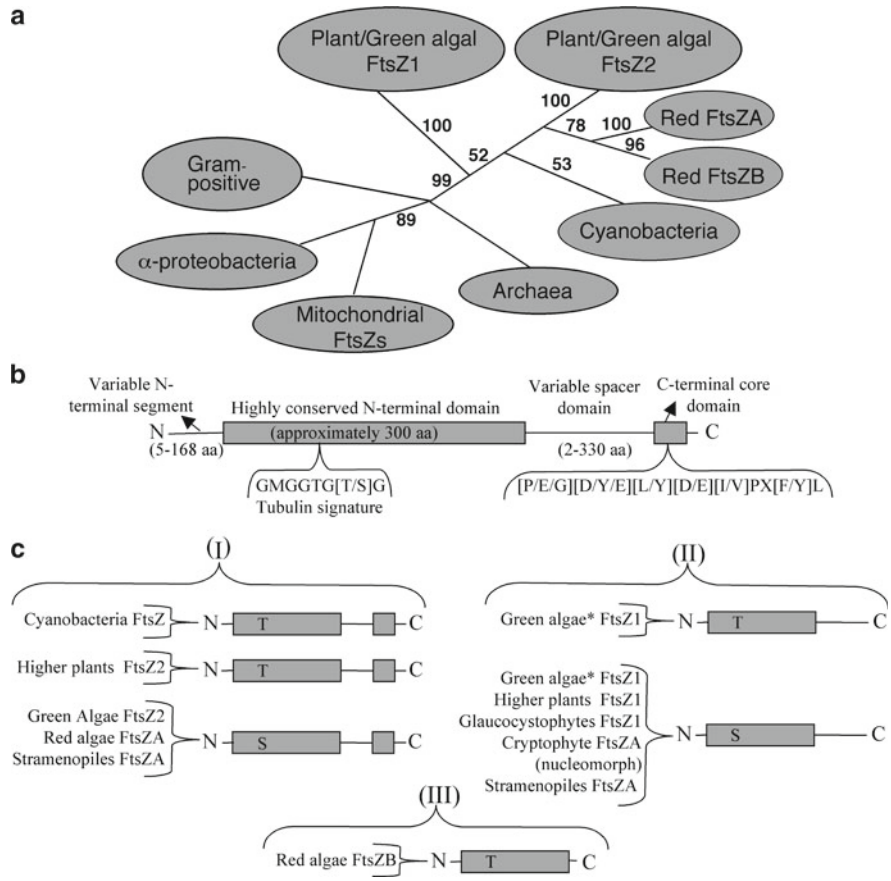

Fig. 2.4. The plastid division protein FtsZ. (**a**) Phylogenetic relationship of bacterial, archaeal and eukaryotic (mitochondrial and plastidial) FtsZ proteins inferred from maximum likelihood analysis. The values at the node represent bootstrap probabilities for the neighbor-joining method based on Kimura distance (Miyagishima et al., 2004). (**b**) General domain composition of FtsZ, showing the four regions outlined in the text and the conserved tubulin signature as well as the C-terminus motif. (**c**) Comparison of the three groups of plastid FtsZ according to their domain compositions. Group I proteins have the variable spacer domain and the C-terminal core domain, group II FtsZs lack the C-terminal core domain but possess the variable spacer region and group III FtsZs lack both the variable spacer domain and the core domain. The *residues serine* (S) or *threonine* (T) in the tubulin signature are shown. *In group II, green algae such as *Chlamydomonas reinhardtii*, *Nannochloris bacillaris*, *Volvox carteri* have the threonine residue, while *Marchantia polymorpha* and *Oestreococcus tauri* harbor a serine residue in the tubulin motif.

It was in the nuclear genome of *Arabidopsis thaliana* that the first eukaryotic *FtsZ* gene was found (Osteryoung and Vierling, 1995). Since then, phylogenetic analyses of FtsZ protein sequences indicate that FtsZ proteins, in higher and lower plants (the green lineage plastids), are divided into two different protein groups called FtsZ1 and FtsZ2 (Osteryoung et al., 1998; Osteryoung and McAndrew, 2001; El-Shami et al., 2002) (Fig. 2.4a). The involvement of both proteins in chloroplast division was demonstrated for FtsZ1 by gene-disruption experiments in the moss *Physcomitrella patens* (Strepp et al., 1998), and in *A. thaliana* by the expression of antisense FtsZ1 and FtsZ2-1 RNAs (Osteryoung et al., 1998). The resulting mutants and transformants are characterized by cells harboring as few as one greatly enlarged chloroplast. Conservation of mechanisms with the bacterial FtsZ was shown by correcting the thermosensitive defect of an *ftsZ E. coli* mutant by expressing FtsZ1 (Gaikwad et al., 2000). The red lineage plastids can also be divided into two phylogenetic groups: (1) The red FtsZA group that includes FtsZs from secondary plastids of red algal origin, and (2) the red FtsZB group (Miyagishima et al., 2004). As expected

from their endosymbiotic origins, mitochondria contain FtsZ proteins that are most closely related to those of α-proteobacteria, the progenitors of mitochondria, whereas chloroplast FtsZs are most closely related to those of cyanobacteria (Fig. 2.4a). Secondary structure alignments between α/β-tubulin and *Methanococcus jannaschii* FtsZ1 (Nogales et al., 1998), and phylogenetic analysis (Vaughan et al., 2004), revealed the presence of four domains in the FtsZ protein. These domains comprise a variable N-terminal segment, a highly conserved core domain, a variable spacer, and a C-terminal core peptide (Fig. 2.4b). The only function actually known for at least part of the N-terminal segment is the targeting of the protein to organelles in eukaryotic cells. The core region of approximately 300 amino acids contains the tubulin signature motif and is indispensable for GTP binding and hydrolysis, which is required for polymerization of FtsZ into protofilaments. The length and amino acid composition of the spacer region is highly variable and is not required for FtsZ polymerization. The C-terminus motif, which in prokaryotes is thought to be involved in protein-protein interaction but not required for FtsZ auto-assembly (Ma and Margolin, 1999; Osteryoung and McAndrew, 2001), is known to bind to ZipA (FtsZ interacting protein A) and FtsA, two membrane-associated proteins (Wang et al., 1997; Din et al., 1998; Liu et al., 1999; Ma and Margolin, 1999; Hale et al., 2000; Mosyak et al., 2000; Yan et al., 2000).

Comparison of the chloroplast C-terminal sequences, including the variable spacer domain and the C-terminal core domain (Miyagishima et al., 2004), revealed three distinct FtsZ groups (Fig. 2.4c). Sequences in group I, which include green lineage FtsZ2 and red FtsZA (algae and Stramenopiles) have the C-terminal core domain and are more closely related to the cyanobacteria sequences, suggesting that they could be the ancestral genes. Sequences in group II, which include green lineage FtsZ1 and secondary plastids FtsZA, lack the C-terminal core domain but contain the spacer domain. Only the red algae FtsZB, lacking the C-terminal core domain and the spacer domain, are actually present in group III. Both the phylogenetic and domain analyses suggest that green *FtsZ1* and red *FtsZB*, emerged via duplication of *FtsZ2* and *FtsZA* genes, respectively – with the subsequent loss of the C-terminal core domain. The function of the C-terminal core domain in the green FtsZ2 and the red FtsZA is not known, given that there are no homologs of known bacterial binding partners. Nevertheless, the same function might be conserved, since the expression of plant FtsZ2 severely disrupts bacterial division (the expression of FtsZ1 has less effect) and the effect is dependent on the presence of the C-terminal core domain, thus suggesting binding to the FtsZ partners FtsA and ZipA in *E. coli* (El-Kafafi et al., 2005).

In Bacteria, both FtsA and ZipA are able to link FtsZ to the membrane. FtsA is more widely conserved, although it is not present in actinomycetes, cyanobacteria, mycoplasma or Archaea. ZipA is only found in bacteria closely related to *E. coli*. The *A. thaliana arc6* mutation (Table 2.2) was mapped to a nuclear gene product acting as a positive regulator of Z-ring formation (Vitha et al., 2003) that is orthologous to cyanobacterial *ftn2* (filamentous transposon 2) (Koksharova and Wolk, 2002). The protein, an integral inner envelope membrane protein, with the N-terminal J-domain residing in the stroma, while the C-terminal region is present in the intermembrane space, interacts specifically with the C-terminal core domain present in FtsZ2. Such a protein could function to tether FtsZ filaments to the membrane. *Arc6* giant chloroplasts harbor numerous short FtsZ filaments instead of the intact FtsZ ring visualized in wild-type plants and excessive FtsZ polymerization is observed in ARC6 over-expressing plants (Vitha et al., 2003). These phenotypes suggest a role for ARC6 in bundling FtsZ filaments as also proposed for ZipA in bacteria (RayChaudhuri, 1999; Hale et al., 2000). *ARC6* homologs are found in the nuclear genome of all higher plants analyzed and appear to be restricted to chlorophytes since orthologues have been found to date only in the nuclear genome of *Chlamydomonas reinhardtii* and *Ostreococcus tauri*.

2. The Topological Proteins and Indirect Regulators of the Z-Ring

The Min system required to prevent formation of DNA-less "minicells", with two or three proteins in bacteria, inhibits unwanted formation of the Z-ring at the cell poles. In *E. coli*, this system consists of the FtsZ assembly inhibitor MinC

(Hu et al., 1999), and the MinD and MinE proteins, which oscillate from one cell pole to the other (Raskin and de Boer, 1999). MinD is an ATPase that binds to the membrane in its ATP form and is released from the membrane on ATP hydrolysis. MinE drives MinD off the membrane by binding to MinD and stimulating its ATP hydrolysis. Because MinC binds to MinD, which is mostly localized at the membranes near the cell poles, MinC is sequestered away from the cell midpoint, allowing FtsZ assembly in the center of the cell and inhibiting cell division at the cell pole. Although the Min proteins are widely conserved, they are not present in all organisms. However, the genes are present in bacteria that branch close to the root of the phylogenetic tree of life (*Thermatoga maritima* and *Aquifex aeolicus*), suggesting that they were present in ancient bacteria but were subsequently lost in some lineages such as *Caulobacter crescentus*. On the other hand, *Bacillus subtilis* lacks MinE but contains an unrelated protein DivIVA that is responsible for spatial regulation of MinCD (for a recent review, see Lutkenhaus, 2007).

No *MinC* homologs have been identified in eukaryotes to date, although targeted overexpression of *E. coli* MinC protein in higher plants results in abnormal chloroplasts (Tavva et al., 2006). However, homologs of *MinD* and *MinE* were first found in the plastid genomes of the green alga *Chlorella vulgaris* (Wakasugi et al., 1997) and the cryptomonad *Guillardia theta* (Douglas and Penny, 1999). Since then, nuclear *MinD* in *A. thaliana* (Colletti et al., 2000; Kanamaru et al., 2000), marigold (Moehs et al., 2001) as well as in other plants such as rice, grape, tobacco, poplar, sorghum and the moss *P. patens* (Hayashida et al., 2005) have been identified. Nuclear genes encoding MinE have also been found in the *A. thaliana* genome (Itoh et al., 2001; Reddy et al., 2002), in rape, rice, grape, barley and the moss *P. Patens* (Hayashida et al., 2005), suggesting that their presence in the nuclear genome is a general feature in higher plants. With the completion of new chloroplast and nuclear genomes in different algae, additional *MinD* and *MinE* have been identified. Interestingly, the localization of both genes is variable, and depending on the organisms the proteins are encoded in the plastid or as the result of lateral gene transfer (LGT) in the nuclear genome. In the charophytes, the immediate land plant ancestors, *MinD* and *MinE* have not been found in the chloroplast genome of *Chaetosphaeridium globosum* (Coleochaetales) and *Chlorokybus atmophyticus,* thus suggesting that they are probably present, as in higher plants, in the nucleus. In *Mesostigma viride*, which has been recently shown to group in the same clade as *Chlorokybus atmophyticus* (Lemieux et al., 2007) and is regarded as representing the earliest divergence of the Streptophyta, MinD, but not MinE is plastid encoded (Lemieux et al., 2000). This result suggests that the LGT of *MinD* to the nucleus occurred after the divergence of the Chlorophyta and Streptophyta. In *Chlamydomonas reinhardtii,* a Chlorophyceae, both MinD (Liu et al., 2007) and MinE are encoded in the nucleus and targeted to the chloroplast. Albeit deriving from red algae whose sequenced plastid and nuclear genomes encode neither MinD nor MinE, secondary plastids genomes in the cryptophytes *Guillardia theta* and *Rhodomonas salina* contain both *MinD* and *MinE*, thus suggesting that the loss of both genes in red algae occurred after the secondary endosymbiosis event. Interestingly, we have also identified a homolog of *MinD* in the plastid genome of *Emiliania huxleyi*, which belongs to the haptophyte group (our unpublished result). Neither *MinD* nor *MinE* orthologues are found in the plastid and nuclear genomes of *Thalassiosira pseudonana* (Heterokonts). The function of both proteins has been investigated in *A. thaliana*. Changes in expression of *MinD* and *MinE* result in asymmetrical plastid division (Colletti et al., 2000; Dinkins et al., 2001; Maple et al., 2002; Fujiwara et al., 2004). Both proteins dimerize (Table 2.4) (Maple et al., 2005) and the interaction of MinE with MinD is required for stimulation of the Ca^{2+}-dependent ATPase activity of MinD, thus highlighting some differences with prokaryotes concerning MinD ATPase activity stimulation (Aldridge and Moller, 2005). Dissection of the assembly of the plastid MinD and MinE complex, and determination of the interdependency of complex assembly and localization in plants, revealed some aspects of the Min assembly dynamics and spatial regulation of the Z ring in chloroplasts (Maple and Moller, 2007b). The differences in domain structure and divergence in amino acid sequence reflect the adaptation of the plastid Min complex to function within its specific environment.

In a new environment, novel proteins need to be engineered in order to replace missing proteins such as MinC, or to fulfill new functions. In *A. thaliana*, ARC3, a chimeric protein consisting of an N-terminal region with homology to FtsZ and a C-terminal domain containing a MORN (membrane occupation and recognition nexus) repeat, linked by a unique sequence, represents such a new protein (Shimada et al., 2004). The MORN repeat is found in multiple copies in several proteins, which are present in almost all species including cyanobacteria. It is also possible that ARC3 is of symbiotic origin, arising by fusion of FtsZ with a MORN repeat containing protein. ARC3 was located at the site of chloroplast division in a ring-shape structure at the early and middle stages of the process (Shimada et al., 2004), but not associated with the outer membrane as previously postulated but as part of the stromal division machinery (Maple et al., 2007). Consistent with this localization, ARC3 interaction through the N-terminal FtsZ-like domain with the C-terminal specific region of FtsZ1 was demonstrated (Table 2.4), indicating that the evolution of a distinct FtsZ1 protein family in green chloroplast organisms was an important step in the evolution of the division machinery. In addition, ARC3 has been shown to interact with *A. thaliana* MinD and MinE (Table 2.4), and *arc3* mutants to exhibit multiple Z-rings within a single enlarged chloroplast, as observed in *arc11/MinD* mutants, thus suggesting a role in mediating correct Z-ring placement (Glynn et al., 2007; Maple et al., 2007). Through its interaction with FtsZ1, ARC3 could inhibit FtsZ1 polymerization, which has been shown to be able to polymerize in vitro, but not FtsZ2 (El-Kafafi et al., 2005), preventing Z-ring formation at unwanted division sites, in a similar way as MinC acts in bacteria (Maple and Moller, 2007a, c).

Giant Chloroplast 1 (GC1, also called AtSulA) was identified as a homolog of cyanobacterial genes (*slr1223* in *Synechocystis* sp. PCC 6803, and *all2390* in *Anabaena* sp. PCC 7120) annotated as *sulA* homologs (Maple et al., 2004; Raynaud et al., 2004). In *E. coli*, SulA (suppressor of lon A) is a component of the SOS response (the so-called "save our souls" inducible DNA repair system widely present in bacteria), inhibiting cell division by interacting with FtsZ until DNA damages are repaired. No interaction between GC1 and AtFtsZ1 or AtFtsZ2 has been observed in the two-hybrid system of yeast (Maple et al., 2004; Raynaud et al., 2004), GC1 being the only one to be able to dimerize. When all2390 was over-expressed in *Anabaena* sp. PCC 7120, no clear inhibition of cell division was observed, although alteration in cell morphology was visible, when the expression of bacterial SulA in *Anabaena* sp. PCC 7120 inhibited cell division by preventing the formation of FtsZ rings (Sakr et al., 2006). GC1 has been shown to be anchored to the stromal surface of the chloroplast inner envelope membrane (Maple et al., 2004), but contradictory results have been published concerning its positive or negative function in plastid division. The annotated SulA in cyanobacteria shares only weak sequence identity to *E. coli* SulA. When Slr1223, or All2390, is used as a probe to search the database, proteins of unknown function belonging to the UPF0105 family rather than SulA are found. These proteins, including YfcH in *E. coli,* harbor a domain of unknown function (DUF1323), which appears towards the C-terminus of proteins of the NAD-dependent epimerase/dehydratase family. Interestingly, many proteins in which this domain is found are involved in cell division suggesting that GC1 belongs to this new protein family, whose function has to be determined.

B. The Host-Cell Derived Division Machinery

In addition to the Inner-PD ring and the FtsZ ring observed on the stromal side of the chloroplast, a PD ring was also observed on the cytosolic side of the envelope (Table 2.1), suggesting a second division machinery on the outer surface of the chloroplast. ARC5 (Table 2.2), the first *ARC* gene product to be identified (Gao et al., 2003), shares similarity with the dynamin superfamily of large mechano-chemical GTPases, which mediate membrane fission and fusion events in eukaryotes and have been implicated in endocytosis and mitochondrial division (Praefcke and McMahon, 2004). At about the same time of this discovery, the homolog of *ARC5* was discovered on the genome of the red alga *Cyanidioschyzon merolae* by a similarity search with dynamin sequences (Miyagishima et al., 2003). ARC5, renamed DRP5B (dynamin related protein 5B; Hong et al., 2003), was shown to be localized in the cytosol

Table 2.4. Plastid division protein interactions

	FtsZ1	FtsZ2	MinD (ARC11)	MinE	ARC6	ARC3	GC1 (AtSulA)	PDV1
FtsZ1	+ YTH[1] FRET[1] BiFC[1]							
FtsZ2	+ YTH[1] FRET[1] BiFC[1]	+ YTH[1] FRET[1] BiFC[1]						
MinD (ARC11)	– [1]	– [1]	+ YTH[2] FRET[2] BiFC[1]					
MinE	– [1]	– [1]	+ YTH[1] FRET[1] BiFC[1]	+ YTH[1] FRET[1] BiFC[1]				
ARC6	– [1]	+ YTH[1] BiFC[1]	– [1]	– [1]	+ BiFC[1]			
ARC3	+ YTH[3] BiFC[3]	– [3]	+ YTH[3] BiFC[3]	+ YTH[3] BiFC[3]	– [3]	+ YTH[3] BiFC[3]		
GC1 (AtSulA)	– [4, 5]	– [4, 5]	– [1]	– [1]	– [1]	– [3]	+ YTH[1]	
PDV1					–[6]			
PDV2					+ YTH[6]			
DRP5B (ARC5)								–[7]

In vivo studies have shown that ARC6 and ARC3 are associated with FtsZ1 and FtsZ2 in a stable complex (McAndrew et al., 2008)

References : (1) Maple et al. (2005), (2) Fujiwara et al. (2004), (3) Maple et al. (2007), (4) (Maple et al. (2004)); (5) Raynaud et al. (2004), (6) Glynn et al. (2008), (7) Miyagishima et al. (2006)

Symbols used: (+) observed in *YTH* yeast two-hybrid analysis, *FRET* fluorescence resonance energy transfer, *BiFC* bimolecular fluorescence complementation assays. (–) not observed in YTH

and to form a ring at the chloroplast division site. In this alga, the related protein associates with the chloroplasts only during the division phase and is shown to form a ring at the chloroplast division site at the late stage of division. Similarly to its function observed in endocytosis, DRP5B may generate the mechanical force allowing the outer chloroplast membrane pinching at late stages of the division process. In *A. thaliana*, at least 16 dynamin proteins have been identified and grouped into 6 functional subfamilies (DRP1 to DRP6) on the basis of their phylogeny and of the presence of functional motifs in the proteins (Hong et al., 2003). Since dynamin was found only in eukaryotic cells, it was assumed that DRP5B represents the contribution of the host cell to the chloroplast division machinery. The discovery that a dynamin-like protein, BDLP (bacterial dynamin-like protein), is present in the filamentous cyanobacterium *Nostoc punctiforme* questions the evolutionary origins of dynamins (Low and Lowe, 2006). Sequence alignments of BDLP with other dynamin family members show that BDLP is most closely related to the mitofusin class of dynamins, which includes *Drosophila melanogaster* mitochondrial FZO (fuzzy onions) homologs and *A. thaliana* chloroplast FZL (FZO-like protein). FZL has been shown to be a determinant of thylakoid and chloroplast morphology (Gao et al., 2006), and BDLP may have a similar function in *N. punctiforme* suggesting conserved functions for these proteins after the symbiosis event occurs. Phylogenetic analysis shows that DRP5B is more closely related to human and yeast dynamins (Hong et al., 2003) than to *A. thaliana* FZL and cynobacterial BDLP. A recent phylogenetic analysis reveals a close relationship of DRP5B with three of the five *Dictyostelium discoideum* (Amoebozoa) dynamins involved in cytokinesis (Miyagishima et al., 2008), thus confirming that DRP5B represents the host cell contribution to the chloroplast division machinery.

In *A. thaliana*, the recruitment of DRP5B to the division site is mediated by PDV1 and PDV2 (plastid division protein 1 and 2) (Miyagishima et al., 2006). Both proteins have a single predicted transmembrane helix and PDV1, similar to DRP5B, localizes at the division site as a discontinuous ring. DRP5B is not required for PDV1 ring assembly and the DRP5B ring forms in absence of either PDV1 or PDV2, but not in the absence of both proteins. PDV1 and PDV2 are therefore required for the proper localization of DRP5B at the division site, but if one protein alone is sufficient for DRP5B localization, both proteins are required for correct plastid division to occur (Miyagishima et al., 2006). In contrast, FtsZ ring formation is not disrupted in the plants that are not expressing any of these cytosolic proteins, suggesting that these proteins act after FtsZ ring formation.

V. Assembly of the Plastid Division Machineries

As deduced from the above studies with the characterization of the proteins participating directly in the mechanism of chloroplast division, a temporal mechanism is involved in chloroplast division, relying on the sequential transition of the FtsZ, PD and dynamin rings (Miyagishima et al., 2001c). A sequential mechanism has also been proposed to occur in the formation of the bacterial division machinery, which usually harbors a single FtsZ ring with a few exceptions such as *Streptococcus pneumoniae* having two different rings, the FtsZ ring and another one formed by the high-molecular-weight PBPs (penicillin-binding proteins) (Morlot et al., 2004). In Bacteria, the division ring is assembled outwards, starting from the cytoplasmic components and then progressing toward the outer membrane by recruiting the membrane and periplasmic division proteins (Romberg and Levin, 2003; Margolin, 2005; Vicente et al., 2006). In *E. coli*, the division ring is nucleated by the assembly of FtsZ, and the other proteins are incorporated sequentially: The flow is as follows: FtsZ > [FtsA, ZapA, ZipA] > (FtsE, FtsX) > FtsK > FtsQ > (FtsB, FtsL) > FtsW > FtsI > FtsN > AmiC > EnvC, where the proteins within brackets are independent of each other but dependent on FtsZ and those within parentheses assemble simultaneously (Vincente et al., 2006) (Fig. 2.5a).

A similar pattern of division, starting in the stroma and progressing to the cytosolic side has been proposed for chloroplast division (Fig. 2.5b). The first event is the localization of FtsZ (FtsZ1 and FtsZ2 in green plastids) at the future division site upon the concerted action of chloroplast MinD, MinE, and possibly ARC3, to form the

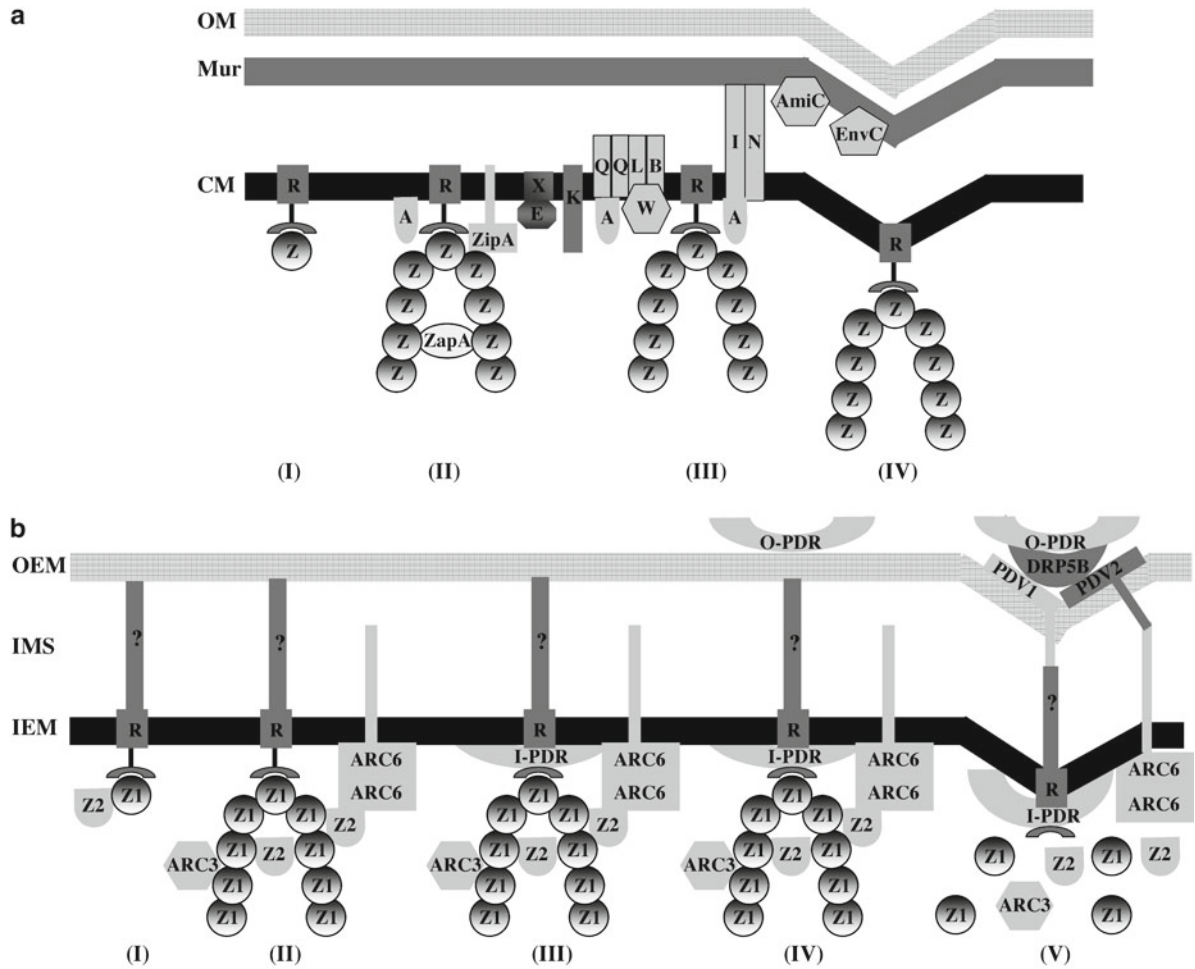

Fig. 2.5. Schematic representation of bacterial and plastidial division machineries. (**a**) Sequences of events during *E. coli* division. Stage I: FtsZ locates at the unknown cell membrane receptor (R). Stage II: assembly of the FtsZ ring dependent on either FtsA or ZipA or both, and ZapA. Stage III: Recruitment of FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, FtsW and then FtsI, FtsN, AmiC and EnvC. Stage IV: cytokinesis. Protein names have been abbreviated by excluding "Fts" from them. *OM* outer membrane, *Mur* murein, *CM* cytoplasmic membrane. (**b**) Sequences of events during chloroplast division in plants. Stage I: FtsZ1 (Z1) together with FtsZ2 (Z2) locate at the plastid division site at the unknown inner envelope membrane receptor (R). Stage II: Assembly of FtsZ1 ring dependent on FtsZ2, ARC6 and ARC3. Stage III, formation of the inner-plastid dividing ring (I-PDR). Stage IV: formation of the outer-plastid dividing ring (O-PDR). Stage V: formation of the dynamin ring (DRP5B), membrane constriction, which is followed by FtsZ ring disassembly and final pinching off for complete cytokinesis. The unknown FtsZ1 receptor is indicated with a question mark (?). *OEM* outer envelope membrane, *IMS* inner membrane space, *IEM* inner envelope membrane.

Z-ring. Association of the ring to the membrane could be mediated by ARC6, the bitopic inner envelope membrane protein, which has a membrane anchor. The N-terminus of ARC6 resides in the stroma (Vitha et al., 2003) and interacts with FtsZ2 but not FtsZ1 (Table 2.4) via the C-terminal core domain, thus tethering FtsZ2 to the membrane and allowing the recruitment of FtsZ1. The association of FtsZ2, but not FtsZ1, with the inner membrane, together with FtsZ2-FtsZ1 interaction (Table 2.4) and the in vitro properties of FtsZ2 in promoting FtsZ1 polymerization, support this model (El-Kafafi et al., 2005). Once the FtsZ ring is assembled, the inner PD ring forms first, followed by the middle ring (when present), and the outer PD ring and the chloroplast begins to constrict (Miyagishima et al., 2001a, 2001c, 2003). During the late constriction stage, following the localization of PDV1 and PDV2 to the outer envelope membrane, the dynamin ring forms on

the cytosolic side of the outer PD ring. The additional middle ring, found in the intermembrane space in *C. merolae*, has not been observed in plant chloroplasts. Whether FtsZ2 fulfills this function in green plants is not yet known, but FtsZ2 has been found to be associated with both the inner and outer membrane in spinach and could form a physical link from the inside to the outside of the chloroplast, thus coordinating the inner and outer division machineries (El-Kafafi et al., 2005). This hypothesis is supported by the finding that membrane-free machineries, containing both FtsZ and dynamins from *C. merolae* do exist (Yoshida et al., 2006). In *A. thaliana*, in addition to FtsZ2, such linking proteins include ARC6 and PDV2, which are transmembrane proteins, extending in the intermembrane space. These two proteins interact and this interaction is required for full chloroplast division activity in *A. thaliana* (Glynn et al., 2007; 2008). In analogy with the bacterial protein flow in bacteria, the protein flow in plastid division could be as follows: FtsZ1, FtsZ2 > ARC6, ARC3 > inner-PD ring > outer-PD ring > (PDV2, PDV1) > DRP5B, where proteins within parentheses assemble simultaneously.

VI. Conclusions

What took millions of years for nature to achieve, man has been able to decipher, at least partly, in a few decades. This achievement was made possible because of the constant scientific progress made during these last few centuries. The progress made in the observation of cells and the consequent discovery of the endosymbiotic origin of organelles were the starting point of the exciting discoveries that were to follow. The judicious choice of few models, such as the higher plant *Arabidopsis thaliana*, allowing forward and reverse genetics, the red unicellular alga *Cyanidioschyzon merolae*, harboring one mitochondrion and one plastid per cell, and the multicellular moss *Physcomitrella patens*, competent for homologous recombination, allowed in less than two decades the elaboration of a model for chloroplast division. The main feature of this model was the discovery that chloroplast division is mediated by two distinct division machineries, one inside the chloroplast of bacterial origin and the second one, outside the chloroplast, of host origin. As in bacteria, a sequential recruitment of the components operates from the inside to the outside of the chloroplast. With a few exceptions, such as ARC3 that can be considered as a novel protein involved in plastid division, many proteins involved in the process can be traced to the origin of the organisms that gave rise to the endosymbiotic organisms.

However, many questions remain unanswered and need to be addressed: Why do chloroplasts need four or five rings in order to divide and what are the components of the inner, middle and outer PD rings? Are all the rings required for constriction and how are they coordinated? ARC6 and PDV2, which extend in the intermembrane space, together with FtsZ2, found associated with the outer membrane, could play a role in linking the stromal and cytosolic division machineries. How do plastids divide in organisms lacking some of the key proteins, and how do organisms lacking MinD and MinE, such as the red algae and heterokonts, select their division site?

A major question in complex green organisms concerns how plastid division is controlled during development. The characterization of proteins not directly involved in the mechanism of division, but interacting or co-localizing with the participating proteins, is a starting point for elucidating these mechanisms in *A. thaliana*. The prereplication factor AtCDT1 is involved not only in nuclear DNA replication but also in plastid division by means of an interaction with ARC6 (Raynaud et al., 2005). In *A. thaliana*, the mechanosensitive channels of small conductance-like proteins (MSLs), co-localize with MinE and may control chloroplast division at the level of the individual chloroplast (Haswell and Meyerowitz, 2006) and *Crumpled Leaf* (CRL) could be involved in cell division, cell differentiation and plastid division (Asano et al., 2004). Chloroplast DNA replication may also play a role in plastid division, since *crinkled leaves 8*, a mutation in the large subunit of ribonucleotide reductase, leads to defects in leaf development and chloroplast division (Garton et al., 2007). If new proteins are involved in the control of plastid division, plastid division proteins might be involved in new functions. In the moss *Physcomitrella patens*, different FtsZ1 isoforms are present and one of them, FtsZ1-2, is not only located in the chloroplast but also in the cytoplasm, assembling into rings in both cell compartments, thus suggesting that this protein might connect cell and organelle division

at least in this organism (Kiessling et al., 2004). Recently, the second FtsZ1-1 isoform has been found, upon over-expression of a GFP (green fluorescent protein) fusion, in strands interconnecting chloroplasts, thus raising the question of whether the protein is a structural component of the stromules (Gremillon et al., 2007). It was also suggested that the FtsZ2 isoforms could be involved in the maintenance of chloroplast integrity, with plastoskeleton function (Kiessling et al., 2000; Reski, 2002) as also recently suggested for FtsZ in a filamentous cyanobacteria (Klint et al., 2007).

In higher plants, FtsZ isoforms may also have additional functions. FtsZ1 was recently shown to be associated with the thylakoids in *A. thaliana*. This association is developmentally regulated, suggesting a function of plant FtsZ1 in thylakoid organization during leaf development. These results highlight new functions for plastid FtsZs (El-Kafafi et al., 2008). Further studies to characterize the functions of the new proteins impairing plastid division, and the new functions acquired by plastid division proteins, will help to connect plastid division together with other processes in plant development.

Additional plant-specific proteins involved in the chloroplast division machinery in plants have been described. The plant-specific protein MCD1 determines the site of chloroplast division in concert with bacteria-derived MinD thus suggesting that the plant cell evolved a component to modulate the cyanobacteria-derived Min system so as to regulate chloroplast FtsZ ring positioning (Nakanishi et al., 2009). A novel chloroplast division site determinant factor sharing homology with ARC6, but only detected in vascular plants, has been characterized in two independent studies. This protein named PARC6 (Glynn et al., 2009) or CDP1 (Zhang et al., 2009) was shown to interact with ARC3. Unlike ARC6 promoting FtsZ assembly (Vitha et al., 2003), PARC6 inhibits FtsZ assembly and is required for PDV1 localisation, but not for PDV2 localisation or ARC5 recruitment (Glynn et al., 2009).

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References

Abdallah F, Salamini F and Leister D (2000) A prediction of the size and evolutionary origin of the proteome of chloroplasts of *Arabidopsis*. Trends Plant Sci 5: 141–142

Aldridge C and Moller SG (2005) The plastid division protein AtMinD1 is a Ca2+−ATPase stimulated by AtMinE1. J Biol Chem 280: 31673–31678

Archibald JM and Keeling PJ (2002) Recycled plastids: a 'green movement' in eukaryotic evolution. Trends Genet 18: 577–584

Asano T, Yoshioka Y, Kurei S, Sakamoto W and Machida Y (2004) A mutation of the *CRUMPLED LEAF* gene that encodes a protein localized in the outer envelope membrane of plastids affects the pattern of cell division, cell differentiation, and plastid division in *Arabidopsis*. Plant J 38: 448–459

Beech PL and Gilson PR (2000) FtsZ and organelle division in Protists. Protist 151: 11–16

Bhattacharya D, Yoon HS and Hackett JD (2004) Photosynthetic eukaryotes unite: endosymbiosis connects the dots. Bioessays 26: 50–60

Bhattacharya D, Archibald JM, Weber AP and Reyes-Prieto A (2007) How do endosymbionts become organelles? Understanding early events in plastid evolution. Bioessays 29: 1239–1246

Block MA, Douce R, Joyard J and Rolland N (2007) Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. Photosynth Res 92: 225–244

Boasson R and Laetsch WM (1969) Chloroplast replication and growth in tobacco. Science 166: 749–751

Bouck BJ (1962) Chromatophore development, pits, and other fine structure in the red alga, *Lomentaria baileyan*a (Harv.) Farlow. J Cell Biol 12: 553–569

Brocks JJ, Logan GA, Buick R and Summons RE (1999) Archean molecular fossils and the early rise of eukaryotes. Science 285: 1033–1036

Cavalier-Smith T (2006) Cell evolution and earth history: stasis and revolution. Philos Trans R Soc Lond B Biol Sci 361: 969–1006

Chaly N and Possingham JV (1981) Structure of constricted proplastids in meristematic plant tissues. Biol Cell 41: 203–210

Chaly N, Possingham JV and Thomson WW (1980) Chloroplast division in spinach leaves examined by scanning electron microscopy and freeze-etching. J Cell Sci 46: 87–96

Chida Y and Ueda K (1991) Division of chloroplasts in a green alga, *Trebouxia potteri*. Ann Bot 67: 435–442

Colletti KS, Tattersall EA, Pyke KA, Froelich JE, Stokes KD and Osteryoung KW (2000) A homologue of the bacterial cell division site-determining factor MinD mediates placement of the chloroplast division apparatus. Curr Biol 10: 507–516

Dangeard P (1947) Cytologie Végétale et Cytologie Générale. Paul Lechevalier, Paris

Delwiche CF (1999) Tracing the thread of plastid diversity through the tapestry of life. Am Nat 154: S164–S177

Din N, Quardokus EM, Sackett MJ and Brun YV (1998) Dominant C-terminal deletions of FtsZ that affect its ability to localize in *Caulobacter* and its interaction with FtsA. Mol Microbiol 27: 1051–1063

Dinkins R, Reddy MS, Leng M and Collins GB (2001) Overexpression of the *Arabidopsis thaliana MinD1* gene alters chloroplast size and number in transgenic tobacco plants. Planta 214: 180–188

Doolittle WF (2000) The nature of the universal ancestor and the evolution of the proteome. Curr Opin Struct Biol 10: 355–358

Douglas SE (1998) Plastid evolution: origins, diversity, trends. Curr Opin Genet Dev 8: 655–661

Douglas SE and Penny SL (1999) The plastid genome of the cryptophyte alga, *Guillardia theta*: complete sequence and conserved synteny groups confirm its common ancestry with red algae. J Mol Evol 48: 236–244

Douglas SE, Murphy CA, Spencer DF and Gray MW (1991) Cryptomonad algae are evolutionary chimaeras of two phylogenetically distinct unicellular eukaryotes. Nature 350: 148–151

Doutreligne J (1935) Note sur la structure des chloroplastes. Porc Akad Wetensch Amsterd 38: 886–896

Duckett JG and Ligrone R (1993a) Plastid-dividing rings in ferns. Ann Bot 72: 619–627

Duckett JG and Ligrone R (1993b) Plastid-dividing rings in the liverwort *Odontoschisma denudatum* (Mart) Dum. (Jungermanniales, Hepaticae). Gio Bot Ital 127: 318–319

Dyall SD, Brown MT and Johnson PJ (2004) Ancient invasions: from endosymbionts to organelles. Science 304: 253–257

El-Kafafi S, Mukherjee S, El-Shami M, Putaux JL, Block MA, Pignot-Paintrand I, Lerbs-Mache S and Falconet D (2005) The plastid division proteins, FtsZ1 and FtsZ2, differ in their biochemical properties and sub-plastidial localization. Biochem J 387: 669–676

El-Kafafi S, Karamoko M, Pignot-Paintrand I, Grunwald D, Mandaron P, Lerbs-Mache S and Falconet D (2008) Developmentally regulated association of plastid division protein FtsZ1 with thylakoid membranes in *Arabidopsis thaliana*. Biochem J 409: 87–94

El-Shami M, El-Kafafi S, Falconet D and Lerbs-Mache S (2002) Cell cycle-dependent modulation of *FtsZ* expression in synchronized tobacco BY2 cells. Mol Genet Genomics 267: 254–261

Esser C and Martin W (2007) Supertrees and symbiosis in eukaryote genome evolution. Trends Microbiol 15: 435–437

Fasse-Franzisket (1955) Die Teilung der Proplastiden und Choroplasten by *Agapanthus umbellatus* l'hérit. Protoplasma 45: 194–227

Fischer WW (2008) Biogeochemistry: Life before the rise of oxygen. Nature 455: 1051–1052

Forterre P and Gribaldo S (2007) The origin of modern terrestrial life. HFSP Journal 1: 156–168

Forterre P and Philippe H (1999) Where is the root of the universal tree of life? Bioessays 21: 871–879

Fujiwara MT, Nakamura A, Itoh R, Shimada Y, Yoshida S and Moller SG (2004) Chloroplast division site placement requires dimerization of the ARC11/AtMinD1 protein in Arabidopsis. J Cell Sci 117: 2399–2410

Gaikwad A, Babbarwal V, Pant V and Mukherjee SK (2000) Pea chloroplast FtsZ can form multimers and correct the thermosensitive defect of an *Escherichia coli ftsZ* mutant. Mol Gen Genet 263: 213–221

Gantt E and Arnott HJ (1963) Chloroplast division in the gametophyte of the fern *Matteucia struthiopteris* (L.) Todaro. J Cell Biol 19: 446–448

Gao H, Kadirjan-Kalbach D, Froehlich JE and Osteryoung KW (2003) ARC5, a cytosolic dynamin-like protein from plants, is part of the chloroplast division machinery. Proc Natl Acad Sci USA 100: 4328–4333

Gao H, Sage TL and Osteryoung KW (2006) FZL, an FZO-like protein in plants, is a determinant of thylakoid and chloroplast morphology. Proc Natl Acad Sci USA 103: 6759–6764

Garton S, Knight H, Warren GJ, Knight MR and Thorlby GJ (2007) *crinkled leaves 8* – a mutation in the large subunit of ribonucleotide reductase – leads to defects in leaf development and chloroplast division in *Arabidopsis thaliana*. Plant J 50: 118–127

Gibbs SP (1978) The chloroplast of *Euglena* may have evolved from symbiotic green algae. Can J Bot 56: 2883–2889

Glynn JM, Miyagishima SY, Yoder DW, Osteryoung KW and Vitha S (2007) Chloroplast division. Traffic 8: 451–461

Glynn JM, Froehlich JE and Osteryoung KW (2008) *Arabidopsis* ARC6 coordinates the division machineries of the inner and outer chloroplast membranes through interaction with PDV2 in the intermembrane space. Plant Cell 20: 2460–2470

Glynn JM, Yang Y, Vitha S, Schmitz AJ, Hemmes M, Miyagishima SY and Osteryoung KW (2009) PARC6, a novel chloroplast division factor, influences FtsZ assembly and is required for recruitment of PDV1 during chloroplast division in *Arabidopsis*. Plant J 59: 700–711

Gray MW, Burger G and Lang BF (1999) Mitochondrial evolution. Science 283: 1476–1481

Green PB (1964) Cinematic observations on the growth and division of chloroplasts in *Nitella*. Am J Bot 51: 334–342

Gremillon L, Kiessling J, Hause B, Decker EL, Reski R and Sarnighausen E (2007) Filamentous temperature-sensitive

Z (FtsZ) isoforms specifically interact in the chloroplasts and in the cytosol of *Physcomitrella patens*. New Phytol 176: 299–310

Gris JBA (1857) Recherches microscopiques sur la chlorophylle. Ann Sci Nat Bot Ser IV 7: 179–219

Gunning B, Koenig F and Govindjee (2006) A dedication to pionners of research on chloroplast structure. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp xxiii–xxxi. Springer, Dordrecht

Hale CA, Rhee AC and de Boer PA (2000) ZipA-induced bundling of FtsZ polymers mediated by an interaction between C-terminal domains. J Bacteriol 182: 5153–5166

Hashimoto H (1986) Double ring structure around the constricting neck of dividing plastids of *Avena sativa*. Protoplasma 135: 166–172

Hashimoto H (1992) Involvement of actin filaments in chloroplast division of the alga *closterium ehrengergii*. Protoplasma 167: 88–96

Hashimoto H (1997) Electron-opaque annular structure girdling the constriction isthmus of the dividing chloroplasts of *Heterosigma akashiwo* (Raphidophyceae, Chromophyta). Protoplasma 197: 210–216

Hashimoto H (2003) Plastid division: its origins and evolution. Int Rev Cytol 222: 63–98

Hashimoto H (2005) The ultrastructural features and division of secondary plastids. J Plant Res 118: 163–172

Hashimoto H and Possingham JV (1989) Division and DNA distribution in ribosome-deficient plastids of the barley mutant "albostrians". Protoplasma 149: 20–23

Haswell ES and Meyerowitz EM (2006) MscS-like proteins control plastid size and shape in *Arabidopsis thaliana*. Curr Biol 16: 1–11

Hayashida A, Takechi K, Sugiyama M, Kubo M, Itoh RD, Takio S, Fujita T, Hiwatashi Y, Hasebe M and Takano H (2005) Isolation of mutant lines with decreased numbers of chloroplasts per cell from a tagged mutant library of the moss *Physcomitrella patens*. Plant Biol (Stuttg) 7: 300–306

Heitz E (1936) Untersuchungen über den Bau der Plastiden. I. Die Gerichteten Chlorophyllscheiben der Chloroplasten. Planta 26: 134–163

Holland HD (2006) The oxygenation of the atmosphere and oceans. Philos Trans R Soc Lond B Biol Sci 361: 903–915

Hollande AC and Hollande G (1941) La structure des chloroplastes. Cell and Tissue Res 31: 648–652

Hong Z, Bednarek SY, Blumwald E, Hwang I, Jurgens G, Menzel D, Osteryoung KW, Raikhel NV, Shinozaki K, Tsutsumi N and Verma DP (2003) A unified nomenclature for *Arabidopsis* dynamin-related large GTPases based on homology and possible functions. Plant Mol Biol 53: 261–265

Hu Z, Mukherjee A, Pichoff S and Lutkenhaus J (1999) The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. Proc Natl Acad Sci USA 96: 14819–14824

Iino M and Hashimoto H (2003) Intermediate features of cyanelle division of *Cyanophora paradoxa* (Glaucocystophyta) between cyanobacterial and chloroplast division. J Phycol 39: 561–569

Itoh R, Fujiwara M, Nagata N and Yoshida S (2001) A chloroplast protein homologous to the eubacterial topological specificity factor minE plays a role in chloroplast division. Plant Physiol 127: 1644–1655

Kameya T and Takahashi N (1971) Division of chloroplast in vitro. Jap J Genet 46: 153–157

Kanamaru K, Fujiwara M, Kim M, Nagashima A, Nakazato E, Tanaka K and Takahashi H (2000) Chloroplast targeting, distribution and transcriptional fluctuation of AtMinD1, a Eubacteria-type factor critical for chloroplast division. Plant Cell Physiol 41: 1119–1128

Kausche GA and Ruska H (1940) Zur Frage der Chloroplastenstruktur. Naturwiss 28: 303–304

Keeling PJ (2004) Diversity and evolutionary history of plastids and their hosts. Am J Bot 91: 1481–1493

Kiessling J, Kruse S, Rensing SA, Harter K, Decker EL and Reski R (2000) Visualization of a cytoskeleton-like FtsZ network in chloroplasts. J Cell Biol 151: 945–950

Kiessling J, Martin A, Gremillon L, Rensing SA, Nick P, Sarnighausen E, Decker EL and Reski R (2004) Dual targeting of plastid division protein FtsZ to chloroplasts and the cytoplasm. EMBO Rep 5: 889–894

Kirk JTO and Tilney-Bassett RAE (1978) The plastids: Their Chemistry, Structure, Growth and Inheritance. Elsevier/North Holland Biochemical Press, Amsterdam

Klint J, Rasmussen U and Bergman B (2007) FtsZ may have dual roles in the filamentous cyanobacterium *Nostoc/Anabaena* sp. strain PCC 7120. J Plant Physiol 164: 11–18

Koksharova OA and Wolk CP (2002) A novel gene that bears a DnaJ motif influences cyanobacterial cell division. J Bacteriol 184: 5524–5528

Kulandaivelu G and Gnanam A (1985) Scanning electron microscope evidence for a budding mode of chloroplast multiplication in higher plants. Physiol Plant 63: 299–302

Kuroiwa T (1989) The nuclei of cellular organelles and the formation of daughter organelles by the "plastid dividing ring". Bot Mag 102: 291–329

Kuroiwa T (2000) The discovery of the division apparatus of plastids and mitochondria. J Electron Microsc (Tokyo) 49: 123–134

Kuroiwa T, Kuroiwa H, Sakai A, Takahashi H, Toda K and Itoh R (1998) The division apparatus of plastids and mitochondria. Int Rev Cytol 181: 1–41

Kuroiwa H, Mori T, Takahara M, Miyagishima SY and Kuroiwa T (2002) Chloroplast division machinery as revealed by immunofluorescence and electron microscopy. Planta 215: 185–190

Kusunoki S and Kawasaki Y (1936) Beobachtungen über die Chloroplastenteilung by Einigen Blutenpflanzen. Cytologia 7: 530–534

Larkum AW, Lockhart PJ and Howe CJ (2007) Shopping for plastids. Trends Plant Sci 12: 189–195

Leech RM and Pyke K (1988) Chloroplast division in higher plants with particular reference to wheat. In: Boffey SA and Lloyd D (eds) The division and Segregation of Organelles, pp 31–62. Cambridge University Press, Cambridge, UK

Leech RM, Thomson WW and Platt-Aloia KA (1981) Observations of the mechanisms of chloroplast division in higher plants. New Phytol 87: 1–9

Lemieux C, Otis C and Turmel M (2000) Ancestral chloroplast genome in Mesostigma viride reveals an early branch of green plant evolution. Nature 403: 649–652

Lemieux C, Otis C and Turmel M (2007) A clade uniting the green algae *Mesostigma viride* and *Chlorokybus atmophyticus* represents the deepest branch of the Streptophyta in chloroplast genome-based phylogenies. BMC Biol 5: 1–17

Lindsay MR, Webb RI, Strous M, Jetten MS, Butler MK, Forde RJ and Fuerst JA (2001) Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. Arch Microbiol 175: 413–429

Liu WZ, Hu Y, Zhang RJ, Zhou WW, Zhu JY, Liu XL and He YK (2007) Transfer of a eubacteria-type cell division site-determining factor *CrMinD gene* to the nucleus from the chloroplast genome in *Chlamydomonas reinhardtii*. Chin. Sci. Bull. 52: 2514–2521

Liu Z, Mukherjee A and Lutkenhaus J (1999) Recruitment of ZipA to the division site by interaction with FtsZ. Mol Microbiol 31: 1853–1861

Low HH and Lowe J (2006) A bacterial dynamin-like protein. Nature 444: 766–769

Luck BT and Jordan EG (1980) The mitochondria and plastids during microsporogenesis in *Hyacinthoides non-scripta* (L.) Chouard. Ann Bot (Lond) 45: 511–514

Lumière A (1919) Le Mythe des Symbiotes. Masson & Cie, Paris

Lutkenhaus J (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. Annu Rev Biochem 76: 539–562

Lutkenhaus J and Addinall SG (1997) Bacterial cell division and the Z ring. Ann Rev Biochem 66: 93–116

Ma X and Margolin W (1999) Genetic and functional analyses of the conserved C-terminal core domain of *Escherichia coli* FtsZ. J Bacteriol 181: 7531–7544

Maple J and Moller SG (2007a) Plastid division: evolution, mechanism and complexity. Ann Bot (Lond) 99: 565–579

Maple J and Moller SG (2007b) Interdependency of formation and localisation of the Min complex controls symmetric plastid division. J Cell Sci 120: 3446–3456

Maple J and Moller SG (2007c) Plastid division coordination across a double-membraned structure. FEBS Lett 581: 2162–2167

Maple J, Chua NH and Moller SG (2002) The topological specificity factor AtMinE1 is essential for correct plastid division site placement in *Arabidopsis*. Plant J 31: 269–277

Maple J, Fujiwara MT, Kitahata N, Lawson T, Baker NR, Yoshida S and Moller SG (2004) GIANT CHLOROPLAST 1 is essential for correct plastid division in *Arabidopsis*. Curr Biol 14: 776–781

Maple J, Aldridge C and Moller SG (2005) Plastid division is mediated by combinatorial assembly of plastid division proteins. Plant J 43: 811–823

Maple J, Vojta L, Soll J and Moller SG (2007) ARC3 is a stromal Z-ring accessory protein essential for plastid division. EMBO Rep 8: 293–299

Margolin W (2005) FtsZ and the division of prokaryotic cells and organelles. Nat Rev Mol Cell Biol 6: 862–871

Marin B, Nowack EC and Melkonian M (2005) A plastid in the making: evidence for a second primary endosymbiosis. Protist 156: 425–432

Marrison JL, Rutherford SM, Robertson EJ, Lister C, Dean C and Leech RM (1999) The distinctive roles of five different *ARC* genes in the chloroplast division process in Arabidopsis. Plant J 18: 651–662

Martin W and Kowallik KV (1999) Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche'. Eur J Phycol 34: 287–295

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M and Penny D (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci USA 99: 12246–12251

Matsuzaki M, Kikuchi T, Kita K, Kojima S and Kuroiwa T (2001) Large amounts of apicoplast nucleoid DNA and its segregation in *Toxoplasma gondii*. Protoplasma 218: 180–191

McAndrew RS, Olson BJ, Kadirjan-Kalbach DK, Chi-Ham CL, Vitha S, Froehlich JE and Osteryoung KW (2008) In vivo quantitative relationship between plastid division proteins FtsZ1 and FtsZ2 and identification of ARC6 and ARC3 in a native FtsZ complex. Biochem J 412: 367–378

McFadden GI and Van Dooren GG (2004) Evolution: red algal genome affirms a common origin of all plastids. Curr Biol 14: R514–516

McFadden GI, Gilson PR and Waller RF (1995) Molecular phylogeny of chlorarachniophytes based on plastid rRNA and rbcL sequences. Archiv Protistenk 145: 231–239

Menke W (1940) Untersuchungen über den Feinbau des Protoplasmas mit dem Universal-Elektronenmikroskop. Protoplasma 35: 115–130

Mereschkowsky C (1905) Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biol. centralblatt 25:

593–604. English translation in Martin W, Kowallik KV (1999) Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche'. Eur J Phycol 1934: 1287–1295

Meyer A (1883) Das Chlorophyllkorn in Chemischer, Morphologischer und Biologischer Beziehung. Felix S, Leipzig

Misumi O, Yoshida Y, Nishida K, Fujiwara T, Sakajiri T, Hirooka S, Nishimura Y and Kuroiwa T (2008) Genome analysis and its significance in four unicellular algae, *Cyanidioshyzon merolae*, *Ostreococcus tauri*, *Chlamydomonas reinhardtii*, and *Thalassiosira pseudonana*. J Plant Res 121: 3–17

Mita T and Kuroiwa T (1988) Division of plastids by a plastid-dividing ring in *Cyanidium caldarium*. Protoplasma Suppl 1: 133–152

Mita T, Kanbe T, Tanaka T and Kuroiwa T (1986) A ring structure around the dividing plane of the *Cyanidium calderium* chloroplast. Protoplasma 130: 211–213

Miyagishima SY and Kuroiwa T (2006) The mechanism of plastid division: the structure and origin of the plastid division apparatus. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 103–121. Springer, Dordrecht

Miyagishima SY, Ito M, Toda K, Takabayashi A, Kuroiwa H and Kuroiwa T (1998) Identification of a triple ring structure involve in plastid division in the primitive red alga *Cyanidioschyzon merolae*. J Electron Microsc 47: 269–272

Miyagishima SY, Itoh R, Toda K, Kuroiwa H and Kuroiwa T (1999) Real-time analyses of chloroplast and mitochondrial division and differences in the behavior of their dividing rings during contraction. Planta 207: 343–353

Miyagishima SY, Kuroiwa H and Kuroiwa T (2001a) The timing and manner of disassembly of the apparatuses for chloroplast and mitochondrial division in the red alga *Cyanidioschyzon merolae*. Planta 212: 517–528

Miyagishima SY, Takahara M and Kuroiwa T (2001b) Novel filaments 5 nm in diameter constitute the cytosolic ring of the plastid division apparatus. Plant Cell 13: 707–721

Miyagishima SY, Takahara M, Mori T, Kuroiwa H, Higashiyama T and Kuroiwa T (2001c) Plastid division is driven by a complex mechanism that involves differential transition of the bacterial and eukaryotic division rings. Plant Cell 13: 2257–2268

Miyagishima SY, Nishida K, Mori T, Matsuzaki M, Higashiyama T, Kuroiwa H and Kuroiwa T (2003) A plant-specific dynamin-related protein forms a ring at the chloroplast division site. Plant Cell 15: 655–665

Miyagishima SY, Nozaki H, Nishida K, Nishida K, Matsuzaki M and Kuroiwa T (2004) Two types of FtsZ proteins in mitochondria and red-lineage chloroplasts: the duplication of FtsZ is implicated in endosymbiosis. J Mol Evol 58: 291–303

Miyagishima SY, Froehlich JE and Osteryoung KW (2006) PDV1 and PDV2 mediate recruitment of the dynamin-related protein ARC5 to the plastid division site. Plant Cell 18: 2517–2530

Miyagishima SY, Kuwayama H, Urushihara H and Nakanishi H (2008) Evolutionary linkage between eukaryotic cytokinesis and chloroplast division by dynamin proteins. Proc Natl Acad Sci U S A 105: 15202–15207

Miyake NH and Taniguchi T (1995) Ultrastructural changes of chloroplasts in peanut mesophyll protoplasts treated with electric fields. Jpn J Crop Sci 64: 131–138

Modrusan Z and Wrischer M (1990) Studies on chloroplast division in young leaf tissues of some higher plants. Protoplasma 154: 1–7

Moehs CP, Tian L, Osteryoung KW and Dellapenna D (2001) Analysis of carotenoid biosynthetic gene expression during marigold petal development. Plant Mol Biol 45: 281–293

Moreira D, Le Guyader H and Philippe H (2000) The origin of red algae and the evolution of chloroplasts. Nature 405: 69–72

Morlot C, Noirclerc-Savoye M, Zapun A, Dideberg O and Vernet T (2004) The D,D-carboxypeptidase PBP3 organizes the division process of *Streptococcus pneumoniae*. Mol Microbiol 51: 1641–1648

Mosyak L, Zhang Y, Glasfeld E, Haney S, Stahl M, Seehra J and Somers WS (2000) The bacterial cell-division protein ZipA and its interaction with an FtsZ fragment revealed by X-ray crystallography. EMBO J 19: 3179–3191

Mühlethaler K (1960) Die Struktur der Grana- und Stroma-Lamellen in Chloroplasten. Z Wissensch Mikroskopie 8: 444–452

Nakanishi H, Suzuki K, Kabeya Y and Miyagishima SY (2009) Plant-specific protein MCD1 determines the site of chloroplast division in concert with bacteria-derived MinD. Curr Biol 19: 151–156

Nass S and Nass MMK (1963) Intramitochondrial fibers with DNA characteristics. J Cell Biol 19: 613–628

Nisbet EG and Sleep NH (2001) The habitat and nature of early life. Science 409: 1083–1091

Nogales E, Wolf SG and Downing KH (1998) Structure of the alpha beta tubulin dimer by electron crystallography. Nature 391: 199–203

Nägeli C (1846) Bläschenförmige Gebilde im Inhalte der Pflanzenzelle. Z Wiss Bot 3/4: 94–128

Ogawa S, Ueda K and Noguchi T (1995) Division apparatus of the chloroplast in *Nannochloris Bacillaris* (Chlorophyta). J Phycol 31: 132–137

Oross JW and Possingham JV (1989) Ultrastructural features of the constricted region of dividing plastids. Protoplasma 150: 131–138

Osteryoung KW and Vierling E (1995) Conserved cell and organelle division. Nature 376: 473–474

Osteryoung KW and McAndrew RS (2001) The plastid division machine. Annu Rev Plant Physiol Plant Mol Biol 52: 315–333

Osteryoung KW, Stokes KD, Rutherford SM, Percival AL and Lee WY (1998) Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial *ftsZ*. Plant Cell 10: 1991–2004

Palmer JD (2003) The symbiotic birth and spread of plastids: how many times and whodunit? J Phycol 39: 4–11

Poole AM and Penny D (2007a) Evaluating hypotheses for the origin of eukaryotes. Bioessays 29: 74–84

Poole AM and Penny D (2007b) Eukaryote evolution: engulfed by speculation. Nature 447: 913

Portier P (1918) Les Symbiotes. Masson et Cie, Paris

Possingham JV and Lawrence ME (1983) Controls to plastid division. Int Rev Cytol 84: 1–56

Possingham JV and Rose RJ (1976) Chloroplast replication and chloroplast DNA synthesis in spinach leaves. Proc. Roy. Soc. London B 193: 295–305

Possingham JV and Saurer W (1969) Change in chloroplast number per cell during leaf development in spinach. Planta 86: 186–194

Praefcke GJ and McMahon HT (2004) The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol 5: 133–147

Pyke KA (1999) Plastid division and development. Plant Cell 11: 549–556

Pyke KA and Leech RM (1991) Rapid image analysis screening procedure for identifying chloroplast number mutants in mesophyll cells of *Arabidopsis thaliana* (L.) Heynh. Plant Physiol 96: 1193–1195

Pyke KA and Leech RM (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. Plant Physiol 99: 1005–1008

Pyke KA and Leech RM (1994) A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana*. Plant Physiol 104: 201–207

Pyke KA and Page AM (1998) Plastid ontogeny during petal development in *Arabidopsis*. Plant Physiol 116: 797–803

Pyke KA, Rutherford SM, Robertson EJ and Leech RM (1994) *arc6*, a fertile *Arabidopsis* mutant with only two mesophyll cell chloroplasts. Plant Physiol 106: 1169–1177

Randolf LF (1922) Cytology of chlorophyll type of maize. Bot Gaz 73: 337–375

Raskin DM and de Boer PA (1999) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. Proc Natl Acad Sci USA 96: 4971–4976

Rasmussen B, Fletcher IR, Brocks JJ and Kilburn MR (2008) Reassessing the first appearance of eukaryotes and cyanobacteria. Nature 455: 1101–1104

RayChaudhuri D (1999) ZipA is a MAP-Tau homolog and is essential for structural integrity of the cytokinetic FtsZ ring during bacterial cell division. EMBO J 18: 2372–2383

Raynaud C, Cassier-Chauvat C, Perennes C and Bergounioux C (2004) An Arabidopsis homolog of the bacterial cell division inhibitor SulA is involved in plastid division. Plant Cell 16: 1801–1811

Raynaud C, Perennes C, Reuzeau C, Catrice O, Brown S and Bergounioux C (2005) Cell and plastid division are coordinated through the prereplication factor AtCDT1. Proc Natl Acad Sci USA 102: 8216–8221

Reddy MS, Dinkins R and Collins GB (2002) Overexpression of the Arabidopsis thaliana *MinE1* bacterial division inhibitor homologue gene alters chloroplast size and morphology in transgenic Arabidopsis and tobacco plants. Planta 215: 167–176

Reski R (2002) Rings and networks: the amazing complexity of FtsZ in chloroplasts. Trends Plant Sci 7: 103–105

Reumann S, Inoue K and Keegstra K (2005) Evolution of the general protein import pathway of plastids (review). Mol Membr Biol 22: 73–86

Reyes-Prieto A, Weber APM and Bhattacharya D (2007) The origin and establishment of the plastid in algae and plants. Annu Rev Genet 41: 147–168

Ridley SM and Leech RM (1970) Division of chloroplasts in an artificial environment. Nature 227: 463–465

Ris H and Plaut W (1962) Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas*. J Cell Biol 19: 383–391

Ris H and Singh RN (1961) Electron microscope studies on blue-green algae. J Biophys Biochem Cytol 9: 63–80

Robertson EJ, Pyke KA and Leech RM (1995) *arc6*, an extreme chloroplast division mutant of *Arabidopsis* also alters proplastid proliferation and morphology in shoot and root apices. J Cell Sci 108: 2937–2944

Robertson EJ, Rutherford SM and Leech RM (1996) Characterization of chloroplast division using the *Arabidopsis* mutant *arc5*. Plant Physiol 112: 149–159

Romberg L and Levin PA (2003) Assembly dynamics of the bacterial cell division protein FtsZ: poised at the edge of stability. Annu Rev Microbiol 57: 125–154

Rutherford SM (1996) The genetic and physical analysis of mutants of chloroplast number and size in *Arabidopsis thaliana.* PhD Thesis. Department of biology, University of York, York, UK

Sagan L (L Margulis) (1967) On the origin of mitosing cells. J Theor Biol 14: 225–275

Sakr S, Jeanjean R, Zhang CC and Arcondeguy T (2006) Inhibition of cell division suppresses heterocyst development in *Anabaena* sp. strain PCC 7120. J Bacteriol 188: 1396–1404

Sapp J (1994) Evolution by Association: A History of Symbiosis. Oxford University Press, New York and Oxford

Sato N (2006) Origin and evolution of plastids: genomic view on the unification and diversity of plastids. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 75–102. Springer, Dordrecht

Schimper AFW (1883) Über die Entwickelung der Chlorophyllkörner und Farbkörper. Bot Zeit 41: 105–112

Schimper AFW (1885) Untersuchungen über die Chlorophyllkörner und die ihnen Homologen Gebilde. Jb Wiss Botan 16: 1–247

Schmitz FKJ (1883) Die Chromatophoren der Algen. Verh Naturhist Ver preuß Rheinland und Westfalen 40: 1–180

Schopf JW (2006) Fossil evidence of Archaean life. Philos Trans R Soc Lond B Biol Sci 361: 869–885

Scott NS, Cain P and Possingham JV (1982) Plastid DNA levels in albino and green leaves of the "albostrians" mutant of *Hordeum vulgare*. Z Pflanzenphysiol 108: 187–191

Sharp LW (1934) Introduction to Cytology. McGraw-Hill book company, New York

Shimada H, Koizumi M, Kuroki K, Mochizuki M, Fujimoto H, Ohta H, Masuda T and Takamiya K (2004) ARC3, a chloroplast division factor, is a chimera of prokaryotic FtsZ and part of eukaryotic phosphatidylinositol-4-phosphate 5-kinase. Plant Cell Physiol 45: 960–967

Simpson CL and Stern DB (2002) The treasure trove of algal chloroplast genomes. Surprises in architecture and gene content, and their functional implications. Plant Physiol 129: 957–966

Staff IA and Parthasarathy MV (1984) The possibility of cell plate-induced plastid division in a flowering plant. New Phytol 97: 77–82

Steahelin LA (2003) Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes. Photosynth Res 76: 185–196

Strepp R, Scholz S, Kruse S, Speth V and Reski R (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. Proc Natl Acad Sci USA 95: 4368–4373

Striepen B, Crawford MJ, Shaw MK, Tilney LG, Seeber F and Roos DS (2000) The plastid of *Toxoplasma gondii* is divided by association with the centrosomes. J Cell Biol 151: 1423–1434

Strugger S (1950) Über den Bau der Proplastiden und Chloroplasten. Naturwissenschaften 37: 166–167

Strugger S (1957) Elektronenmikroskopische Beobachtungen über die Teilung der Proplastiden im Urmeristem der Wurzelspitze von *Allium cepa*. Z Naturforsch 12b: 280–283

Summons RE, Jahnke LL, Hope JM and Logan GA (1999) 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. Nature 400: 554–557

Suzuki KI and Ueda R (1975) Electron microscope observations on plastid division in root meristematic cells of *Pisum sativum* L. Bot Mag Tokyo 88: 319–321

Tavva VS, Collins GB and Dinkins RD (2006) Targeted overexpression of the *Escherichia coli* MinC protein in higher plants results in abnormal chloroplasts. Plant Cell Rep 25: 341–348

Tewinkel M and Volkmann D (1987) Observations on dividing plastids in the protonema of the moss *Funaria hygrometrica*. Planta 172: 309–320

Trécul AAL (1858) Des formations vésiculaires dans les cellules végétales. Ann Sci Nat Bot Ser IV 8: 20 –163, 205–382

Ueda R, Tominga S and Tanuma T (1970) Cinematographic observations on the chloroplast division in *Mnium* leaf cells. Science Report of the Tokyo Daigaku, Section B 14: 129–137

Vaughan S, Wickstead B, Gull K and Addinall SG (2004) Molecular evolution of FtsZ protein sequences encoded within the genomes of archaea, bacteria, and eukaryota. J Mol Evol 58: 19–29

Ventura GT, Kenig F, Reddy CM, Schieber J, Frysinger GS, Nelson RK, Dinel E, Gaines RB and Schaeffer P (2007) Molecular evidence of Late Archean archaea and the presence of a subsurface hydrothermal biosphere. Proc Natl Acad Sci USA 104: 14260–14265

Vicente M, Rico AI, Martinez-Arteaga R and Mingorance J (2006) Septum enlightenment: assembly of bacterial division proteins. J Bacteriol 188: 19–27

Vitha S, Froehlich JE, Koksharova O, Pyke KA, Van Erp H and Osteryoung KW (2003) ARC6 is a J-domain plastid division protein and an evolutionary descendant of the cyanobacterial cell division protein Ftn2. Plant Cell 15: 1918–1933

von Wettstein D (1954) Formwechsel und Teilung der Chromatophoren von *Fucus vesiculosus.* Z Naturforsch 9B: 476–481

Wakasugi T, Nagai T, Kapoor M, Sugita M, Ito M, Ito S, Tsudzuki J, Nakashima K, Tsudzuki T, Suzuki Y, Hamada A, Ohta T, Inamura A, Yoshinaga K and Sugiura M (1997) Complete nucleotide sequence of the chloroplast genome from the green alga *Chlorella vulgaris*: the existence of genes possibly involved in chloroplast division. Proc Natl Acad Sci USA 94: 5967–5972

Wallin IE (1927) Symbionticism and the Origin of Species. Williams and Wilkins, Baltimore

Wang X, Huang J, Mukherjee A, Cao C and Lutkenhaus J (1997) Analysis of the interaction of FtsZ with itself, GTP, and FtsA. J Bacteriol 179: 5551–5559

Weatherill K, Lambiris I, Pickett-Heaps J, Deane JA and Beech PL (2007) Plastid division in *Mallomonas* (Synurophyceae, Heterokonta). J Phycol 43: 535–541

Whatley JM (1988) Mechanism and morphology of plastid divison. In: Boffey SA and Lloyd D (eds) The Division and Segregation of Organelles, pp 63–83. Cambridge University Press, Cambridge, UK

Woese C (1998) The universal ancestor. Proc Natl Acad Sci USA 95: 6854–6859

Woese CR and Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74: 5088–5090

Yan K, Pearce KH and Payne DJ (2000) A conserved residue at the extreme C-terminus of FtsZ is critical for the FtsA-FtsZ interaction in *Staphylococcus aureus*. Biochem Biophys Res Commun 270: 387–392

Yang Y, Glynn JM, Olson BJ, Schmitz AJ and Osteryoung KW (2008) Plastid division: across time and space. Curr Opin Plant Biol 11: 577–584

Yoder DW, Kadirjan-Kalbach D, Olson BJ, Miyagishima SY, Deblasio SL, Hangarter RP and Osteryoung KW (2007) Effects of mutations in Arabidopsis FtsZ1 on plastid division, FtsZ ring formation and positioning, and FtsZ filament morphology in vivo. Plant Cell Physiol 48: 775–791

Yoon HS, Hackett JD, Ciniglia C, Pinto G and Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21: 809–818

Yoshida Y, Kuroiwa H, Misumi O, Nishida K, Yagisawa F, Fujiwara T, Nanamiya H, Kawamura F and Kuroiwa T (2006) Isolated chloroplast division machinery can actively constrict after stretching. Science 313: 1435–1438

Yuasa A (1949) Studies in the cytology of Pteridophyta. Jpn J Genet 24: 166–173

Zhang M, Hu Y, Jia J, Li D, Zhang R, Gao H and He Y (2009) CDP1, A novel component of chloroplast division site positioning system in *Arabidopsis*. Cell Res 19: 877–886

Chapter 3

Chlorophyll Biosynthesis in Higher Plants

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Summary

Chlorophyll (Chl) is essential for light harvesting and energy transduction in photosynthesis. The Chl biosynthesis pathway in higher plants is complex and is mediated by more than 17 enzymes. The formation of Chl can be subdivided into four parts: (1) synthesis of 5-aminolevulinic acid (ALA), the precursor

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of Chl and heme; (2) formation of a pyrrole ring porphobilinogen from the condensation reaction of two molecules of ALA and assembly of four pyrroles leading to the synthesis of the first closed tetrapyrrole having inversion of ring D, i.e., uroporphyrinogen III; (3) synthesis of protoporphyrin IX via several decarboxylation and oxygenation reactions, and (4) insertion of Mg to the protoporphyrin IX (PPIX) moiety steering it to the Mg-branch of tetrapyrrole synthesis leading to the formation of Chl. In higher plants, tetrapyrrole synthesis occurs in plastids, where it is initiated by the reduction of the glutamyl moiety of glutamyl-tRNA to glutamate-1-semialdehyde. The first branch point in the pathway is the methylation of uroporphyrinogen III that directs it toward the synthesis of siroheme, an essential component of nitrite reductase and sulfite reductase, whereas decarboxylation steers it towards PPIX synthesis. A second branch point of the tetrapyrrole biosynthesis pathway is Fe insertion to PPIX leading to the synthesis of hemes. Mg-insertion to the PPIX moiety leads to the synthesis of Mg-protoporphyrins and chlorins. During the day when the ATP levels are high, the magnesium branch of the pathway is favoured, as Mg-chelatase needs ATP for the Mg-PPIX synthesis. In this chapter, we discuss the mechanism of Chl biosynthesis; heterogeneity of the monovinyl and divinyl protochlorophyllide pool; regulation of Chl biosynthesis; the intraplastidic Chl biosynthesis route, and the evolution of Chl biosynthesis.

Abbreviations: ALA – 5-aminolevulinic acid; ALAD – Aminolevulinic dehydratase; CAO – Chlorophyllide *a* oxygenase; CBR – Chlorophyll *b* reductase; Chl – Chlorophyll; Chlide – Chlorophyllide; CHLG – Chlorophyll synthase; CHLP – geranyl-geranyl reductase; CPOX – Coproporphyrinogen oxidase; DV-Pchlide – Divinyl protochlorophyllide; DVR – Divinyl reductase; FLU – Negative regulator of the chlorophyll biosynthesis pathway; FD – Feredoxin; GA – Gibbralic acid; GGPP – Geranyl geranyl pyrophosphate; GluRS – Glutamyl-tRNA synthetase; GluTR – Glutamyl-tRNA reductase; GSA – Glutamate 1-semialdehyde; GSA-AT – Glutamate 1-semialdehyde aminotransferase; GUN – Genome uncoupled; LHCII – Light-harvesting complex II; Lin2 – Lesion initiation 2; lip1 – Light-independent photomorphogenesis 1; MgCh – Magnesium chelatase; MPE – Mg-protoporphyrin IX monomethylester; MPEC – Mg-protoporphyrin IX monomethylester cyclase; MTF – Mg-protoporphyrin IX methyltransferase; MV-Pchlide – Monovinyl protochlorophyllide; PBG – Porphobilinogen; PBGD – Porphobilinogen deaminase; PC – Plastocyanin; Pchlide – Protochlorophyllide; PhPP – Phytyl diphosphate; PLBs – Prolamellar bodies; POR – Protochlorophyllide oxidoreductase; PPIX – Protoporphyrin IX; PPOX – Protoporphyrinogen oxidase; PQ – Plastoquinone; Protogen IX – Protoporphyrinogen IX; PS II – Photosystem II; SAM – *S*-adenosyl- methionine; SDR – Short chain dehydrogenases/reductases; UROD – Uroporphyrinogen III decarboxylase; Urogen III – Uroporphyrinogen III; UROS – Uroporphyrinogen III synthase

I. Introduction

Chlorophyll (Chl), the most abundant pigment, ubiquitously distributed in all plant species, is essential for light harvesting and energy transduction in photosynthesis. The major site of Chl biosynthesis in higher plants is the plastid. All enzymes of the Chl biosynthetic pathway are nuclear encoded and post-translationally imported into chloroplasts. Chl synthesis is synchronized with the formation of other pigments such as carotenoids and with pigment-binding proteins; Chl synthesis also incorporates coordination between chloroplast and nucleus. In higher plants, most of the genes responsible for the Chl biosynthesis pathway are identified and the enzymatic steps of the pathway are characterized (Fig. 3.1). For a background on plastids, on Chls, on carotenoids, and on Chl biosynthesis in chloroplasts, the readers are referred to books edited by Wise and Hoober (2006), Grimm et al. (2006), Frank et al. (1999) and Rebeiz et al. (2010), respectively.

Throughout this chapter nuclear genes will be designated in uppercase italics and their corresponding protein as uppercase normal font; mutant plants will be in lowercase italics. In some cases a lowercase letter may form part of the accepted abbreviation for a nuclear-encoded enzyme (e.g.,

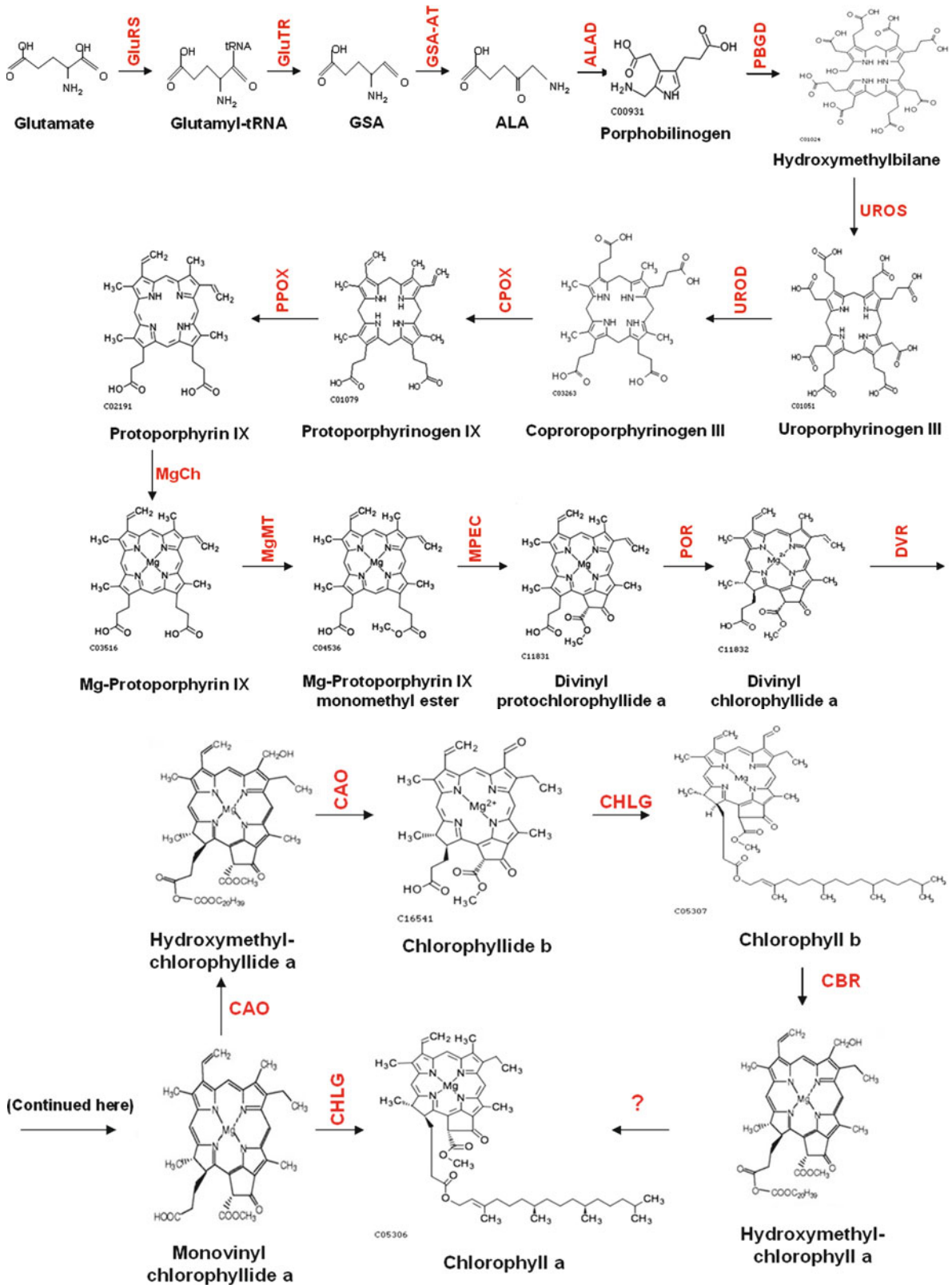

Fig. 3.1. Chlorophyll biosynthesis pathway in higher plants. All abbreviations in this figure are defined in the *Abbreviations* list for this chapter.

Glutamyl-tRNA synthetase is abbreviated GluTR and its corresponding gene is *GluTR*). In these cases the lower case letter has been retained and the reader should consult the *Abbreviations* list to clarify individual examples as required.

II. Enzymes Involved in Chlorophyll Biosynthesis

A. Glutamyl-tRNA Synthetase

Glutamyl-tRNA synthetase (GluRS), also known as aminoacyl-tRNA synthetase, ligates glutamate to $tRNA^{GLU}$ (Huang et al., 1984; Kannagara et al., 1984, 1994) (Fig. 3.1). GluRS belongs to the class I aminoacyl-tRNA synthetases. Aminoacyl-tRNA synthetases generally catalyze the formation of an aminoacyl-AMP from an amino acid and ATP, prior to the aminoacyl transfer to tRNA. However, GluRS avoids the aminoacyl-AMP formation in the absence of tRNA.

In eukaryotic cells two different kinds of GluRS are present, one inside the chloroplast and the other in the cytosol. Both enzymes are encoded by nuclear DNA, and one of the enzymes is imported into the chloroplast where it ligates glutamate to $tRNA^{GLU}$ (Schon et al., 1988). In barley, $tRNA^{GLU}$ is encoded by chloroplast DNA. It contains the UUC glutamate anticodon and is involved in aminolevulinic acid (ALA) synthesis (Schon et al., 1986).

GluRS has been purified from barley (Bruyant and Kannagara, 1987), wheat (Ratinaud et al., 1983), tobacco (Kim et al., 2005) and *Arabidopsis thaliana* (Day et al., 1998). Virus-induced gene silencing of *GluRS* in tobacco results in a severe leaf-yellowing phenotype (Kim et al., 2005). Reduction of the amount of GluRS leads to reduced number and size of the chloroplasts and reduced Chl content.

B. Glutamyl-tRNA Reductase

Glutamyl-tRNA reductase (GluTR), the second enzyme of the pathway, reduces the activated α-carboxyl group of glutamyl-tRNA (Glu-tRNA) in the presence of NADPH and releases glutamate 1-semialdehyde (GSA) (Fig. 3.1). Pyridine nucleotides are required for this reaction (Hoober et al., 1988). The GluTR protein was purified from barley and consisted of five identical subunits of 54 kDa each (Pontoppidan and Kannangara, 1994).

This enzyme is subject to feedback regulation by heme and appears to be a major control point of porphyrin biosynthesis (Kannangara et al., 1988). Micromolar concentrations of Zn^{2+}, Cu^{2+} and Cd^{2+} inhibit barley GluTR (Pontoppidan and Kannangara, 1994). In green barley plants, this enzyme is stimulated by GTP (Kannagara et al., 1988).

GluTR is encoded by the *HEMA* gene. The cDNA for GluTR has been isolated from barley (Bougri and Grimm, 1996); cucumber (Tanaka et al. 1996), and *A. thaliana* (Kumar et al., 1996; Matsumoto et al., 2004). It has two isoforms in barley and in cucumber, whereas in *A. thaliana* it has three isoforms. In cucumber and *A. thaliana* plants, the *HEMA1* gene is expressed in photosynthetic tissues and is induced by illumination, but no transcripts are detectable in roots. Gene expression of *HEMA1*, and the corresponding protein abundance, increases in response to light treatment of dark-grown seedlings suggesting that increased demand for Chl biosynthesis stimulates its expression and the gene promoter may have light-responsive elements (Mohanty et al., 2006). Light-induced expression of *HEMA1* indicates the involvement of phytochrome (McCormac et al. 2001; McCormac and Terry, 2002a, b). On the other hand, *HEMA2* is preferentially expressed in non-photosynthetic tissues, and its expression is not altered with illumination. A third *HEMA* gene, *HEMA3*, has been identified in *A. thaliana*, but its expression is very low under all experimental conditions tested (Matsumoto et al., 2004). Northern blot analysis of *BHA1* (a cDNA clone from barley encoding GluTR) shows that the transcripts are present in roots and are elevated after cytokinin treatment, whereas *BHA 13* (another cDNA clone from barley encoding GluTR) is not present in roots. The *BHA1* levels show oscillations with a circadian rhythm. The *HEMA1* mRNA accumulates in response to a demand for Chl synthesis in photosynthesising tissues, whereas *HEMA2* mRNA is expressed in response to the demand of synthesis of porphyrins other than Chl (Bougri and Grimm, 1996).

A. thaliana plants, expressing antisense *HEMA1*, show decreased amounts of its mRNA, decreased ALA and reduced Chl content (Kumar and Soll, 2000). The RNAi silencing of genes encoding GluTR results in reduced levels of Chl and heme in young leaves (Hedtke et al., 2007). In *A. thaliana*, GluTR interacts with FLU, a negative

regulator of the Chl biosynthesis pathway (Meskauskiene et al., 2001, Meskauskiene and Apel, 2002). FLU is a nuclear-encoded chloroplastic protein and the *flu* mutant has a higher level of ALA synthesis and protochlorophyllide (Pchlide) accumulation than that of wild-type plants. Probably FLU is a component of a negative regulatory system for ALA synthesis when cells have a high Pchlide content. FLU-like protein is also present in barley (Lee et al., 2003).

C. Glutamate 1-Semialdehyde Aminotransferase

The formation of 5-aminolevulinate from GSA is catalyzed by glutamate 1-semialdehyde aminotransferase (GSA-AT). This enzyme is functionally an aminomutase, which transfers the amino group from carbon 2 of GSA to the neighboring carbon atom i.e., carbon 5 of ALA (Fig. 3.1). During the conversion of GSA to ALA, the amino group from pyridoxamine phosphate is donated to GSA, leading to the formation of an intermediate, 4, 5-diaminovalerate (Hoober et al., 1988). The enzyme then releases an amino group from position 4 of this intermediate, releasing 5-ALA. The enzyme is inhibited by gabaculine (Gough et al., 1992). With the exception of barley, most GSA-AT enzymes require either pyridoxamine 5′ phosphate or pyridoxal 5′ phosphate as a cofactor for the enzymatic conversion of GSA into ALA (Kannangara et al., 1988).

The *GSAT* gene encoding GSA-AT has been isolated from barley (Kannangara et al., 1994), *A. thaliana* (*GSAT1* and *GSAT2*) (Ilag et al., 1994), tomato (Polking et al., 1995); soybean (Sangwan and O'Brian, 1993), tobacco (*GSAT1* and *GSAT2*) (Höfgen et al., 1994) and brasicca (Tsang et al., 2003).

In *A. thaliana* light is reported to stimulate transcription of this gene (Ilag et al., 1994). The *GSAT* gene expression is also activated by the hormone kinetin (Yaronskaya et al., 2006). The gene expression of *GSAT*, and the protein abundance of GSAT-AT, increases when etiolated seedlings are transferred to light demonstrating that it is a light-inducible gene and significantly contributes to Chl synthesis (Mohanty et al., 2006). In soybean the *GSAT* gene is also light inducible. It contains a light-regulated cis element (containing GAGA) that is found to be involved in transcriptional control (Frustaci et al., 1995). Its message abundance is high in soybean leaves (Sangwan and O'Brian, 1993) whereas it is absent in roots (Frustaci et al., 1995). Antisense tobacco and brassica plants, expressing *GSAT*, show reduction in Chl levels (Höfgen et al., 1994; Tsang et al., 2003).

D. 5-Aminolevulinic Acid Dehydratase

5-Aminolevulinic acid dehydratase (ALAD), also known as porphobilinogen (PBG) synthase, is a homooctameric metalloenzyme that catalyzes the condensation of two ALA molecules to form PBG (Fig. 3.1). The mechanism of action of ALAD was first proposed by Shemin (1976). The aldol condensation between two ALA molecules involves the initial binding of two substrate molecules and a five-membered heterocyclic ring of PBG is formed with the help of a lysine and a histidine residue (Jordan and Shemin, 1980; Spencer and Jordan, 1994, 1995).

This enzyme has been isolated from wheat (Nandi and Waygood, 1967), tobacco (Shetty and Miller, 1969), radish (Shibata and Ochiai, 1977), spinach (Liedgens et al., 1980), pea (Smith, 1988) and tomato (Polking et al., 1995). Spinach enzyme is found to be a hexamer with molecular weight of 300 kDa (Liedgens et al., 1980) while radish leaves were found to have two isozymes of ALAD (Tchuinmogne et al., 1992). Enzymes from radish cotyledons showed a pH optimum of 8.0 (Shibata and Ochiai, 1977) and a requirement of Mg^{2+} and Mn^{2+} for activity. Using an artificially synthesized ALAD in pea, Kervinen et al. (2000) showed that this enzyme is most active at slightly alkaline pH and shows a maximal binding of three Mg (II) per subunit. The enzyme from tobacco leaves and radish cotyledons was found to be inhibited by Zn^{2+} and Fe^{2+} (Shetty and Miller, 1969; Shibata and Ochiai, 1977); furthermore, arsenic inhibits ALAD activity in maize leaves (Jain and Gadre, 2004), while $PbCl_2$ and $CdCl_2$ inhibit ALAD in *Amaranthus lividus* (Bhattacharjee and Mukherjee, 2003). The activity of ALAD significantly decreases during senescence (Hukmani and Tripathy, 1994) and in chill- and heat-stressed plants (Tewari and Tripathy, 1998).

The gene encoding ALAD has been isolated from pea (Boese et al., 1991), spinach (Schaumburg et al., 1992), soybean (Kaczor et al., 1994) and tomato (Polking et al., 1995). In pea, expression of *ALAD* was high in dark-grown as compared to light-grown tissues (Li et al., 1991). ALAD was detectable in embryonic leaves whether the plants were grown in darkness or under continuous white-light illumination (He et al., 1994).

In pea, *ALAD* transcript abundance was found to be highly dependent on leaf developmental age: the transcript abundance increased with increasing age until the leaf was fully expanded and after that the mRNA levels decreased sharply (He et al., 1994). However, a significant amount of protein was detected even in the matured leaves despite the mRNA expression of *ALAD* being extremely low. Additionally, even though the steady-state level of the *ALAD* mRNA was slightly higher in the dark than in the light, the corresponding protein level was significantly lower in the dark (He et al., 1994). The expression of *ALAD* was not regulated by light in soybean and its expression was 2–3 fold higher in symbiotic tissues as compared to the uninfected roots (Kaczor et al., 1994). On the other hand, in cucumber and wheat *ALAD* expression increased upon transfer of etiolated seedlings to light (Mohanty et al., 2006).

E. Porphobilinogen Deaminase

The enzyme porphobilinogen deaminase (PBGD) is a soluble chloroplastic protein (Castelfranco et al., 1988) that catalyzes the formation of the linear tetrapyrrole, hydroxymethylbilane, from four molecules of PBG (Fig. 3.1). PBGD has been isolated and purified from pea (Spano and Timko, 1991), wheat germ and spinach leaves (Higuchi and Bogorad, 1975) and *A. thaliana* (Jones and Jordan, 1994). Molecular weights from all these sources ranged from 34 kDa to 44 kDa.

The PBGD enzyme from pea chloroplasts was inhibited by Fe^{2+}, Mn^{2+} and Zn^{2+}, whereas Ca^{2+} and Mg^{2+} were only weakly inhibitory at physiological concentrations (Spano and Timko, 1991). PBGD is also inactivated by arginine-, histidine- and lysine-specific reagents as well as by the substrate analogue 2-bromoporphobilinogen (Jones and Jordan, 1994). The enzyme is heat stable and maintains its activity at temperatures ranging from 55^0C to 70^0C. Furthermore, PBGD activity rapidly declines during senescence (Hukmani and Tripathy, 1994) and is reduced by chill- or heat-stress (Tewari and Tripathy, 1998, 1999).

The *PBGD* gene has been isolated and cloned from pea (Witty et al., 1993) and *A. thaliana* (Lim et al., 1994). In *A. thaliana*, PBGD was found to be expressed both in the leaves and the roots (Lim et al., 1994). The steady-state level of *PBGD* mRNA was slightly higher in the dark than in the light, even though the protein level was significantly lower in dark (He et al., 1994). The *PBGD* transcript abundance was found to be highly dependent on leaf developmental age, i.e., the transcript abundance increased with increased age until the leaf was fully expanded and after that the mRNA levels decreased sharply (He et al., 1994). However, a significant amount of protein was detected even in matured leaves despite the mRNA expression of *PBGD* being extremely low.

Sequence comparison from different species shows that specific Arg and Cys residues are well conserved and these are implicated in catalysis and dipyrromethane cofactor binding (Witty et al., 1993). The synthesis and activity of PBGD are regulated by light and cell types (Smith, 1988; Shashidhara and Smith, 1991; Spano and Timko, 1991; He et al., 1994).

F. Uroporphyrinogen III Synthase

In concert with PBDG, the uroporphyrinogen III synthase (UROS) enzyme catalyses the formation of uroporphyrinogen III (Urogen III) from hydroxymethylbilane, a product of PBGD activity. This enzyme helps in maintaining the formation of biologically active isomer III by inverting the ring D; in its absence, hydroxymethylbilane spontaneously cyclizes to uroporphyrinogen I (Urogen I). Inversion of ring D probably involves the production of a spiro-cyclic intermediate (Crockett et al., 1991). This enzyme has been purified from wheat germ (Higuchi and Bogorad, 1975). The enzyme was found to be heat labile and the activity was enhanced by Na^+ and K^+. The enzymes PBGD and UROS may be present as a complex (Tsai et al., 1987). Tan et al. (2008) have isolated the *UROS* gene from *A. thaliana* and shown it to have in vitro activity. The localization of the protein in the chloroplast was confirmed by an in vitro protein import study and confocal microscopy (Tan et al., 2008). The barley *uros* mutant showed a necrotic phenotype in a developmental manner because of Urogen I accumulation (Ayliffe et al., 2009). The mutation in *UROS* also suppressed the expression of genes involved in the light reactions of photosynthesis (Ayliffe et al., 2009).

G. Uroporphyrinogen III Decarboxylase

The uroporphyrinogen III decarboxylase (UROD) enzyme catalyzes stepwise decarboxylation of Urogen III to yield coproporphyrinogen III (Coprogen III). The enzyme catalyzes decarboxylation of all four carboxyl residues of Urogen III to yield coproporphyrinogen. The order of Urogen III decarboxylation is substrate concentration dependent and under normal conditions enzymatic decarboxylation begins at the ring-D acetate group in a clockwise manner (Luo and Lim, 1993). Although all four isomers of uroporphyrinogen are accepted by the enzyme, aromatic porphyrins are not decarboxylated (Castelfranco and Beale, 1981). The discrimination between isomers Urogen I and Urogen III in conversion into coproporphyrinogen occurs principally at the first step. Porphyrins, especially oxidation products of the substrates, have been shown to inhibit the activity of UROD (Smith and Francis, 1981). The enzyme activity is inhibited by metals such as Fe^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+} and Mg^{2+} (Chen and Miller, 1974), but is stimulated by ATP (Manohara and Tripathy, 2000).

The *UROD* gene has been isolated from tobacco and barley (Mock et al., 1995). The in vitro translational product of *UROD* was imported into pea chloroplasts and processed to 39 kDa (Mock et al., 1995). Martins et al. (2001) reported the first crystal structure of a plant (tobacco) UROD. The expression of *UROD* and the corresponding protein expression both increase during illumination in the case of barley (Mock et al., 1995) and cucumber (Mohanty et al., 2006). Transgenic tobacco plants with reduced activity of UROD accumulate uroporphyrin and other photosensitizing tetrapyrrole intermediates. When UROD is antisensed, uroporphyrin accumulates in theses plants (Mock and Grimm, 1997; Shalygo et al., 1998). These plants also display increased pathogenesis-related protein expression (Mock et al., 1999). *UROD* impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize (Hu et al., 1998).

H. Coproporphyrinogen Oxidase

Coproporphyrinogen oxidase (CPOX) catalyses the oxidative decarboxylation of propionate side chains on rings A and B of coprogen III to yield protoporphyrinogen IX (Protogen IX). In aerobic organisms, oxygen is utilized as the sole electron acceptor for enzymatic activity. The enzyme activity was found to be activated by $Fe^{2+}$and Mn^{2+} and inhibited by EDTA and o-phenanthroline (Hsu and Miller, 1970). The corresponding gene, *CPOX*, was isolated and characterized from soybean (Madsen et al., 1993), tobacco and barley (Kruse et al., 1995a, b), *A. thaliana* (Ishikawa et al., 2001) and maize (Williams et al., 2006).

The *CPOX* mRNA is highly expressed in soybean root nodules, but less in leaves. However, no mRNA for *CPOX* was detectable in soybean roots (Madsen et al., 1993). The level of mRNA reached its maximum in developing cells and decreased drastically when cells were completely differentiated. The *CPOX* gene expression is not affected by light or heat stress in wheat and cucumber; however, the gene expression is downregulated by chill stress (Mohanty et al., 2006). In vitro protein import assays of tobacco and barley CPOX protein showed that it was imported into the pea chloroplast and accumulated in its stroma. There are two isoforms of CPOX (Cpx1and Cpx2) found in maize. The Cpx1 fused with green fluorescent protein showed that it was localized in the plastid, whereas in the case of Cpx2, it appeared to localize to mitochondria (Williams et al., 2006). However, mitochondria lack CPOX activity (Smith, 1988).

Mock et al. (1999) have shown that tobacco plants containing antisense RNA for *CPOX* are more resistant to tobacco mosaic virus. Tobacco plants containing antisense *CPOX* RNA showed decreased enzyme levels of CPOX and were characterized by growth retardation and necrosis, showing that these plants were damaged due to oxidative stress (Kruse et al., 1995a). An *A. thaliana* mutant defective in the *LIN2* gene encoding CPOX develops lesions on leaves, in a developmentally regulated and light-dependent manner (Ishikawa et al., 2001).

I. Protoporphyrinogen Oxidase

Protoporphyrinogen oxidase (PPOX) catalyzes the oxygen-dependent aromatization of protogen IX to protoporphyrin IX (PPIX). This enzyme catalyses the six-electron oxidation of protogen using a flavin cofactor, and molecular oxygen as

terminal electron acceptor (Poulson and Polglasse, 1974). Protogen is unstable and spontaneously undergoes oxidation in the presence of oxygen and its oxidation is enhanced by light (Jacobs and Jacobs, 1979). PPOX is active only if there are no polar groups on rings A and B and is quite stable towards acids and bases. Koch et al. (2004) reported the crystal structure of mitochondrial PPOX from tobacco and revealed that it contains a FAD-binding domain, a substrate-binding domain and a membrane-binding domain (Koch et al., 2004). PPOX forms a loosely associated dimer that folds into a FAD-binding and substrate-binding domain. The substrate-binding domain of PPOX also helps in forming a complex with the ferrochelatase enzyme. PPOX has been purified from barley etioplasts (Jacobs and Jacobs, 1987) and localized in the envelope (stromal side) and thylakoid membranes (stromal side) of chloroplasts (Matringe et al., 1992a; Che et al., 2000).

Manohara and Tripathy (2000) showed that envelope and thylakoid membranes failed to synthesize PPIX from the substrate ALA, whereas the stromal fraction could synthesize a little of it; however, when all three components where mixed together, the PPIX synthesizing capacity increased. Moreover, the synthesizing capacity was reduced by the addition of oxidizing agents, and the reaction was reversed in the presence of reductants like dithiothreitol (DTT). ATP increased PPIX synthesis (Manohara and Tripathy, 2000).

PPOX has been isolated from spinach, tobacco and *A. thaliana* (Narita et al., 1996; Lermontova et al., 1997; Che et al., 2000; Watanabe et al., 2001). In all the above plant species, PPOX was encoded by two genes namely *PPOX1* and *PPOX2* that were found in the chloroplast and mitochondria, respectively. In *A. thaliana*, the transcripts of plastidal PPOX were very high in leaves, whereas they were low in roots and floral buds (Narita et al., 1996). In tobacco, both transcripts were expressed synchronously throughout tobacco plant development during diurnal and circadian growth (Lermontova et al., 1997). The spinach PPOX1 preferentially located itself on the stromal side of the thylakoid membrane and on the inner envelope membrane (Che et al., 2000). The spinach *PPOX2* codes for two proteins of molecular mass of 59 kDa (PPOX2 L) and of 55 kDa (PPOX2 S), by using two in-frame start codons. PPOX2 L is associated with the chloroplast inner envelope membrane and PPOX2 S is associated with the inner mitochondrial membranes (Watanabe et al., 2001).

PPOX is highly resistant to proteases (trypsin, endoproteinase Glu-C, or carboxypeptidases A, B and Y) because the protein is folded into an extremely compact form (Arnould and Camadro, 1998). However, structurally bicyclic herbicides, i.e., diphenyl ether-type herbicides, were shown to inhibit PPOX activity in chloroplasts (Camadro et al., 1991; Matringe et al., 1992b). Dayan et al. (2008) have shown that the fungal toxin Cyperin inhibits PPOX activity. The PPOX activity substantially decreases in response to chilling-stress (Tewari and Tripathy, 1998). Transgenic rice plants expressing a *Bacillus subtilis* PPOX gene (Lee et al., 2000), soybean plants overexpressing mitochondrial PPOX (Warabi et al., 2001) and tobacco plants overexpressing the plastidial PPOX of *A. thaliana* (Lermontova and Grimm, 2000) have been shown to confer resistance to acifluorfen herbicide. However, overexpression of human PPOX in rice resulted in accumulation of PPIX and as a result necrotic spots were observed (Jung et al., 2008). In tobacco, PPOX antisense plants had more necrotic leaf lesions under low- than under high-light growth conditions (Lermontova and Grimm, 2006**)**.

J. Mg-Chelatase

Mg-chelatase catalyzes the insertion of Mg^{2+} into PPIX to form Mg-protoporphyrin (Mg-PPIX). In photosynthetic organisms, Mg-chelatase has three subunits (CHLI, CHLD and CHLH) and catalyses the insertion of Mg^{2+} in two steps; an ATP-dependent activation that is followed by an ATP-dependent chelation step (Walker and Weinstein, 1994; Walker and Willows, 1997). The optimal ATP concentration for activation is found to be higher than that of the chelation step. Out of its three subunits, CHLI is an ATPase and its ATPase activity is repressed when it forms a complex with CHLD (Jensen et al., 1999). The N-terminal halves of subunits CHLD and CHLI share high sequence similarity that suggests the CHLD subunit is also an AAA + -protein (ATPases Associated with diverse cellular Activities); however, the ATPase activity has not been detected from CHLD (Jensen et al., 1999).

The activation step requires interaction of subunits CHLD and CHLI. Six CHLI subunits are

assembled into a hexameric ring structure, which is a Mg^{2+} and ATP-dependent process, and the hexameric ring formed by six CHLD subunits is an ATP-independent process. Both the hexameric rings form the Mg–ATP–I–D complex. Subsequently, the CHLH subunit along with the Mg–ATP–I–D complex forms the holocomplex that hydrolyses ATP to release Mg^{2+}. The Mg^{2+} is subsequently coordinated into the porphyrin macrocycle to form Mg-PPIX (Walker and Willows, 1997). It has been proposed that after the formation of Mg-PPIX, the complex disassembles. The accumulation of Mg-PPIX and pheophorbide inhibits Mg-chelatase activity in pea (Popperl et al., 1997). Mg-chelatase activity and the expression of the genes encoding this enzyme are upregulated by light (Mohanty et al., 2006). However, the activity of Mg-chelatase is severely downregulated by low and high temperatures (Tewari and Tripathy, 1998).

The *CHLI* gene has been cloned from soybean (Nakayama et al., 1995); barley (Jensen et al., 1996); *A. thaliana* (Gibson et al., 1996; Rissler et al., 2002); maize (Sawers et al., 2006) and rice (Zhang et al., 2006). This protein is localized in the stroma. The *CHLI* mRNA is induced by light (Gibson et al., 1996; Jensen et al., 1996; Nakayama et al., 1998) and constitutively expressed in matured leaves and also regulated by a diurnal rhythm but not regulated by a circadian rhythm (Matsumoto et al., 2004). In *A. thaliana*, most of the *chlI* homozygous mutants have a pale green phenotype (Rissler et al., 2002). A second *CHII* gene, *CHII-2* has been identified from *A. thaliana* (Rissler et al., 2002). By using the *A. thaliana* T-DNA knockout mutant line of *chlI*, it was observed that the second *CHII* gene also contributes to Chl synthesis. Recently it was also observed that *A. thaliana CHLI2* can substitute for *CHLI1* (Huang and Li, 2009). Using transformants of tobacco with sense and antisense mRNA for *CHLI*, it has been shown that both elevated and decreased levels of *CHLI* mRNA and CHLI protein led to reduced Mg-chelatase activity and in these plants Chl synthesis was also reduced (Papenbrock et al., 2000a). CHLI could be a target for chloroplastic thioredoxin and the in vivo reduction process is light dependent (Ikegami et al., 2007).

The N-terminus of CHLD shows structural similarities with the AAA domain of CHLI and therefore it is believed it contributes towards complex formation and interaction with CHLH (Fodje et al., 2001). The *CHLD* cDNA sequence has been isolated and cloned from tobacco (Papenbrock et al., 1997) and rice (Zhang et al., 2006). The *CHLD* expression changes with respect to the diurnal changes in tobacco (Papenbrock et al., 1999). Virus-induced gene silencing of *CHLH* in tobacco led to lowering of *CHLD* and *CHLI* mRNAs along with less Chl content (Hiriart et al., 2002).

In tobacco, *CHLH* is strongly expressed in young leaves and less expressed in mature leaves and only traces of both transcripts were found in flowering organs (Kruse et al., 1997). *CHLH* expression was found to be light inducible in soybean and rice and the transcript levels were under the control of a circadian oscillation (Nakayama et al., 1998; Jung et al., 2003). The *CHLH* transcripts undergo diurnal variation in *A. thaliana* and tobacco (Gibson et al., 1996; Papenbrock et al., 1999). Depending upon the concentration of Mg^{2+} in lysis buffer, the CHLH protein migrated between stroma and the envelope membranes and was localized in the envelope membrane at very high concentrations of Mg^{2+} (Nakayama et al., 1998).

Mutants of *CHLH* have been isolated from *A. thaliana* (Mochizuki et al., 2001). Transgenic tobacco plants expressing antisense RNA for Mg-chelatase *CHLH* were Chl deficient (Papenbrock et al., 2000b). In these plants, less PPIX and heme accumulated, and a decrease in ALA synthesizing capacity was seen. Virus-induced gene silencing of *CHLH* in tobacco led to lowering of *CHLD* and *CHLI* mRNAs along with less Chl content (Hiriart et al., 2002). The rice *CHLH* mutants also showed a Chl-deficient phenotype (Jung et al., 2003; Zhang et al., 2006). In *A. thaliana*, the Mg-chelatase subunit CHLH is also regulated by retrograde signaling (Mochizuki et al., 2001). Mutation in the *CHLH* gene had repressed expression of *LHCB*. *A. thaliana* protein GUN4 regulates Mg-chelatase activity (Larkin et al., 2003; Davison et al., 2005), and promotes the interactions between CHLH and chloroplast membranes (Adhikari et al., 2009).

K. S-Adenosyl-L-Methionine:Mg Protoporphyrin IX Methyltransferase

S-adenosyl-L-methionine:Mg-PPIX methyltransferase (SAM-MgProtoMTF) catalyzes the

conversion of Mg-PPIX to Mg-protoporphyrin monomethyl ester (MPE) by transferring a methyl group to the carboxyl group of the C13-propionate side chain of Mg-PPIX (Gibson et al., 1963) where, SAM acts as a methyl group donor. This enzyme belongs to the broad family of SAM-dependent methyltransferases (Kagan and Clarke, 1994), which contains the SAM-binding domain, a seven-stranded β-sheet (Jones, 1999). The gene (*CHLM*) encoding for the SAM-MgProtoMTF has been isolated from *A. thaliana* (Block et al., 2002) and tobacco (Alawady and Grimm, 2005). It was seen that the tobacco methyltransferase physically interacts with the CHLH subunit of Mg-chelatases (Alawady et al., 2005).

The *A. thaliana* CHLM protein contains an N-terminal plastid transit sequence. The mature protein (without transit peptide) contains two functional regions, the N-terminal hydrophobic region that enhances the association of the protein with the envelope and thylakoid membranes and the C-terminal region that binds to Ado-met (Block et al., 2002). The *A. thaliana chlm* T-DNA mutant shows albino phenotype, there is accumulation of Mg-PPIX and reduction in major Chl protein complexes (Pontier et al., 2007). Downregulation of the CHLM protein in antisense *CHLM* tobacco plants results in reduced ALA-synthesis and Mg-chelatase activities (Alawady and Grimm, 2005).

L. Mg-Protoporphyrin IX Monomethylester Cyclase

Mg-protoporphyrin IX monomethylester cyclase catalyzes the formation of an isocyclic ring E of the Mg-protoporphyrins and converts MPE to Pchlide. There are two pathways for the formation of the isocyclic ring, i.e., aerobic cyclization and anaerobic cyclization. The former pathway is predominant in plants, green algae and cyanobacteria where the ketone oxygen of divinyl Pchlide (DV-Pchlide) is derived from molecular oxygen (Walker et al., 1989). The pH optimum of the cyclase activity is approximately 9.0 and the enzyme activity was found to be inhibited by CN^- and N_3^- (Whyte and Castelfranco, 1993). The studies of the cyclase reaction with the two barley mutants *xantha l* and *viridis K* revealed the need of at least two plastidal proteins (a membrane bound protein and a soluble protein) for the cyclization reaction (Walker et al., 1991; Walker and Willows, 1997). Biochemical and genetic studies have demonstrated that the gene responsible for the *xantha-l* mutant encodes a membrane-bound cyclase subunit and it needs a soluble fraction for the cyclization reaction (Rzeznicka et al., 2005).

The gene responsible for the aerobic cyclization reaction has been isolated and characterized from different plants i.e., *CHL27* from *A. thaliana* and *XANTHA l* from barley (Tottey et al., 2003; Rzeznicka et al., 2005). Antisense *A. thaliana* and tobacco plants with reduced amounts of CHL27 show chlorotic leaves with reduced abundance of all Chl proteins and accumulate MPE (Tottey et al., 2003; Peter et al., 2010). The *A. thaliana chl27* T-DNA mutant is pale green with an elevated Chl *a*/*b* ratio, and has unstacked thylakoid membranes, reduced LHCII protein and the photosynthetic activity is reduced due to a damaged Photosystem II (PS II) reaction center (Bang et al., 2008; Hansson and Jensen, 2009). In tobacco plants the co-supression of the *NTZIP gene*, which includes coding for a diiron motif, resulted in a reduced Chl level and lower photosynthetic activity (Liu et al., 2004).

M. Protochlorophyllide Oxidoreductase

Protochlorophyllide oxidoreductase (POR) is the only light-requiring enzyme of the Chl biosynthesis pathway. It catalyses the conversion of Pchlide to chlorophyllide (Chlide) by using light as a substrate along with Pchlide and NADPH. POR converts Pchlide to Chlide, by adding two hydrogen atoms at C17 and C18 on ring D. In the POR catalytic cycle, a ternary enzyme-NADPH-Pchlide complex is formed. Light energy absorbed by the Pchlide in the complex may produce torsional strain in the molecule that provides a favorable condition for hydride/hydrogen transfer from NADPH (Begley and Young, 1989). POR is a member of a large family of enzymes known as short chain dehydrogenases/reductases (SDR) (Wilks and Timko, 1995) which generally catalyze NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers. A tyrosine (Tyr) and a lysine (Lys) residue are both conserved throughout all members of the SDR family. In POR, it was also seen that Tyr and Lys residues are important for its activity (Wilks and Timko, 1995; Lebedev et al., 2001). The Tyr may be

deprotonated, acting as a general acid to facilitate hydride transfer to or from NAD(P)+/H (Bohren et al., 1994). The proton at the C-18 position of Pchlide is derived from Tyr and the hydride transferred to the C-17 position is derived from the *pro-S* face of NADPH. The close proximity of the Lys residue is thought to allow the deprotonation step to occur at physiological pH by lowering the apparent pK_a of the phenolic group of the Tyr (Wilks and Timko, 1995**)**. The mutation of either Tyr275 or Lys279 did not completely abolish the catalytic activity of POR. However, mutation of either residue impairs formation of the ground state ternary enzyme-substrate complex, indicating their key role in substrate binding. Both residues have multiple roles in catalysis, involving formation of the ground state ternary enzyme-substrate complex, stabilization of a Pchlide excited state species and proton transfer to the reaction intermediate formed after the light reaction (Menon et al., 2009). Recently it has been demonstrated that a light-activated conformational change of the protein is necessary to activate catalysis (Heyes et al., 2008; Sytina et al., 2008). The fact that POR is light activated means the enzyme–substrate complex can be formed in the dark. This has recently been exploited by studying Pchlide reduction at low temperatures to trap intermediates in the reaction pathway (Heyes et al., 2002, 2003; Heyes and Hunter, 2004). As a result, the reaction has been shown to consist of at least three distinct steps: an initial light-driven step, followed by a series of 'dark' reactions. An initial photochemical step can occur below 200 K (Heyes et al., 2002), whereas two 'dark' steps were identified for *Synechocystis* sp. PCC 6803 POR, which can only occur close to or above the 'glass transition' temperature of proteins (Heyes et al., 2003**)**. First, $NADP^+$ is released from the enzyme and then replaced by NADPH, before release of the Chlide product and subsequent binding of Pchlide have taken place (Heyes and Hunter, 2004). Monovinyl protochlorophyllide (MV-Pchlide) and DV-Pchlide don't influence differentially the enzyme kinetics or the steps involved in the reaction pathway (Heyes et al., 2006). The secondary structure analysis of POR reveals that it has 33% alpha helix, 19% beta-sheets, 20% turn and 28% random coil. A hydrophobic loop-region has been suggested to be involved in membrane anchoring (Birve et al., 1996). Mutation studies by Dahlin et al. (1999) found that mutation in predicted α-helical regions of the protein showed the least effect on enzyme activity, whereas mutations in the predicted β-sheet regions showed an adverse effect on enzyme function. The replacement of charged amino acids by alanine in the N- and C-terminal regions of the mature protein did not affect POR assembly, whereas mutations within the central core created protein incapable of proper attachment to the thylakoid.

POR is nuclear encoded, translated as a precursor protein in the cytosol and ultimately transported into plastids (Apel, 1981). It is a peripheral membrane protein that accumulates to high level in prolamellar bodies (PLBs), where it forms a ternary complex with Pchlide and NADPH (Oliver and Griffiths, 1982) and is present at low levels in the thylakoid membranes of developing and mature plastids. It is observed that the Cys residues of POR are crucial for its membrane association (Aronsson et al., 2001) and for NADPH and pigment binding (Townley et al., 2001; Reinbothe et al., 2006). The association of POR with Pchilde results in three different spectral forms of Pchlide based on their fluorescence emission maximum (in nm): Pchlide F631 (due to the pigment structural arrangements), Pchlide F644 (due to association of POR), and Pchlide F655 (due to localization in PLBs and/or prothylakoids) (Böddi et al., 1992, 1993). Spectroscopic studies of dark-grown bean seedlings produced the idea of two forms of Pchlide, a main component with a red absorption band at 650 nm and a minor component absorbing at 636 nm (Shibata, 1957). On the basis of flash illumination, two kinds of Pchlide can be categorized: one is transformed into Chlide and is called photoactive Pchlide, whereas the other remains unchanged and is called nonphotoactive Pchlide. The latter is assembled into various complexes with different molecular structure and spectral properties (Masuda and Takamiya, 2004; Schoefs and Franck, 2003). Plastids isolated from dark-grown wheat seedlings exhibit a smaller 77 K fluorescence emission peak at 632 nm due to nonphototransformable Pchlide and a larger peak at 657 nm due to phototransformable Pchlide. The non-phototransformable Pchlide emitting at 632 nm is due to a monomeric Pchlide complex or esterified Pchlide i.e., protochlorophyll (Lindsten et al. 1988), which spontaneously dimerizes to form

$(POR\text{-}Pchlide\text{-}NADPH)_2$. The short-wavelength, monomeric Pchlide is not flash-photoactive: instead it regenerates the long wavelength Pchlide forms (Schoefs and Franck, 1993; He et al., 1994; Schoefs et al., 1994, 2000a, b). The dimer has the absorption maximum at 638 nm and emission maximum at 645 nm (Lebedev and Timko, 1999). The dimeric POR-Pchlide-NADPH complex further polymerizes to form 16-mer or larger aggregates of POR-Pchlide-NADPH complex i.e., $(POR\text{-}Pchlide\text{-}NADPH)_n$ having absorption maximum at 650 nm and emission maximum at 657 nm (Böddi et al., 1989; Wiktorsson et al., 1993) and is flash photoactive (Böddi et al., 1991). However, long-term illumination i.e., more than a minute usually converts non-active Pchlide to photo-active Pchlide. Unpublished observations from our laboratory demonstrate that the photo-transformable Pchlide (F657) rapidly decreases when 5-day-old etiolated seedlings are transferred to 42°C in the dark for 24 h. In heat-stressed seedlings the Shibata shift is substantially arrested while in chill-stress conditions, the same is partially affected.

Full-length cDNA clones of *POR* were isolated from barley (Holtorf et al., 1995; Schulz et al., 1989), oat (Darrah et al., 1990), pea (Spano et al., 1992), wheat (Teakle and Griffiths, 1993), *A. thaliana* (Armstrong et al., 1995; Benli et al., 1991; Oosawa et al., 2000), tobacco (Masuda et al., 2002), cucumber (Kuroda et al., 1995) and banana (Coemans et al., 2005). The high degree of sequence similarity among PORs from different taxonomic group implies a common mechanism of enzyme action.

A characteristic feature of *POR* accumulating in darkness is its sensitivity to illumination. The *POR* mRNA expression was also decreased (Santel and Apel, 1981). Red and far-red light treatment also inhibits *POR* mRNA expression indicating that *POR* expression is controlled by phytochrome (Apel, 1981; Batschauer and Apel 1984; Mosinger et al., 1985). The negative effect of light on the POR enzyme and its mRNA was observed in different dicotyledons like bean, pea, tomato and *A. thaliana* (Forreiter et al., 1991; Spano et al., 1992; Armstrong et al., 1995) and in the monocotyledonous plants maize and barley (Forreiter et al., 1991; Holtorf et al., 1995). However, some flowering plants have isoforms of POR. In *A. thaliana*, (Armstrong et al., 1995; Oosawa et al., 2000; Su et al., 2001; Pattanayak and Tripathy, 2002), barley (Holtorf et al., 1995; Holtorf and Apel, 1996a, b) and tobacco (Masuda et al., 2002) there are different PORs present. The N-terminus of PORA and PORB of barley etioplasts have recently been characterized (Ploscher et al., 2009). In *A. thaliana* there are three isoforms of POR, namely PORA, PORB and PORC. These three isoforms are differentially regulated by light. The level of *PORA* mRNA and protein decreases on illumination of etiolated plants (Holtroff and Apel, 1996a) while that of *PORC* increases and was dominantly expressed in both mature and immature tissues (Oosawa et al., 2000). *PORB* transcript and PORB protein levels remain constant in both dark and on illumination (Armstrong et al., 1995, Holtrof et al., 1995; Holtroff and Apel, 1996a). Both *PORB* and *PORC* of *A. thaliana* exhibit diurnal fluctuation but only the *PORB* mRNA of *A. thaliana* exhibits circadian regulation (Su et al., 2001). *PORC* mRNA and PORC protein expression also increased under high light intensity (Su et al., 2001; Masuda et al., 2003). In cucumber the levels of the *POR* mRNA increased in etiolated cotyledons when they were illuminated with continuous light (Kuroda et al., 1995; Fusada et al., 2000). The plant hormone cytokinin regulates cucumber *POR* gene expression by binding to the *cis*-elements present at the 5' region of the *POR* promoter (Fusada et al., 2005). In tobacco, two POR isoforms have been isolated, the expression of which was not negatively regulated by light, persisted in mature green tissue and showed diurnal fluctuations with a similar oscillation phase (Masuda et al., 2002).

A plant specific downstream element in the 3' untranslated region of the *PORA* transcript confers *PORA* mRNA instability, whereas it was not responsible for *PORB* mRNA degradation (Holtorf and Apel, 1996a). *POR* gene expression in cucumber is regulated by phytohormone, particularly by cytokinins and abscissic acid (Kuroda et al., 2001). In the *lip1* mutant of pea, cytokinins restored the formation of PLB and photoactive Pchlide in the dark (Seyedi et al., 2001a), but in *A. thaliana* its application results in loss of PLBs (Chory et al., 1994). In lupine, *POR* expression is also regulated by cytokinins and abscissic acid (Kusnetsov et al., 1998).

POR gene expression is also organ specific. *A. thaliana PORB* and *PORC* are expressed in all photosynthetic tissues of the mature plants but

not in root (Armstrong et al., 1995; Oosawa et al., 2000). Cucumber *POR* expression is also observed in photosynthetic tissues (Kuroda et al., 1995). Plant age also plays a crucial role in *POR* gene expression. In *A. thaliana* and barley *PORA* expression is only observed in young seedlings whereas *PORB* is expressed both in young and matured green tissue (Armstrong et al., 1995; Schunmann and Ougham, 1996). In *A. thaliana* both *PORB* and *PORC* expression is observed in green tissue (Oosawa et al., 2000; Su et al., 2001). In the leaves of dark-grown seedlings, the highest level of expression is observed 8–10 days post germinated seedlings (Spano et al., 1992). The transcript level of pea *POR* did not decrease after 48 h of light exposure. But immunoblot analysis showed there was no POR protein after 48 h of light exposure. These results suggested that pchlide reductase activity in pea is primarily regulated post-transcriptionally, most likely at the level of translation initiation/elongation or protein turnover (Spano et al., 1992).

Degradation of PORA is specific and controlled by nuclear-encoded proteases. The mechanism of light-activation of protease expression is unknown. Mapleston and Griffiths (1980) observed that POR activity decreased after illumination. Reinbothe et al. (1995) showed that the barley precursor of PORA and Pchlide (pPORA-Pchlide) complex was resistant to protease treatment and independent of the presence or absence of NADPH. In contrast, the pPORA-Chlide complex was rapidly degraded. The pPORA protein without its substrate or products was less sensitive to proteolysis than the pPORA-Chlide complex suggesting that both substrate binding and product formation had caused differential changes in protein conformation (Reinbothe et al., 1995). PORB was not degraded by the protease. The PORA degrading protein is assumed to be nuclear encoded, energy dependent and a plastid localized protein in barley (Reinbothe et al., 1995). But the study of post-import degradation of radiolabeled barley pPORA and pPORB on incubation with stroma enriched fractions from etiolated and light grown barley or wheat did not show any protease activity (Dahlin et al., 2000).

In vivo functions of each POR isoform have been extensively studies in *A. thaliana* seedlings. In the *det340* (de-etiolated) mutant of *A. thaliana*, *PORA* is constitutively down regulated, resulting in a lack of PORA and photoactive Pchlide-F655 in dark-grown seedlings and the plants are susceptible to photooxidative damage at extremely low light intensities (Lebedev et al., 1995). The photoprotective mechanism of PORA is also described by Buhr et al. (2008). *A. thaliana* etiolated seedlings grown under continuous far-red light are unable to green when subsequently transferred to white light, which is called far-red blocking of the greening process. This process involves depletion of PORA, partial depletion of PORB and the concomitant loss of PLBs resulting in photo-oxidative damage (Barnes et al., 1996; Runge et al., 1996). From these studies, PORA has been proposed to play a special role in the formation of POR ternary complexes containing photoactive Pchlide-F655, PLB assembly, and protection against photo-oxidative damage caused by non-photoactive Pchlide (Reinbothe et al., 1999). However, overexpression of *PORA* and *PORB* in specific mutants overcame the photo-oxidative damage (Sperling et al., 1997, 1998). Franck et al. (2000) examined in detail redundant roles of PORA and PORB in etioplast differentiation by manipulating the total POR content and the PORA to PORB ratio of *A. thaliana* seedlings using antisense and overexpression approaches.

It was recently observed that overexpression of a cyanobacterial POR protein in the *A. thaliana porA* mutant could restore PLB formation. However, the amount of photoactive Pchlide in the etioplasts of the complementing lines was retained at a low level as in the parent *PORA* knockdown mutant (Masuda et al., 2009). The *lip1* mutant of pea lacked PLBs but could store PLBs if treated with cytokinin (Seyedi et al., 2001a): but unlike the *A. thaliana* mutant, it did not undergo photooxidative damage (Seyedi et al., 2001b). The physiological function of specific POR isoforms in vivo has been well characterized in knockout mutants of *A. thaliana* (Frick et al., 2003; Masuda et al., 2003). Single *POR* mutants display no obvious phenotypes at the whole plant or chloroplast ultrastructural levels, except that *PORB* mutants have less extensive etioplast inner membranes. However, the *PORB/PORC* double mutant, which displayed a seedling-lethal *xantha* phenotype at the cotyledon stage, contained only a small amount of Chl *a*, and possessed chloroplasts with mostly unstacked thylakoid membranes (Frick et al., 2003). Masuda et al. (2003)

focused on the greening process of *por* mutants, and showed that the etiolated *PORB* mutant seedling was able to green to a similar extent as the wild type, and the greening of the *porc* mutant was repressed under high light conditions.

From a molecular evolutionary perspective, the light-dependent POR (LPOR) enzymes are extraordinarily highly conserved. Comparative analysis of complete plastid genome sequences indicate that LPOR genes were lost from the plastid at some point during early evolution (Martin et al., 1998, 2002), and analysis of LPOR proteins in species of conifer show evidence for loss of enzyme activity (Kusumi et al., 2006). The discovery of genes for LPOR in the plastid genomes of diverse cryptophyte algae suggests that these genes have been lost relatively recently.

N. Divinyl Reductase

In all photosynthetic organisms Pchlide and Chlide are originally formed as 3,8-divinyl derivates. The 8-vinyl reductase reduces the 8-vinyl group on the tetrapyrrole to an ethyl using NADPH as the reductant. This enzymatic activity has been detected in isolated chloroplasts of barley (Tripathy and Rebeiz, 1988), plastid membranes from cucumber (Parham and Rebeiz, 1995), and also in solubilized crude extracts derived from etiolated barley leaves (Kolossov and Rebeiz, 2001). It has been demonstrated in vitro that the monovinyl (MV) and divinyl (DV) Chl biosynthesis reactions may operate in parallel (Tripathy and Rebeiz, 1986). However, the mutant of maize (*Zea mays*) that accumulated only DV-Chl instead of MV-Chl and capable of photosynthetic growth with DV-Chl suggests that a single gene product is responsible for the reduction of the vinyl group of Chlide (Bazzaz, 1981). Nagata et al. (2005) followed by Nakanishi et al. (2005) isolated a mutant of *A. thaliana* which accumulates DV-Chl. By map-based cloning they found out that the gene is 8-vinyl reductase. The recombinant protein was successfully tested for the conversion of the C8-vinyl group of Chlide to an ethyl group on ring B. The 3,8-divinyl-chlide *a* is the major substrate of divinyl reductase (DVR) (Nagata et al., 2007). The mutant is pale green and the Chl *a*/*b* ratio varies in between 6 and 10 depending on the developmental stage and growth conditions. This mutant is capable of photosynthesizing and growing under low-light conditions (70–90 μmole photons m^{-2} s^{-1}); but rapidly dies under high light conditions (1,000 μmole photons m^{-2} s^{-1}) (Nagata et al., 2005). The thylakoid membranes were organized in a disorderly fashion having no distinct grana stacks in the mutant but no distinct differences in the size and the number of chloroplasts between the wild type and the mutant were observed. Starch granules were not found in the mutant chloroplasts, suggesting the reduction of photosynthetic activity in the mutant (Nakanishi et al., 2005). The transcript level of *DVR* expression is high in leaves, stems and flower buds, and low in roots.

O. Chlorophyllide a Oxygenase

Chlorophyllide *a* oxygenase (CAO) converts chlorophyllide (Chlide) *a* to Chlide *b*. During conversion of Chlide *a* to Chlide *b* the electron is transferred from the Rieske center to the mononuclear iron with subsequent activation of molecular oxygen for oxygenation of the Chlide *a* methyl group (Beale and Weinstein, 1990; Porra et al., 1993). Chlide *b* is synthesized by oxidation/conversion of the methyl group on the D ring of the porphyrin molecule to a formyl group at that position. The CAO enzyme contains domains for a [2Fe-2S] Rieske center and for a mononuclear nonheme iron-binding site and has a tyrosine radical (Eggink et al., 2004). The conserved Rieske center and non-heme-iron binding motifs of CAO are likely to be involved in electron transport from ferredoxin to molecular oxygen. The recombinant CAO protein catalyzes Chlide *a* to Chlide *b* in the presence of NADPH and reduced ferredoxin (Oster et al., 2000). However, Pchlide *a* is not a substrate for the CAO enzyme (Oster et al., 2000).

The *CAO* gene was first isolated from *Chlamydomonas reinhardtii* by Tanaka et al. (1998) and has also been isolated from *A. thaliana* (Espineda et al., 1999) and rice (Lee et al., 2005). Both transcript and protein levels of CAO increased when *A. thaliana* plants were transferred from moderate to shade light (Harper et al., 2004). Rice has two CAO isoforms encoded by *OsCAO1* and *OsCAO2* that are differentially regulated in light and dark. The *OsCAO1* transcript is less in the dark and induced by light whereas the *OsCAO2* mRNA levels are higher in dark conditions, and its transcripts are reduced by exposure to light (Lee et al., 2005).

Overexpression of the *CAO* gene in *A. thaliana* led to an increase in the Chl *b* level leading to reduction of the Chl *a*:*b* ratio from 2.85 to 2.65 in full green rosette leaves and at the same time there is 10–20% increase in antenna size (Tanaka et al., 2001). Overexpression of *A. thaliana CAO* in *Synechosystis* sp. PCC 6803 resulted in production of Chl *b* up to about 10% of total Chl content and the resulting Chl *b* pigments efficiently incorporated into the Photosystem I Chl-protein complex (Satoh et al., 2001). Simultaneous overexpression of both *CAO* and *LHCII* genes in *Synechosystis* sp. PCC 6803 resulted in an increase in Chl *b* content up to 80% of total Chl (Xu et al., 2001). Overexpression of *CAO* in tobacco plants resulted in a decreased Chl *a*/*b* ratio i.e., from 3.38 in wild-type plants to 2.33 in transgenic plants when grown in high light (Pattanayak et al., 2005). High light grown transgenic *A. thaliana* plants also showed a decreased Chl *a*/*b* ratio under high light (Tanaka and Tanaka, 2005). When the *CAO* gene of *Prochlorothrix holandica* was overexpressed in *A. thaliana* it was observed that approximately 40% of Chl *a* of the core antenna complexes was replaced by Chl *b* in both photosystems (Hirashima et al., 2006). The CAO sequence has been classified into four parts, the N-terminal sequence predicted to be a transit peptide, the subsequent conserved sequence unique in land plants (A-domain), a less-conserved sequence (B-domain) and the C-terminal conserved sequence common in chlorophytes and prochlorophytes (C-domain) (Nagata et al., 2004). The C-domain is sufficient for catalytic activity and the N-terminal 'A' domain confers protein instability by sensing the presence of Chl *b* and regulates the accumulation of the CAO protein (Yamasato et al., 2005). Chloroplast Clp protease is involved in regulating Chl *b* biosynthesis through the destabilization of CAO in response to the accumulation of Chl *b* (Nakagawara et al., 2007). The B domain alone is not involved in the regulation of CAO protein levels (Sakuraba et al., 2007). Further work on domain analysis also indicated that transgenic *A. thaliana* plants overexpressing the A-domain-deleted CAO accumulated an excess amount of Chl *b* during greening and the etiolated transgenic plants either died or were retarded when exposed to continuous light immediately after etiolation (Yamasato et al., 2008). This was most likely due to deregulated Chl b synthesis that reduced the energy transfer rate between photosynthetic pigments (Sakuraba et al., 2010).

P. Chlorophyll b Reductase

Chl *b* reductase catalyzes the conversion of Chl *b* to Chl *a*. It reduces the formyl group of Chl *b* to a hydroxymethyl group. It was observed that barley etioplasts had Chlide *b* reductase activity and the enzyme needs NADPH and reduced ferredoxin for its activity (Scheumann et al., 1996; 1999). The gene encoding Chl *b* reductase was isolated from rice and it belongs to a family of short-chain dehydrogenase/reductases (Kusaba et al., 2007). It encodes a protein of 504 amino acids and contains a dinucleotide binding motif (TGXXXGXG) and a catalytic site (YXXXK) and uses NADPH as a cofactor. Interestingly, two genes for Chl *b* reductase were found in the genomes of *A. thaliana* and rice (Kusaba et al., 2007; Sato et al., 2009). It was also observed that disruption of the genes encoding Chl *b* reductase in *A. thaliana* resulted in non-degradation of Chl *b* and LHCII (Horie et al., 2009).

The interconversion of Chl *b* and Chl *a* form a "chlorophyll cycle" which starts with Chlide *a* (Fig. 3.2). It has been observed that in presence of recombinant CAO enzyme, the Chlide *a* gets converted to Chlide *b* where NADPH, molecular oxygen and ferredoxin are used (Oster et al., 2000). In this in vitro assay, even a small amount of 7-hydroxymehtyl Chlide *a* was formed. When the 7-hydroxymehtyl Chlide *a* was used as a substrate for the in vitro enzymatic assay, the recombinant enzyme also efficiently converted 7-hydroxymehtyl Chlide *a* to Chlide *b* (Oster et al., 2000). Then, Chl synthase converts Chlide *b* into Chl *b*. Chl b is then converted to hydroxymethyl Chl *a* by the enzyme Chl *b* reductase (Kusaba et al., 2007). The enzyme converts the formyl group of Chl *b* to a hydroxymethyl group using NADPH as a reductant. 7-Hydroxymethyl-Chl *a* reductase catalyzes the reduction of the hydroxylmethyl group of 7-hydroxymethyl Chl *a* to a methyl group to form Chl *a*. The gene for this enzyme is yet to be identified.

Q. Geranyl-Geranyl Reductase

Geranyl-geranyl reductase catalyses the reduction of geranyl geranyl diphosphate to phytyl diphosphate. The cDNA encoding a pre-geranyl-geranyl reductase from *A. thaliana* has been isolated and characterized (Keller et al., 1998). The recombinant protein catalyzed the reduction

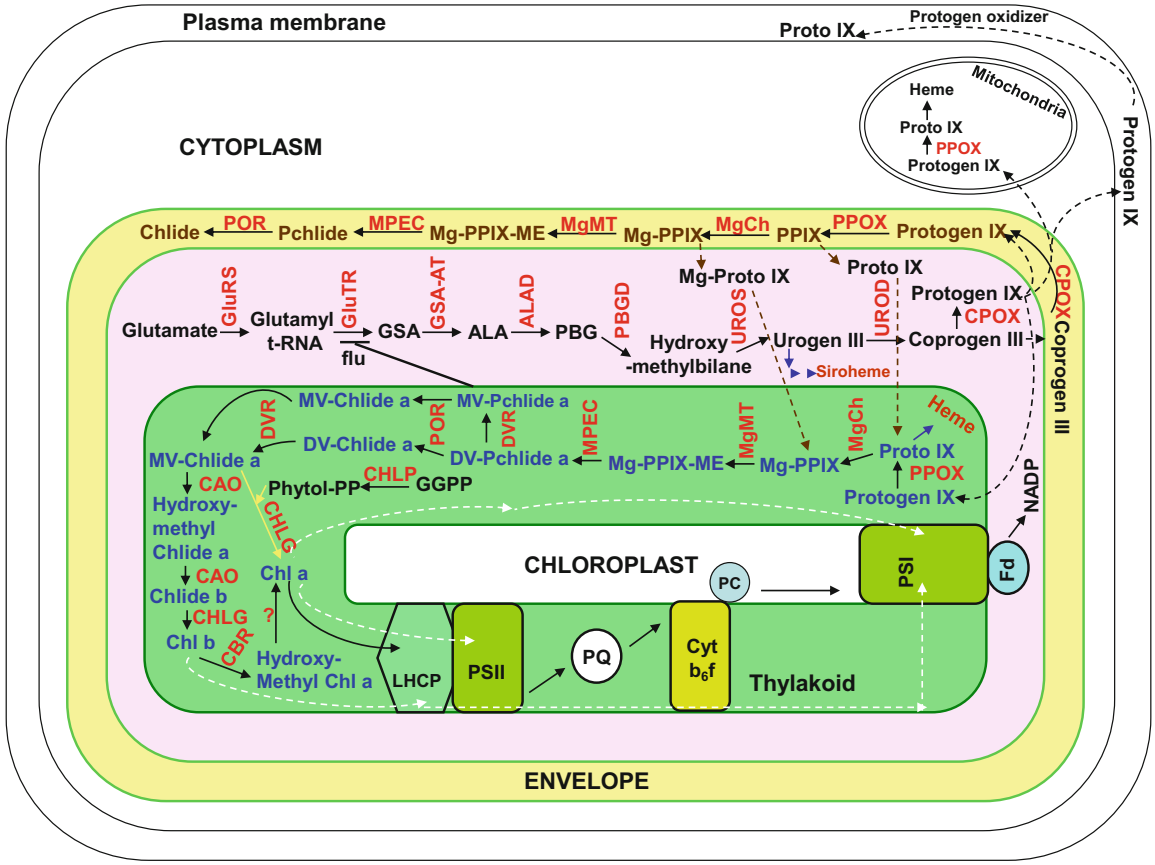

Fig. 3.2. Intraplastidic chlorophyll biosynthesis route in green plants. All abbreviations in this figure are defined in the *Abbreviations* list for this chapter.

of geranyl-geranyl-Chl *a* into phytyl-Chl *a* as well as the reduction of free geranyl-geranyl diphosphate into phytyl diphosphate, suggesting that this is a multifunctional gene. The transcript level is up-regulated during etioplast to chloroplast and chloroplast to chromoplast development (Keller et al., 1998).

The decrease in the expression of *CHLP* coding for geranyl-geranyl reductase affects the Chl and tocopherol contents in tobacco (Tanaka et al., 1999). The reduced tochopherol and Chl contents in *CHLP* antisense plants resulted in the reduction of electron transport chains and PS II activity. There is also more lipid peroxidation product in *CHLP* antisense plants. Havaux et al. (2003) found the accumulation of xanthophylls cycle pigments in *CHLP* antisense plants which could be a compensatory mechanism for tochopherol deficiency. The *CHLIP* transcript levels in peach was abundant in Chl-containing tissues and flower organs but barely detected in roots and mesocarp of the ripening fruits (Giannino et al., 2004).

R. Chlorophyll Synthase

Chlorophyll synthase catalyzes the esterification of Chlide *a* and Chlide *b* to Chl (Rüdiger et al., 1980). Pchlide is not the substrate for this enzyme, which indicates that reduction of the 17, 18 double bond on ring D is essential for esterification (Benz and Rüdiger, 1981b). Compounds which have the 13(2)-carbomethoxy group at the same side of the macrocycle as the propionic side chain of ring D are neither substrates nor competitive inhibitors (Helfrich et al., 1994). Only compounds having the 13(2)-carbomethoxy group at the opposite site are substrates for the enzyme. Esterification kinetics of Chlide is a rapid phase, leading to esterification of 15% of total Chlide within 15–30 s, followed by a lag-phase

of nearly 2 min and a subsequent main phase (Schmid et al., 2002; Domanskii et al., 2003). It has been shown that the conversion of Chlide to Chl is a four-step process including three intermediates i.e., Chlide geranylgeraniol, Chlide dihydrogeranylgeraniol and Chlide tetrahydrogeranylgeraniol before the formation of Chlide phytol or Chl (Shoefs and Bertrand, 2000).

In etioplasts, geranyl-geranyl pyrophosphate (GGPP) is used as a substrate (Rüdiger et al., 1980), while in chloroplasts the preferential substrate is phytyl diphosphate (PhPP) (Soll et al., 1983). Chl synthase in chloroplast thylakoid membranes incorporates phytol in the presence of ATP and a stromal kinase (Benz and Rüdiger, 1981a). The enzyme was not affected by the developmental stage of the plastids. In etiolated wheat, the enzyme was found in latent form in PLBs (Lindsten et al., 1990).

The *CHLG* gene, encoding Chl synthase, was isolated from *A. thaliana*, *Avena sativa*, rice and tobacco. (Gaubier et al. 1995; Schmid et al., 2001; Wu et al., 2007; Shalygo et al., 2009). In *A. thaliana*, the *CHLG* transcript is only detected in green or greening tissues (Gaubier et al., 1995), whereas in *A. sativa*, this gene is expressed equally both in dark- and light-grown seedlings (Schmid et al., 2001). Sequence analysis of EST cDNAs from rice yielded a putative Chl synthase homolog (Scolnik and Bartley, 1996); however, the biochemical properties and physiological functions remained unknown until Wu et al. (2007) characterized a rice mutant with inactivated *CHLG*. The young rice Chl synthase mutant plants show yellow-green leaves with decreased Chl synthesis (Wu et al., 2007). In the mutated plants, there is accumulation of tetrapyrrole intermediates, reduced expression of *lhcb1* and delayed chloroplast development. However, the antisense expression of the tobacco *CHLG* gene did not result in the accumulation of Chlide, but there was reduced ALA synthesizing capacity (Shalygo et al., 2009).

III. Intraplastidic Route of Chlorophyll Biosynthesis

Chloroplasts have two limiting envelope membranes, the outer membrane in contact with the cytoplasm of the cell and the inner membrane surrounding the stroma. The inner envelope membrane is essential for the biosynthesis of plastid components such as glycolipids and prenylquinones (Douce and Joyard, 1990). In addition, the envelope membrane plays a key role in the sorting of plastid proteins that are coded by the nuclear genome. The envelope also plays a significant role in Chl degradation. Chlorophyllase and Mg-dechelatase are present in the inner envelope membrane (Matile et al., 1996, 1999).

Chl is bound to pigment-protein complexes of thylakoid membranes. Chl and its precursors are essential for chloroplast development and nuclear gene expression (Eichacker et al., 1990; Jilani et al., 1996; Kropat et al., 1997, 2000). Interplay of envelope, stroma and thylakoids is shown for PPIX (Proto IX in Fig. 3.2) synthesis and enzymes responsible for conversion of ALA to PPIX i.e., ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen decarboxylase and coproporphyrinogen oxidase are mostly located in the stromal phase (Manohara and Tripathy, 2000). All subsequent steps of Chl biosynthesis are catalyzed by membrane-bound or membrane-associated enzymes (Joyard et al., 1990; Matringe et al., 1992a; Manohara and Tripathy, 2000). Pchlide (Pineau et al., 1986) and PPIX (Mohapatra and Tripathy, 2002, 2003, 2007) are present both in the thylakoid and envelope membranes. Out of the total plastidic Pchlide, envelope membranes contained 1.5%, thylakoids have the maximum 98.48% and stroma has a trace fraction of 0.02%. Distribution of the Mg-PPIX and its monoester was 89.0% in thylakoids, 10.0% in stroma and 1.0% in envelope. A substantial fraction (33.77%) of plastidic PPIX was partitioned into stroma. Envelope contained 0.66% and thylakoids had 65.57% of the total plastidic PPIX pool. Had there been trafficking of Pchlide from the envelope to thylakoids or vice versa, significant amounts of Pchlide should have been present in the stroma. Therefore, in the developed chloroplast, Pchlide present in the envelope may be synthesized de novo independent of thylakoids. During greening Pchlide content (mg protein)$^{-1}$ decreased in thylakoids and increased in envelope membranes (Barthelemy et al., 2000).

ALA biosynthetic enzymes are located in the stroma (Kannangara et al., 1994). Enzymes responsible for conversion of ALA to protogen IX i.e., ALA dehydratase, porphobilinogen deaminase,

and uroporphyrinogen decarboxylase are mostly located in the stromal phase (Manohara and Tripathy, 2000). Coproporphyrinogen oxidase activity is predominantly observed in the stroma and a small fraction in the envelope (M.S. Manohara and B.C. Tripathy, unpublished). Once synthesized in the stroma, protogen IX may associate either to the envelope or thylakoid membranes where it is oxidized to PPIX. The presence of PPOX is shown both in the envelope and thylakoid membranes (Matringe et al., 1992a). Part of plastidic protogen IX migrates to mitochondria where it is oxidised by protoporphyrinogen oxidase II to PPIX, the substrate of heme synthesis (Lermontova et al., 1997). A substantial amount of PPIX present in the stroma may be due to its own diffusion from the site of its synthesis i.e., envelope and thylakoids. The next step in Chl biosynthesis is the conversion of PPIX to Mg-PPIX by Mg-chelatase. The association of MPE with envelope membranes suggests that Mg-chelatase is functional in envelope membranes. It is often argued that Chl biosynthetic enzymes present in envelope membranes are protein translocation intermediates. As there is an obligate requirement of three subunits of Mg-chelatase i.e., CHLD, CHLH and CHLI to assemble in a definite proportion to form the functional enzyme (von Wettstein et al., 1995; Kannangara et al., 1997; Papenbrock et al., 1997), it is unlikely that translocation intermediates of Mg-chelatase enzyme could mediate the synthesis of Mg-porphyrin. PPIX is converted to Mg-PPIX in the stroma in close association with inner envelope membrane or thylakoid. This explains the presence of MPE in the envelope membrane, stroma and thylakoids.

It is likely that the amphiphilic tetrapyrrole Mg-PPIX subsequently migrates to both envelope and thylakoids where it is independently esterified to MPE and subsequently metabolised to Pchlide. The POR protein is present both in the envelope and thylakoid membranes and phototransforms Pchlide to Chlide. As Chl synthetase is absent from envelope memembrane, Chl synthesis is not advanced in the envelope membrane. Chlide present in the envelope membrane may play an important role in the stabilization of light-harvesting proteins during their post-translational protein import. Although envelope membranes participate in tetrapyrrole biosynthesis leading to the synthesis of Chlide, the major role is played by the thylakoid membranes during late steps of Chl biosynthesis. The detailed intraplastidic Chl biosynthesis pathway is shown in Fig. 3.2.

IV. Regulation of Mg and Fe Branches of Tetrapyrrole Biosynthesis

In photosynthetic organisms Chl and heme biosynthesis is tightly regulated at various levels in response to environmental adaptation and plant development. The formation of ALA is the key regulatory step and provides adequate amounts of the common precursor molecule for the Mg and Fe branches of tetrapyrrole biosynthesis. ALA formation declines immediately after transition from light to dark and is correlated with an immediate accumulation of Pchlide in darkness. Pathway control prevents accumulation of metabolic intermediates and avoids photo-oxidative damage. Dark repression of ALA formation relies more on rapid post-translational regulation in response to accumulating Pchlide than on changes in nuclear gene expression (Stobart and Ameenbukhari, 1984; 1986; Richter et al., 2010).

PPIX may be acted upon by ferro-chelatase to form Fe-PPIX leading to heme synthesis. This is the second known branch point of tetrapyrrole biosynthesis after methylation of Urogen III leading to siroheme synthesis (Fig. 3.2). Ferrochelatase is inhibited by ATP (Cornah et al., 2002). During the day when ATP levels are higher, the magnesium branch of the pathway would be favored as Mg-chelatase needs ATP for Mg-PPIX synthesis (see below). Conversely, in the night the steady-state level of total heme increases in tobacco plants during the dark period and corresponds to the Fe-chelatase activity (Papenbrock et al., 1999). In *A. thaliana*, the Mg-chelatase subunit CHLH reaches a peak at the beginning of the light phase and ferrochelatase reaches a peak at the end of the light phase, indicating a diurnal regulation of Mg and Fe branches of tetrapyrrole biosynthesis (Harmer et al., 2000). NADPH-dependent thioredoxin reductase could be involved in redox regulation or protection of chlorophyll biosynthetic enzymes (Stenbaek and Jensen, 2010). The most important external modulator of the tetrapyrrole pathway in plants is light. In angiosperms, it plays a direct role in the Chl branch as light-dependent POR

phototransforms most of Pchlide to Chlide and this removes Pchlide-mediated feedback inhibition of ALA biosynthesis leading to increased availability of PPIX for the augmented ATP-dependent (light-dependent) Mg-chelation reaction. DELLAs (gibbralic acid-INSENSITIVE [GAI], REPRESSOR OF GA1-3 [RGA], RGA-LIKE1 [RGL1], RGL2, and RGL3) are a subfamily of transcriptional regulators that repress GA-mediated responses, and GA overcomes this DELLA-mediated restraint by stimulating the polyubiquitination of DELLAs that integrate many other environmental signal inputs in addition to light through their interaction with phytochrome interacting factors (PIFs) (Achard et al., 2006). DELLAs may regulate the levels of POR and protochlorophyllide in the dark to protect etiolated seedlings against photooxidative damage during initial light exposure (Cheminant et al., 2011).

V. Heterogeneity of Chlorophyll Biosynthesis

In higher plants i.e., angiosperms, Chls are synthesized during the day. Most of the DV-Pchlide (Tripathy and Rebeiz, 1985) synthesized during the day is immediately acted upon by POR and phototransformed to DV-Chlide. The latter is immediately fully converted to MV-Chlide (Duggan and Rebeiz, 1982; Nagata et al., 2007) that is subsequently phytylated to form MV-Chl. During the day under steady-state conditions even in the presence of POR and light, not all of the divinyl Pchlide pool is phototransformed to DV-Chlide. This pool of DV-Pchlide is slowly acted upon by divinyl reductase and converted to MV-Pchlide. The latter is subsequently phototransformed by POR to MV-Chlide that is esterified to form MV-Chl. During the night most of the DV-Pchlide pool that accumulates is slowly converted to MV-Pchlide by DVR (Tripathy and Rebeiz, 1988). At day break MV-Pchlide is immediately photo-transformed by POR to MV-Chlide. DVR activity varies between different species. It reduces DV-Pchlide to MV-Pchlide very quickly in barley and wheat but does so very slowly in cucumber and a number of other plant species (Carey et al., 1985). Therefore, various species have different MV- to DV-Pchlide ratios. As DVR speedily converts DV-Chlide to MV-Chlide, we do not see a mixture of MV- and DV-Chlide or MV- and DV-Chl in green plants. This Chl biosynthetic heterogeneity needs to be further understood.

VI. Evolution of Chlorophylls

Porphyrins are found in early rocks and meteorites (Hodgson and Baker, 1964). Synthesis of porphyrins, from pyrroles and aldehydes in an abiotic environment in aqueous clay suspension, has demonstrated the potential for non-enzymatic anabolic events that may have taken place before life originated (Cady and Pinnavaia, 1978). These porphyrins present in early Earth history were available for life when life came into existence from matter. As several porphyrins were needed for the sustenance of life, their synthesis was enhanced by the advent of enzymes (Larkum, 1991). Among tetrapyrroles, uroporphyrinogen, coproporphyrinogen, protoporphyrinogen and protoporphyrin were probably the earliest evolved pigments as they are needed for heme, cytochrome, biliproteins, phytochrome, vitamin B12, and F430 synthesis to support respiration and several metabolic processes (Hodgson and Ponamperuma, 1968). Early reaction centers in anaerobic prokaryotes could be porphyrins synthesized from oxidation of porphyrinogens mediated by UV light. On the early Earth, in the absence of oxygen, a lot of UV light could penetrate the atmosphere and UV light and blue light could have driven early photoreactions.

The Fe^{2+} ion was abundant in the anaerobic conditions early in Earth's history and Fe^{2+} could have been spontaneously inserted into a PPIX moiety to form heme. Formation of Bchl or Chl required the insertion of Mg^{2+} rather than Fe^{2+} to PPIX and this may have taken place non-enzymatically in places of high Mg^{2+} availability leading to the synthesis of Mg-PPIX, a first branch point leading to Bchl or Chl synthesis. Mg-porphyrins were favored over Fe-porphyrins because of their longer excited lifetime and were more efficient in inter-molecular energy transfer. With the advent of enzymes such the bacterial proteins BchlD, BchlH and BchlI or the eukaryotic enzymes CHLD, CHLH and CHLI during

the course of evolution, the enzymatic catalysis of Mg-insertion into a PPIX moiety by the assembly of these proteins to form the holoenzyme Mg-chelatase that required ATP hydrolysis was favored. Moreover, a fifth isocyclic ring was added to synthesize Pchlide, favoring capture of solar energy from a broader visible spectral range. Pchlide under early anaerobic conditions may have been converted to form the chlorin, Chlide by a light-independent Pchlide reductase enzyme made up of 3 subunits i.e., Bchl, BchN and BchB or CHLL, CHLN and CHLB. It is likely that from here Bchl *a* and Chl *a* branches diverged. It is possible that the early oxygenic photosynthetic bacteria i.e., chloroxybacteria evolved from an ancient group of anoxygenic photosynthetic bacteria and contained both Bchl and Chl (Larkum, 1999). Later the Chl branch was favored in the oxygen rich atmosphere.

Chl *d* probably evolved in *Acaryochloris marina* (Miyashita et al., 1996) from early Chl biosynthetic intermediates to harness solar energy at a longer wavelength than that of Chl *a* probably being dictated by its niche. Among prokaryotes, the prochlorophytes contain both Chl *a* and Chl *b* and have a relatively larger proportion of Chl *b* leading to a reduced Chl *a*/*b* ratio typically found in eukaryotic green algae and plants. The early marine environment that had reduced light intensity in deeper layers probably favored the synthesis of more accessory pigments such as Chl *b*. In eukaryotic Chl *b*-containing organisms, including chlorophytes and higher plants, the Chl *a*/*b* ratio is larger than in prochlorophytes due to reduced synthesis of Chl *b*. This was achieved by adding the N-terminal regulatory Chl *b*-sensing A domain to CAO that leads the enzyme into the degradation pathway. This modulates the level of CAO that converts Chlide *a* to Chlide *b* (Nagata et al., 2004; Nakagawara et al., 2007; Tanaka and Tanaka, 2007).

VII. Future Prospects

Although we have a fairly complete understanding of the mechanisms required for Chl biosynthesis, apart from knowing all the genes involved in the synthesis of the cyclopentanone ring (ring E), Chl *d* and conversion of Chl *b* to Chl *a*, our knowledge of Chl biosynthetic heterogeneity, regulation of Chl biosynthesis, their modulated insertion to light-harvesting complexes and different photosystems is far from complete. Chls and their biosynthetic intermediates are directly involved in the generation of $^1O_2$ or O_2^- via type II or type I photosensitization reactions (Chakraborty and Tripathy, 1992; Tripathy et al., 2007). Understanding the regulation of Chl biosynthesis and the insertion Chl into photosynthetic protein complexes will be important for the future minimization of the generation of reactive oxygen species in plants. Besides it will enhance our knowledge of the biology of energy capture and transfer among photosynthetic pigments that could be utilized in artificial photosynthesis research. Evolution of Chl synthesis is little understood and needs to be studied further.

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References

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J and Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311: 91–94

Adhikari ND, Orler R, Chory J, Froehlich JE and Larkin RM (2009) Porphyrins promote the association of genomes uncoupled 4 and a MG-chelatase subunit with chloroplast membranes. J Biol Chem 284: 24783–24796

Alawady AE and Grimm B (2005) Tobacco Mg protoporphyrin IX methyltransferase is involved in inverse activation of Mg porphyrin and proto heme synthesis. Plant J 41: 282–290

Alawady A, Reski R, Yaronskaya E and Grimm B (2005) Cloning and expression of the tobacco CHLM sequence encoding Mg protoporphyrin IX methyltransferase and its interaction with Mg chelatase. Plant Mol Biol 57: 679–91

Apel K (1981) The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Phytochrome-induced decrease of translatable mRNA coding for the NADPH: protochlorophyllide oxidoreductase. Eur J Biochem 120: 89–93

Armstrong GA, Runge S, Frick G, Sperling U and Apel K (1995) Identification of NADPH: protochlorophyllide oxidoreductase A and B: A branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol 108: 1505–1517

Arnould S and Camadro JM (1998) The domain structure of protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. Proc Natl Acad Sci USA 95: 10553–10558

Aronsson H, Sundqvist C, Timko MP and Dahlin C (2001) The importance of the C-terminal region and Cys residues for the membrane association of the NADPH:protochlorophyllide oxidoreductase in pea. FEBS Lett 502: 11–15

Ayliffe MA, Agostino A, Clarke BC, Furbank R, von Caemmerer S and Pryor AJ (2009) Suppression of the barley uroporphyrinogen III synthase gene by a Ds activation tagging element generates developmental photosensitivity. Plant Cell 21: 814–831

Bang WY, Jeong IS, Kim DW, Im CH, Ji C, Hwang SM, Kim SW, Son YS, Jeong J, Shiina T and Bahk JD (2008) Role of *Arabidopsis* CHL27 protein for photosynthesis, chloroplast development and gene expression profiling. Plant Cell Physiol 49:1350–63

Barnes SA, Nishizawa NK, Quaggio RB, Whitelam GC and Chua N-H (1996) Far-red light blocks greening of *Arabidopsis* seedlings via a phytochrome A-mediated change in plastid development. Plant Cell 8: 601–615

Barthélemy X, Bouvier G, Radunz A, Docquier S, Schmid GH and Franck F (2000) Localization of NADPH-protochlorophyllide reductase in plastids of barley at different greening stages. Photosynth Res 64: 63–76

Batschauer A and Apel K (1984) An inverse control by phytochrome of the expression of two nuclear genes in barley (*Hordeum vulgare* L.). Eur J Biochem 143: 593–597

Bazzaz MB (1981) New chlorophyll chromophores isolate from a chlorophyll deficient mutant of maize. Photobiochem Photobiophys 2: 199–207

Begley TP and Young H (1989) Protochlorophyllide reductase. 1. Determination of the regiochemistry and the stereochemistry of the reduction of protochlorophyllide to chlorophyllide. J Am Chem Soc 111: 3095–3096

Beale SI and Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. In: Dailey HA (eds) Biosynthesis of Heme and Chlorophyll. pp 287–291. McGraw-Hill, New York

Benli M, Schulz R and Apel K (1991) Effect of light on the NADPH-Pchlide oxidoreductase (POR) of *A. thaliana*. Plant Mol Biol 16: 615–625

Benz J and Rüdiger W (1981a) Incorporation of 1-14 C-isopentenyldiphosphate, geraniol and farnesol into chlorophyll in plastid membrane fractions of *Avena sativa* L. Z Pflanzenphysiol 102: 95–100

Benz J and Rüdiger W (1981b) Chlorophyll biosynthesis: various chlorophyllides as exogenous substrates for chlorophyll synthetase. Z Naturforsch 36c: 51–57

Bhattacharjee S and Mukherjee AK (2003) Heavy metals alter photosynthetic pigment profiles as well as activities of chlorophyllase and 5-aminolevulinic acid dehydratase (ALAD) in *Amaranthus lividus* seedlings. J Environ Biol 24: 395–399

Birve SJ, Selstam E and Johansson LB (1996) Secondary structure of NADPH: protochlorophyllide oxidoreductase examined by circular dichroism and prediction methods. Biochem J 317: 549–555

Block MA, Tewari AK, Albrieux C, Marechal E and Joyard J (2002) The plant S-adenosyl-L-methionine: Mg-protoporphyrin IX methyl transferase is isolated in both envelope and thylakoid chloroplast membranes. Eur J Biochem 269: 240–248

Böddi B, Lindsten A, Ryberg M and Sundqvist C (1989) On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. Physiol Plant 76: 135–143

Böddi B, Ryberg M and Sundqvist C (1991) The formation of a short-wavelength chlorophyllide form at partial phototransformation of protochlorophyllide in etioplast inner membranes. Photochem Photobiol 53: 667–673

Böddi B, Ryberg M and Sundqvist C (1992) Identification of four universal protochlorophyllide forms in dark-grown leaves by analyses of the 77 K fluorescence emission spectra. J Photochem Photobiol B Biol 12: 389–401

Böddi B, Ryberg M and Sundqvist C (1993) Analysis of the 77 K fluorescence emission and excitation spectra of isolated etioplast inner membranes. J Photochem Photobiol B Biol 21: 125–133

Boese QF, Spano AJ, Li JM and Timko MP (1991) Aminolevulinic acid dehydratase in pea (*Pisum sativum* L.) Identification of an unusual metal binding in the plant enzyme. J Biol Chem 266: 17060–17066

Bohren KM, Grimshaw CE, Lai CJ, Harrison DH, Ringe D, Petsko GA and Gabbay KH (1994) Tyrosine-48 is the proton donor and histidine-110 directs substrate stereochemical selectivity in the reduction reaction of human aldose reductase: enzyme kinetics and crystal structure of the Y48H mutant enzyme. Biochemistry 33: 2021–2032

Bougri O and Grimm B (1996) Members of a low-copy number gene family encoding glutamyl-tRNA reductase are differentially expressed in barley. Plant J 9: 867–878

Bruyant P and Kannangara CG (1987) Biosynthesis of 5-aminolevulinate in greening barley leaves. VII. Purification and characterization of the glutamate-tRNA ligase. Carlsberg Res Comm 52: 99–109

Buhr F, El Bakkouri M, Valdez O, Pollmann S, Lebedev N, Reinbothe S and Reinbothe C (2008) Photoprotective role of NADPH:protochlorophyllide oxidoreductase A. Proc Natl Acad Sci USA 105: 12629–12634

Cady SS and Pinnavaia TJ (1978) Porphyrin intercalation in mice-type silicates. Inorg Chem 17: 1501–1507

Camadro JM, Matringe M, Scalla R and Labbe P (1991) Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. Biochem J 277: 17–21

Carey EE, Tripathy BC and Rebeiz CA (1985) Chloroplast biogenesis 51: Modulation of monovinyl and divinyl protochlorophyllide biosynthesis by light and darkness in vitro. Plant Physiol 79: 1059–1063

Castelfranco PA and Beale SI (1981) Chlorophyll biosynthesis. In: Stumpf PK and Conn EE (eds) The Biochemistry of Plants: A Comprehensive Treatise, Vol 8, pp 375–421, Academic Press, New York

Castelfranco PA, Thayer SS, Wilkinson JQ and Bonner BA (1988) Labeling of porphobilinogen deaminase by radioactive 5-aminolevulinic acid in isolated developing pea chloroplasts. Arch Biochem Biophys 266: 219–226

Chakraborty N and Tripathy BC (1992) Involvement of singlet oxygen in photodynamic damage of isolated chloroplasts of cucumber (*Cucumis sativus* L.) cotyledons. Plant Physiol 98: 7–11

Che FS, Watnabe N, Iwano M, Inokuchi H, Takayama S, Yoshida S and Isogai A (2000) Molecular characterization and subcellular localization of protoporphyrinogen oxidase in spinach chloroplasts. Plant Physiol 124: 59–70

Cheminant S, Wild M, Bouvier F, Pelletier S, Renou JP, Erhardt M, Hayes S, TerryMJ, Genschik P and Achard P (2011) DELLAs regulate chlorophyll and carotenoid biosynthesis to prevent photooxidative damage during seedling deetiolation in Arabidopsis. Plant Cell May 27. [Epub ahead of print]

Chen TC and Miller GW (1974) Purification and characterization of uroporphyrinogen decarboxylase from tobacco leaves. Plant Cell Physiol 15: 993–1005

Chory J, Reinecke D, Sim S, Washburn T and Brenner M (1994) A role of cytokinins in de-etiolation in *Arabidopsis det* mutants have an altered response to cytokinins. Plant Physiol 104: 339–347

Coemans B, Matsumura H, Terauchi R, Remy S, Swennen R and Sagi L (2005) SuperSAGE combined with PCR walking allows global gene expression profiling of banana (*Musa acuminata*), a non-model organism. Theor Appl Genet 111: 1118–1126

Cornah JE, Roper JM, Pal Sing D and Smith AG (2002) Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and non-photosynthetic cells of pea (*Pisum sativum* L.). Biochem J 362: 423–32

Crockett N, Alefounder PR, Battersby AR and Abell C (1991) Uroporphyrinogen III synthase: Studies on its mechanism of action molecular biology and biochemistry. Tetrahedron 47: 6003–6014

Dahlin C, Aronsson H, Wilks HM, Lebedev N, Sundqvist C and Timko MP (1999) The role of protein surface charge in catalytic activity and chloroplast membrane association of the pea NADPH:protochlorophyllide oxidoreductase (POR) as revealed by alanine scanning mutagenesis. Plant Mol Biol 39: 309–323

Dahlin C, Aronsson H, Almkvist J and Sundqvist C (2000) Protochlorophyllide-independent import of two NADPH: Pchlide oxidoreductase proteins (PORA and PORB) from barley into isolated plastids. Physiol Plant 109: 298–303

Darrah PM, Kay SA, Teakle GR and Griffiths WT (1990) Cloning and sequencing of protochlorophyllide reductase. Biochem J 265: 789–798

Davison PA, Schubert HL, Reid JD, Iorg CD, Heroux A, Hill CP and Hunter CN (2005) Structural and biochemical characterization of Gun4 suggests a mechanism for its role in chlorophyll biosynthesis. Biochemistry 44: 7603–7612

Day IS, Golovkin M and Reddy AS (1998) Cloning of the cDNA for glutamyl-tRNA synthetase from *Arabidopsis thaliana*. Biochim Biophys Acta 1399: 219–224

Dayan FE, Ferreira D, Wang YH, Khan IA, McInroy JA and Pan Z (2008) A pathogenic fungi diphenyl ether phytotoxin targets plant enoyl (acyl carrier protein) reductase. Plant Physiol 147: 1062–1071

Domanskii V, Rassadina V, Gus-Mayer S, Wanner G, Schoch S and Rudiger W (2003) Characterization of two phases of chlorophyll formation during greening of etiolated barley leaves. Planta 216: 475–483

Douce R and Joyard J (1990) Biochemistry and function of the plastid envelope. Annu Rev Cell Biol 6: 173–216

Duggan JX and Rebeiz CA (1982) Chloroplast biogenesis. 37. Induction of chlorophyllide *a* (E459F675) accumulation in higher plants. Plant Sci Lett 24: 27–37

Eggink LL, LoBrutto R, Brune DC, Brusslan J, Yamasato A, Tanaka A and Hoober JK (2004) Synthesis of chlorophyll *b*: localization of chlorophyllide a oxygenase and discovery of a stable radical in the catalytic subunit. BMC Plant Biol 4: 5

Eichacker LA, Soll J, Lauterbach P, Rudiger W, Klein RR and Mullet JE (1990) In vitro synthesis of chlorophyll *a* in the dark triggers accumulation of chlorophyll *a* apoproteins in barley etioplasts. J Biol Chem 265:13566–13571

Espineda CE, Linford AS, Devine D and Brusslan JA (1999) The AtCAO gene, encoding chlorophyll *a* oxygenase, is required for chlorophyll *b* synthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96: 10507–10511

Fodje MN, Hansson A, Hansson M, Olsen JG, Gough S, Willows RD and Al-Karadaghi S (2001) Interplay between an AAA module and an integrin I domain may regulate the function of magnesium chelatase. J Mol Biol 311: 111–122

Forreiter C, Van Cleve B, Schmidt A and Apel K. (1991) Evidence for a general light-dependent negative control of NADPH–protochlorophyllide oxidoreductase in angiosperms. Planta 183: 126–132

Franck F, Sperling U, Frick G, Pochert B, Van Cleve B, Apel K and Armstrong GA (2000) Regulation of etioplast pigment–protein complexes, inner membrane architecture, and protochlorophyllide *a* chemical heterogeneity by light-dependent NADPH: protochlorophyllide oxidoreductases A and B. Plant Physiol 124: 1678–1696

Frank HA, Young AJ, Britton G and Cogdell RJ (eds) (1999) The Photochemistry of Carotenoids, Advances in Photosynthesis, Vol 8. Kluwer Academic Publishers, Dordrecht

Frick G, Su Q, Apel K and Armstrong GA (2003) An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH: protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested. Plant J 35: 141–153

Frustaci JM, Sangwan I and O'Brian MR (1995) gsa1 is a universal tetrapyrrole synthesis gene in soybean and is regulated by a GAGA element. J Biol Chem 270: 7387–7393

Fusada N, Masuda T, Kuroda H, Shiraishi T, Shimada H, Ohta H and Takamiya K (2000) NADPH–protochlorophyllide oxidoreductase in cucumber is encoded by a single gene and its expression is transcriptionally enhanced by illumination. Photosynth Res 64: 147–154

Fusada N, Masuda T, Kuroda H, Shimada H, Ohta H and Takamiya K (2005) Identification of a novel cis-element exhibiting cytokinin-dependent protein binding in vitro in the 5'-region of NADPH-protochlorophyllide oxidoreductase gene in cucumber. Plant Mol Biol 59: 631–645

Gaubier P, Wu HJ, Laudie M, Delseny M and Grellet F (1995) A chlorophyll synthetase gene from *Arabidopsis thaliana*. Mol Gen Genet 249: 673–676

Giannino D, Condello E, Bruno L, Testone G, Tartarini A, Cozza R, Innocenti AM, Bitonti MB and Mariotti D (2004) The gene geranylgeranyl reductase of peach (*Prunus persica* [L.] Batsch) is regulated during leaf development and responds differentially to distinct stress factors. J Exp Bot 55: 2063–2073

Gibson LC, Marrison JL, Leech RM, Jensen PE, Bassham DC, Gibson M and Hunter CN (1996) A putative Mg chelatase subunit from *Arabidopsis thaliana* cv C24. Sequence and transcript analysis of the gene, import of the protein into chloroplasts, and in situ localization of the transcript and protein. Plant Physiol 111: 61–71

Gibson KD, Neuberger A and Tait GH (1963) Studies on the biosynthesis of porphyrin and bacteriochlorophyll by *Rhodopseudomonas spheriodes*. 4. S-adenosylmethionine-magnesium protoporphyrin methyltransferase. Biochem J 88: 325–334

Gough SP, Kannangara CG and von Wettestein D (1992) Glutamate 1-semialdehyde aminotransferase as a target for herbicides. In: Boger P and Sandmann G (eds) Target Assays for Modern Herbicides and Related Phytotoxic Compounds, pp 21–27. Lewis Publishers, Chelesa

Grimm B, Porra RJ, Rüdiger W and Scheer H (eds) (2006) Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications, Advances in Photosynthesis and Respiration, Vol 25. Springer, Dordrecht

Hansson A and Jensen PE (2009) Chlorophyll limitation in plants remodels and balances the photosynthetic apparatus by changing the accumulation of photosystems I and II through two different approaches. Physiol Plant 135: 214–228

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA and Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290: 2110–2113

Harper AL, von Gesjen SE, Linford AS, Peterson MP, Faircloth RS, Thissen MM and Brusslan JA (2004) Chlorophyllide *a* oxygenase mRNA and protein levels correlate with the chlorophyll *a*/*b* ratio in *Arabidopsis thaliana*. Photosynth Res 79: 149–159

Havaux M, Lutz C and Grimm B (2003) Chloroplast membrane photostability in chlP transgenic tobacco plants deficient in tocopherols. Plant Physiol 132: 300–310

He ZH, Li J, Sundqvist C and Timko MP (1994) Leaf development age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). Plant Physiol 106: 537–546

Hedtke B, Alawady A, Chen S, Börnke F and Grimm B (2007) HEMA RNAi silencing reveals a control mechanism of ALA biosynthesis on Mg chelatase and Fe chelatase. Plant Mol Biol 64: 733–742

Helfrich M, Schoch S, Lempert U, Cmiel E and Rudiger W (1994) Chlorophyll synthetase cannot synthesize chlorophyll *a*. Eur J Biochem 219: 267–275

Heyes DJ and Hunter CN (2004) Identification and characterization of the product release steps within the catalytic cycle of protochlorophyllide oxidoreductase. Biochemistry 43: 8265–8271

Heyes DJ, Ruban AV, Wilks HM and Hunter CN (2002) Enzymology below 200 K: the kinetics and thermodynamics of the photochemistry catalyzed by protochlorophyllide oxidoreductase. Proc Natl Acad Sci USA 99: 11145–11150

Heyes DJ, Ruban AV and Hunter CN (2003) Protochlorophyllide oxidoreductase: spectroscopic characterization of the 'dark' reactions. Biochemistry 42: 523–528

Heyes DJ, Kruk J and Hunter CN (2006) Spectroscopic and kinetic characterization of the light-dependent enzyme protochlorophyllide oxidoreductase (POR) using monovinyl and divinyl substrates Biochem J 394: 243–248

Heyes DJ, Menon BR, Sakuma M and Scrutton NS (2008) Conformational events during ternary enzyme-substrate complex formation are rate limiting in the catalytic cycle of the light-driven enzyme protochlorophyllide oxidoreductase. Biochemistry 47: 10991–10998

Higuchi M and Bogorad L (1975) The purification and properties of uroporphyrinogen I synthase and uroporphyrinogen III cosynthase. Interactions between the Enzymes. Ann NY Acad Sci 244: 401–418

Hirashima M, Satoh S, Tanaka R and Tanaka A (2006) Pigment shuffling in antenna systems achieved by expressing prokaryotic chlorophyllide *a* oxygenase in *Arabidopsis*. J Biol Chem 281: 15385–15393

Hiriart JB, Lehto K, Tyystjarvi E, Junttila T and Aro EM (2002) Suppression of a key gene involved in chlorophyll biosynthesis by means of virus-inducing gene silencing. Plant Mol Biol 50: 213–224

Hodgson GW and Baker GL (1964) Evidence for porpyrin in the Orgueil meteorite. Nature 202:125–127

Hodgson GW and Ponnamoeruma C (1968) Prebiotic porphyrin genesis: Porphyrin from electric discharge in methane, ammonia and water vapour. Proc Natl Acad Sci USA 31:153–158

Höfgen R, Axelsen KB, Kannangara CG, Schuttke I, Pohlenz, Willmitzer L, Grimm B and von Wettestein D (1994) A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll biosynthesis with a glutamate 1-semialdehyde aminotransferase antisense gene. Proc Natl Acad Sci USA 91: 1726–1730

Holtorf H and Apel K (1996a) Transcripts of the two NADPH–protochlorophyllide oxidoreductase genes PorA and PorB are differentially degraded in etiolated barley seedlings. Plant Mol Biol 31: 387–392

Holtorf H and Apel K (1996b) The regulation of NADPH–protochlorophyllide oxidoreductase *a* and *b* in green barley plants kept under a diurnal light dark cycle. Planta 199: 289–295

Holtorf H, Reinbothe S, Reinbothe C, Bereza B and Apel K (1995) Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). Proc Natl Acad Sci USA 92: 3254–3258

Hoober JK, Kahn A, Ash DE, Gough SP and Kannagara CG (1988) Biosynthesis of 5-aminolevulinate in greening barley leaves.IX. Structure of the substrate, mode of gabaculine inhibition, and the catalytic mechanism of glutamate-1-semialdehyde aminotransferase. Carlsberg Res Com 53:11–25

Horie Y, Ito H, Kusaba M, Tanaka R and Tanaka A (2009) Participation of chlorophyll *b* reductase in the initial step of the degradation of light-harvesting chlorophyll *a*/*b*-protein complexes in *Arabidopsis*. J Biol Chem 284:17449–17456

Hsu WP and Miller GW (1970) Coproporphyrinogenase in tobacco (*Nicotiana tabacum* L.). Biochem J 117: 215–220

Hu G, Yalpani N, Briggs SP and Johal GS (1998) A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. Plant Cell 10: 1095–1105

Huang DD, Wang WY, Gough SP and Kannangara CG (1984) Delta-Aminolevulinic acid-synthesizing enzymes need an RNA moiety for activity. Science 225: 1482–1484

Huang YS and Li HM (2009) *Arabidopsis* CHLI2 can substitute for CHLI1. Plant Physiol 150: 636–645

Hukmani P and Tripathy BC (1994) Chlorophyll biosynthetic reactions during senescence of excised barley (*Hordeum vulgare* L. cv IB 65) Leaves. Plant Physiol 105: 1295–1300

Ikegami A, Yoshimura N, Motohashi K, Takahashi S, Romano PG, Hisabori T, Takamiya K and Masuda T (2007) The CHLI1 subunit of *Arabidopsis thaliana* magnesium chelatase is a target protein of the chloroplast thioredoxin. J Biol Chem 282: 19282–19291

Ilag LL, Kumar AM and Soll D (1994) Light reduction of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in *Arabidopsis*. Plant Cell 6: 265–275

Ishikawa A, Okamoto H, Iwasaki Y and Asahi T (2001) A deficiency of coproporphyrinogen III oxidase causes lesion formation in *Arabidopsis*. Plant J 27: 89–99

Jacobs NJ and Jacobs JM (1979) Microbial oxidation of protoporphyrinogen: an intermediate in heme and chlorophyll biosynthesis. Arch Biochem Biophys 197: 396–403

Jacobs JM and Jacobs NJ (1987) Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Purification and partial characterization of the enzyme from barley organelles. Biochem J 244: 219–224

Jain M and Gadre RP (2004) Inhibition of 5-amino levulinic acid dehydratase activity by arsenic in excised etiolated maize leaf segments during greening. J Plant Physiol 161: 251–255

Jensen PE, Willows RD, Petersen BL, Vothknecht UC, Stummann BM, Kannangara CG, von Wettstein D and Henningsen KW (1996) Structural genes for Mg-chelatase subunits in barley: Xantha-f, -g, and -h. Mol Gen Genet 250: 383–394

Jensen PE, Gibson LCD and Hunter CN (1999) ATPase activity associated with the magnesium-protoporphyrin IX chelatase enzyme of *Synechocystis* PCC6803: evidence for ATP hydrolysis during Mg^{2+} insertion, and MgATP dependent interaction of ChlI and ChlD subunits. Biochem J 339:127–134

Jilani A, Kar S, Bose S and Tripathy BC (1996) Regulation of the carotenoid content and chloroplast development by levulinic acid. Physiol Plant 96: 139–145

Jones DT (1999) GenTHREADER: an efficient and reliable protein fold recognition method for genomic sequences. J Mol Biol 287: 797–815

Jones RM and Jordan PM (1994) Purification and properties of porphobilinogen deaminase from *Arabidopsis thaliana*. Biochem J 299: 895–902

Jordan PM and Shemin D (1980) Mechanism of action of 5-aminolevulinic acid dehydratase: Stepwise order of addition of the two molecules of 5-aminilevulinic acid in the enzymatic synthesis of porphobilinogen. J Chem Soc Chem Comm 240–242

Joyard J, Block M, Pineau B, Albrieux C and Douce R (1990) Envelope membranes from mature spinach chloroplasts contain a NADPH:protochlorophyllide reductase on the cytosolic side of the outer membrane. J Biol Chem 265: 21820–21827

Jung KH, Hur J, Ryu CH, Choi Y, Chung YY, Miyao A, Hirochika H and An G (2003) Characterization of a rice chlorophyll-deficient mutant using the T-DNA gene-trap system. Plant Cell Physiol 44: 463–472

Jung S, Lee HJ, Lee Y, Kang K, Kim YS, Grimm B and Back K (2008) Toxic tetrapyrrole accumulation in protoporphyrinogen IX oxidase-overexpressing transgenic rice plants. Plant Mol Biol 67: 535–546

Kaczor CM, Smith MW, Sangwan I and O'Brian MR (1994) Plant delta-aminolevulinic acid dehydratase. Expression in soybean root nodules and evidence for a bacterial lineage of the Alad gene. Plant Physiol 104: 1411–1417

Kagan RM and Clarke S (1994) Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. Arch Biochem Biophys 310: 417–427

Kannangara CG, Gough SP, Oliver RP and Rasmussen SK (1984) Biosynthesis of 5-ALA in greening barley leaves. VI. Activation of glutamate by ligation to RNA. Carlsberg Res Com 49: 417–437

Kannagara CG, Gough SP, Bruyant P, Hoober JK, Kahn A and Wettestein DV (1988) $tRNA^{glu}$ as a cofactor on δ-aminolevulinate biosynthesis: Steps that regulatechlorophyll synthesis. Trends Biol Sci 13: 139–143

Kannangara CG, Andersen RV, Pontoppidan B, Willows R and von Wettstein D (1994) Enzymic and mechanistic studies on the conversion of glutamate to 5-aminolaevulinate. Ciba Found Symp 180: 3–20

Kannangara CG, Vothknecht UC, Hansson M and von Wettstein D (1997) Magnesium chelatase: association with ribosome and mutant complementation studies identify barley subunit Xantha-G as a functional counterpart of *Rhodobacter* subunit BchD. Mol Gen Genet 254: 85–92

Keller Y, Bouvier F, D'`Harlingue A and Camara B (1998) Metabolic compartmentation of plastid prenyllipid biosynthesis. Evidence for the involvement of a multifunctional geranylgeranyl reductase. Eur J Biochem 251: 413–417

Kervinen J, Dunbrack Rl Jr, Litwin S, Martins J, Scarrow RC, Volin M, Yeung AT, Yoon E and Jaffe EK (2000) Porphobilinogen synthase from pea: Expression from an artificial gene, kinetic characterisation and novel implication for subunit interactions. Biochemistry 39: 9018–9029

Kim YK, Lee JY, Cho HS, Lee SS, Ha HJ, Kim S, Choi D and Pai HS (2005) Inactivation of organellar glutamyl- and seryl-tRNA synthetases leads to developmental arrest of chloroplasts and mitochondria in higher plants. J Biol Chem 280: 37098–38106

Koch M, Breithaupt C, Kiefersauer R, Freigang J, Huber R and Messerschmidt A (2004) Crystal structure of protoporphyrinogen IX oxidase: a key enzyme in haem and chlorophyll biosynthesis EMBO J 23: 1720–1728

Kolossov VL and Rebeiz CA (2001) Chloroplast biogenesis 84: Solubilization and partial purification of membrane-bound [4-vinyl] chlorophyllide *a* reductase from etiolated barley leaves. Anal Biochem 295: 214–219

Kropat J, Oster U, Rüdiger W and Beck CF (1997) Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-shock genes. Proc Natl Acad Sci USA 94: 14168–14172

Kropat J, Oster U, Rüdiger W and Beck CF (2000) Chloroplast signalling in the light induction of nuclear HSP70 genes requires the accumulation of chlorophyll precursors and their accessibility to cytoplasm/nucleus. Plant J 24: 523–531

Kruse E, Mock HP and Grimm B (1995a) Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and affects the oxidative defense system. EMBO J 14: 3712–3720

Kruse E, Mock HP and Grimm B (1995b) Coproporphyrinogen III oxidase from barley and tobacco-sequence analysis and initial expression studies. Planta 196: 796–803

Kruse E, Mock HP and Grimm B (1997) Isolation and characterization of tobacco (*Nicotiana tabacum*) cDNA clones encoding proteins involved in magnesium chelation into protoporphyrin IX. Plant Mol Biol 35: 1053–1056

Kumar AM and Söll D (2000) Antisense HEMA1 RNA expression inhibits heme and chlorophyll biosynthesis in *Arabidopsis*. Plant Physiol 122: 49–56

Kumar AM, Csankovszki G and Söll D (1996) A second and differentially expressed glutamyl-tRNA reductase gene from *Arabidopsis thaliana*. Plant Mol Biol 30: 419–426

Kuroda H, Masuda T, Ohta H, Shioi Y and Takamiya K (1995) Light-enhanced gene expression of NADPH–protochlorophyllide oxidoreductase in cucumber. Biochem Biophys Res Commun 210: 310–316

Kuroda H, Masuda T, Fusada N, Ohta H and Takamiya K (2001) Cytokinin-induced transcriptional activation of NADPH–protochlorophyllide oxidoreductase gene in cucumber. J Plant Res 114: 1–7

Kusnetsov V, Herrmann RG, Kulaeva ON and Oelmüller R (1998) Cytokinin stimulates and abscisic acid inhibits greening of etiolated *Lupinus luteus* cotyledons by affecting the expression of the light-sensitive protochlorophyllide oxidoreductase. Mol Gen Gene 259: 21–28

Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M and Tanaka A (2007) Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell 19: 1362–1375

Kusumi J, Sato A and Tachidi H (2006) Relaxation of functional constraint on light-independent protochlorophyllide reductase in *Thuja*. Mol Biol Evol 23: 941–948

Larkin RM, Alonso JM, Ecker JR and Chory J (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling. Science 299: 902–906

Larkum AWD (1991) The evolution of chlorophylls. In: Scheer H (ed) Chlorophylls, pp 367–383. CRC Press, Boca Raton

Larkum AWD (1999) The evolution of algae. In: Seckbach J (ed) Enigmatic Microorganisms and Life in Extreme Environments, pp 31–48. Kluwer Academic Publishers, Dordrecht

Lebedev N and Timko MP (1999) Protochlorophyllide oxidoreductase B-catalysed protochlorophyllide photoreduction in vitro: insight into the mechanism of chlorophyll formation in light-adapted plants. Proc Natl Acad Sci USA 96: 9954–9959

Lebedev N, Van Cleve B, Armstrong G and Apel K (1995) Chlorophyll synthesis in a deetiolated (*det340*) mutant of *Arabidopsis* without NADPH–protochlorophyllide (PChlide) oxidoreductase (POR) *a* and photoactive PChlide-F655. Plant Cell 7: 2081–2090

Lebedev N, Karginova O, McIvor W and Timko MP (2001) Tyr275 and Lys279 stabilize NADPH within the catalytic site of NADPH: protochlorophyllide oxidoreductase and are involved in the formation of the enzyme photoactive state. Biochemistry 40: 12562–12574

Lee HJ, Lee SB, hung JS, Han SU, Han O, Guh JO, Jeon JS, An G and Back K (2000) Transgenic rice plants expressing a *Bacillus subtilis* protoporphyrinogen oxidase gene are resistant to diphenyl-ether herbicide acifluorfen. Plant Cell Physiol 41: 743–749

Lee KP, Kim C, Lee DW and Apel K (2003) TIGRINA d, required for regulating the biosynthesis of tetrapyrolles in barley, is an ortholog of FLU gene of *Arabidopsis thaliana*. FEBS Lett 553: 119–124

Lee S, Kim JH, Yoo ES, Lee CH, Hirochika H and An G (2005) Differential regulation of chlorophyll *a* oxygenase genes in rice. Plant Mol Biol 57: 805–818

Lermontova I and Grimm B (2000) Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. Plant Physiol 122: 75–84

Lermontova I and Grimm B (2006) Reduced activity of plastid protoporphyrinogen oxidase causes attenuated photodynamic damage during high-light compared to low-light exposure. Plant J 48: 499–510

Lermontova I, Kruse E, Mock HP and Grimm B (1997) Cloning and characterisation of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. Proc Natl Acad Sci USA 94: 8895–8900

Li J, Spano AJ and Timko MP (1991) Isolation and characetisation of nuclear genes encoding the ALA dehydratase of pea (*Pisum sativum* L.). Plant Physiol 96: 125–127

Liedgens W, Grutzmann R and Schneider HAW (1980) Highly efficient purification of the labile plant enzyme 5-aminolevulinate dehydratase (EC 4.2.1.24) by means of monoclonal antibodies. Z Naturforsch 35c: 958–962

Lim SH, Witty M, Wallace-Cook AD, Ilag LI and Smith AG (1994) Porphobilinogen deaminase is encoded by a single gene in *Arabidopsis thaliana* and is targeted to the chloroplasts. Plant Mol Biol 26: 863–872

Lindsten A, Ryberg M and Sundqvist C (1988) The polypeptide composition of highly purified prolamellar bodies and prothylakoids from wheat (*Tritium aestivum*) as revealed by silver staining. Physiol Plant 72: 167–176

Lindstein A, Welch CJ, Schoch S, Ryberg M, Rüdiger W and Sundqvist C (1990) Chlorophyll synthatase is latent in well preserved prolamellar bodies of etiolated wheat. Physiol Plant 80: 277–285

Liu N, Yang YT, Liu HH, Yang GD, Zhang NH and Zheng CC (2004) NTZIP antisense plants show reduced chlorophyll levels. Plant Physiol Biochem 42: 321–327

Luo J and Lim CK (1993) Order of urogen III decarboxylation on incubation of PBG and urogen III with erythrocyte UDC. Biochem J 289: 529–532

Madsen O, Sandal L, Sandal NN and Marcker KA (1993) A soybean coproporphyrinogen oxidase gene is highly expressed in root nodules. Plant Mol Biol 23: 35–43

Manohara MS and Tripathy BC (2000) Regulation of protoporphyrin IX biosynthesis by intraplastidic compartmentalization and adenosine triphosphate. Planta 212: 52–59

Mapleston RE and Griffiths WT (1980) Light modulation of the activity of protochlorophyllide reductase. Biochem J 189: 125–133

Martin W, Stoebe B, Goremykin V, Hansmann S, Hasegawa M and Kowallik KV (1998). Gene transfer to the nucleus and the evolution of chloroplasts. Nature 393: 162–165

Martin BM, Grimm B, Mock HP, Huber R and Messerschmidt A (2001) Crystal structure and substrate binding modeling of the uroporphyrinogen-III decarboxylase from *Nicotiana tabacum*. Implications for the catalytic mechanism. J Biol Chem 276: 44108–44116

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M and Penny D (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci USA 99: 12246–12251

Masuda T and Takamiya K (2004) Novel insights into enzymology, regulation and physiological functions of light-dependant Protochlorophyllide oxidoreductase in angiosperms. Photosynth Res 81: 1–29

Masuda T, Fusada N, Shiraishi T, Kuroda H, Awai K, Shimada H, Ohta H and Takamiya K (2002) Identification of two differentially regulated isoforms of protochlorophyllide oxidoreductase (POR) from tobacco revealed a wide variety of light and development-dependent regulations of POR gene expression among angiosperms. Photosynth Res 74: 165–172

Masuda T, Fusada N, Oosawa N, Takamatsu K, Yamamoto YY, Ohto M, Nakamura K, Goto K, Shibata D, Shirano Y, Hayashi H, Kato T, Tabata S, Shimada H, Ohta H and Takamiya K (2003) Functional analysis of isoforms of NADPH: protochlorophyllide oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana*. Plant Cell Physiol 44: 963–974

Masuda S, Ikeda R, Masuda T, Hashimoto H, Tsuchiya T, Kojima H, Nomata J, Fujita Y, Mimuro M, Ohta H and Takamiya K (2009) Prolamellar bodies formed by cyanobacterial protochlorophyllide oxidoreductase in *Arabidopsis*. Plant J 58: 952–960

Matringe M, Camadro JM, Block MA, Joyard J, Scalla R, Labbe P and Douce R (1992a) Localisation within the chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether like herbicides. J Biol Chem 267: 4646–4651

Matringe M, Mornet R and Scalla R. (1992b) Characterization of [3 H] acifluorfen binding to purified pea etioplasts and evidence that protogen oxidase specifically binds acifluorfen. Eur J Biochem 209: 861–868

Matile P, Hortensteiner S, Thomas H and Krautler B (1996) Chlorophyll breakdown in senescent leaves. Plant Physiol 12: 1403–1409

Matile P, Hortensteiner S and Thomas H (1999) CHLOROPHYLL DEGRADATION. Annu Rev Plant Physiol Plant Mol Biol 50: 67–95

Matsumoto F, Obayashi T, Sasaki-Sekimoto Y, Ohta H, Takamiya K and Masuda T (2004) Gene expression profiling of the tetrapyrrole metabolic pathway in *Arabidopsis* with a mini-array system. Plant Physiol 135: 2379–2391

McCormac AC and Terry MJ (2002a) Light-signalling pathways leading to the coordinated expression of HEMA1 and Lhcb during chloroplast development in *Arabidopsis thaliana*. Plant J 32: 549–559

McCormac AC and Terry MJ (2002b) Loss of nuclear gene expression during the phytochrome A-mediated far-red block of greening response. Plant Physiol 130: 402–414

McCormac AC, Fischer A, Kumar AM, Soll D and Terry MJ (2001) Regulation of HEMA1 expression by phytochrome and a plastid signal during de-etiolation in *Arabidopsis thaliana*. Plant J 25: 549–561

Menon BR, Waltho JP, Scrutton NS and Heyes DJ (2009) Cryogenic and laser photoexcitation studies identify multiple roles for active site residues in the light-driven enzyme protochlorophyllide oxidoreductase. J Biol Chem 284: 18160–18166

Meskauskiene R and Apel K (2002) Interaction of FLU, a negative regulator of tetrapyrrole biosynthesis, with the glutamyl–tRNA reductase requires the tetratricopeptide repeat domain of FLU. FEBS Lett 532: 27–30

Meskauskiene R, Nater M, Goslings D, Kessler F, op den Camp R and Apel K (2001) FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 98: 12826–12831

Miyashita H, Ikemoto H, Kurano N, Adachi K, Chilara M and Miyachi S (1996) Chlorophyll *d* as a major pigment. Nature 383: 402

Mochizuki N, Brusslan JA, Larkin R, Nagatani A and Chory J (2001) *Arabidopsis* genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastidto- nucleus signal transduction. Proc Natl Acad Sci USA 98: 2053–2058

Mock HP and Grimm B (1997) Reduction of uroporphyrinogen decarboxylase by antisense RNA expression affects activities of other enzymes involved in tetrapyrrole biosynthesis and leads to light dependent necrosis. Plant Physiol 113: 1101–1112

Mock HP, Trainotti L, Kruse E and Grimm B (1995) Isolation, sequencing and expression of cDNA sequences encoding uroporphyrinogen decarboxylase from tobacco and barley. Plant Mol Biol 28: 245–256

Mock HP, Heller W, Molina A, Neubohn B, Sandermann H and Grimm B (1999) Expression of uroporphyrinogen decarboxylase or coproporphyrinogen oxidase antisense RNA in tobacco induces pathogen defence responses conferring increased resistance to tobacco mosaic virus. J Biol Chem 274: 4231–4238

Mohanty S, Grimm B and Tripathy BC (2006) Light and dark modulation of chlorophyll biosynthetic genes in response to temperature. Planta 224: 692–699

Mohapatra A and Tripathy BC (2002) Detection of protoporphyrin IX in envelope membranes of pea chloroplasts. Biochem Biophys Res Commun 299: 751–754

Mohapatra A and Tripathy BC (2003) Developmental changes in sub-plastidic distribution of chlorophyll biosynthetic intermediates in cucumber (*Cucumis sativus* L.). J Plant Physiol 160: 9–15

Mohapatra A and Tripathy BC (2007) Differential distribution of chlorophyll biosynthetic intermediates in stroma, envelope and thylakoid membranes in *Beta vulgaris*. Photosynth Res 94: 401–410

Mosinger E, Batschauer A, Schafer E and Apel K (1985) Phytochrome control of in vitro transcription of specific genes in isolated nuclei from barley (*Hordeum vulgare*). Eur J Biochem 147: 137–142

Nakagawara E, Sakuraba Y, Yamasato A, Tanaka R and Tanaka A (2007) Clp protease controls chlorophyll *b* synthesis by regulating the level of chlorophyllide *a* oxygenase. Plant J 49: 800–809

Nagata N, Satoh S, Tanaka R and Tanaka A (2004) Domain structures of chlorophyllide *a* oxygenase of green plants and *Prochlorothrix hollandica* in relation to catalytic functions. Planta 218: 1019–1025

Nagata N, Tanaka R, Satoh S and Tanaka A (2005) Identification of a vinyl reductase gene for chlorophyll synthesis in *Arabidopsis thaliana* and implications for the evolution of *Prochlorococcus* species. Plant Cell 17: 233–240

Nagata N, Tanaka R and Tanaka A (2007) The major route for chlorophyll synthesis includes [3,8-divinyl]-chlorophyllide a reduction in *Arabidopsis thaliana*. Plant Cell Physiol 48: 1803–1808

Nakanishi H, Nozue H, Suzuki K, Kaneko Y, Taguchi G and Hayashida N (2005) Characterization of the *Arabidopsis thaliana* mutant *pcb2* which accumulates divinyl chlorophylls. Plant Cell Physiol 46: 467–473

Nakayama M, Masuda T, Sato N, Yamagata H, Bowler C, Ohta H, Shioi Y and Takamiya K (1995) Cloning, subcellular localization and expression of CHLI, a subunit of magnesium- chelatase in soybean. Biochem Biophys Res Com 215: 422–428

Nakayama M, Masuda T, Bando T, Yamagata H, Ohta H and Takamiya K (1998) Cloning and expression of the soybean chlH gene encoding a subunit of Mg-chelatase and localization of the Mg^{++} concentration-dependent chlH protein within the chloroplast. Plant Cell Physiol 39: 275–284

Nandi DL and Waygood ER (1967) Biosynthesis of porphyrins in wheat leaves. II. 5-aminolevulinate hydrolyase. Canad J Biochem 45: 327–336

Narita S, Tanaka R, Ito T, Okada K, Taketani S and Inokuchi H (1996) Molecular cloning and characterisation of a cDNA that encodes protoporphyrinogen oxidase of *Arabidopsis thaliana*. Gene 182: 169–175

Oliver RP and Griffiths WT (1982) Pigment-protein complexes of illuminated etiolated leaves. Plant Physiol 70: 1019–1025

Oosawa N, Masuda T, Awai K, Fusada N Shimada H, Ohta H and Takamiya K (2000) Identification and light-induced expression of a novel gene of NADPH-potochlorophyllide oxidoreductase isoform in *Arabidopsis thaliana*. FEBS Lett 474: 133–136

Oster U, Tanaka R, Tanaka A and Rudiger W (2000) Cloning and functional expression of the gene encoding the key enzyme for chlorophyll *b* biosynthesis (CAO) from *Arabidopsis thaliana*. Plant J 21: 305–310

Papenbrock J, Gräfe S, Kruse E, Hanel F and Grimm B (1997) Mg-chelatase of tobacco: identification of a Chl D cDNA sequence encoding a third subunit, analysis of the interaction of the three subunits with the yeast two-hybrid system, and reconstitution of the enzyme activity by co-expression of recombinant CHL D, CHL H and CHL I. Plant J 12: 981–990

Papenbrock J, Mock HP, Kruse E and Grimm B (1999) Expression studies on tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. Planta 208: 264–273

Papenbrock J, Pfundel E, Mock HP and Grimm B (2000a) Decreased and increased expression of the subunit CHL I diminishes Mg chelatase activity and reduced chlorophyll synthesis in transgenic tobacco plants. Plant J 22: 155–164

Papenbrock J, Mock HP, Tanaka R, Kruse E and Grimm B (2000b) Role of magnesium chelatase activity in the early steps of the tetrapyrrole biosynthetic pathway. Plant Physiol 122: 1161–1169

Parham R and Rebeiz CA (1995) Chloroplast biogenesis 72: a [4-vinyl] chlorophyllide *a* reductase assay using divinyl chlorophyllide *a* as an exogenous substrate. Anal Biochem 231: 164–169

Pattanayak GK and Tripathy BC (2002) Catalytic function of a novel protein protochlorophyllide oxidoreductase C of *Arabidopsis thaliana*. Biochem Biophys Res Commun 291: 921–924

Pattanayak GK, Biswal AK, Reddy VS and Tripathy BC (2005) Light-dependent regulation of chlorophyll *b* biosynthesis in chlorophyllide *a* oxygenase overexpressing tobacco plants. Biochem Biophys Res Commun 326: 466–471

Peter E, Rothbart M, Oelze ML, Shalygo N, Dietz KJ and Grimm B (2010) Mg protoporphyrin monomethylester cyclase deficiency and effects on the tetrapyrrole metabolism in different light conditions. Plant Cell Physiol 2010 May 11. [Epub ahead of print]

Pineau B, Dubertret G, Joyard J and Douce R (1986) Fluorescence properties of the envelope membranes from spinach chloroplasts. Detection of protochlorophyllide. J Biol Chem 261: 9210–9215

Polking GF, Hannapel DJ and Gladon RJ (1995) A cDNA clone for 5-aminolevulinic acid dehydratase from tomato (*Lycopersicon esculentum* Mill.). Plant Physiol 107: 1033–1034

Plöscher M, Granvogl B, Reisinger V and Eichacker LA (2009) Identification of the N-termini of NADPH: protochlorophyllide oxidoreductase A and B from barley etioplasts (*Hordeum vulgare* L.) FEBS J 276: 1074–1081

Pontier D, Albrieux C, Joyard J, Lagrange T and Block MA (2007) Knock-out of the magnesium protoporphyrin IX methyltransferase gene in *Arabidopsis*. Effects on chloroplast development and on chloroplast-to-nucleus signaling. J Biol Chem 282: 2297–2304

Pontoppidan B and Kannangara CG (1994) Purification and partial characterization of barley glutamyl-tRNAglu reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. Eur J Biochem 225: 529–537

Popperl G, Oster U, Blos I and Rudiger W (1997) Magnesium chelatase of *Hordeum vulgare* L. is not activated by light but inhibited by pheorphorbide. Z Naturforsch 52c: 144–152

Porra RJW, Schäfer E, Cmiel IK and Scheer H (1993) Derivation of the formyl group oxygen of chlorophyll *b* from molecular oxygen in greening leaves of a higher plant (*Zea mays*). FEBS Lett 371: 21–24

Poulson R and Polglasse WJ (1974) Aerobic and anaerobic coproporphyrinogen oxidase activities in extract from *Saccharomyces cerevisiae*. J Biol Chem 249: 6367–6371

Ratinaud MH, Thomes JC and Julien R (1983) Glutamyl-tRNA synthetases from wheat. Isolation and characterization of three dimeric enzymes. Eur J Biochem 135: 471–477

Rebeiz CA, Benning C, Bohnert HJ, Danielle H, Hoober JK, Lichtenthaler HK, Portis AR and Tripathy BC (eds) (2010) The Chloroplast: Basics and Applications, Advances in Photosynthesis and Respiration, Vol 31. Springer, Dordrecht

Reinbothe C, Apel K and Reinbothe S (1995) A light-induced protease from barley plastids degrades NADPH:protochlorophyllide oxidoreductase complexed with chlorophyllide. Mol Cell Biol 15: 6206–6212

Reinbothe C, Lebedev N and Reinbothe S (1999) A protochlorophyllide light-harvesting complex involved in de-etiolation of higher plants. Nature 397: 80–84

Reinbothe C, Buhr F, Bartsch S, Desvignes C, Quigley F, Pesey H and Reinbothe S (2006) In vitro-mutagenesis of NADPH:protochlorophyllide oxidoreductase B: two distinctive protochlorophyllide binding sites participate in enzyme catalysis and assembly. Mol Genet Genomics 275: 540–552

Richter A, Peter E, Pörs Y, Lorenzen S, Grimm B and Czarnecki O (2010) Rapid dark repression of 5-aminolevulinic acid synthesis in green barley leaves. Plant Cell Physiol 51: 670–681

Rissler HM, Collakova E, DellaPenna D, Whelan J and Pogson BJ (2002) Chlorophyll biosynthesis. Expression of a second chl I gene of magnesium chelatase in *Arabidopsis* supports only limited chlorophyll synthesis. Plant Physiol 128: 770–779

Rudiger W, Benz J and Guthoff C (1980) Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur J Biochem 109: 193–200

Runge S, Sperling U, Frick G, Apel K and Armstrong GA (1996) Distinct roles for light-dependent NADPH:protochlorophyllide oxidoreductases (POR) A and B during greening in higher plants. Plant J 9: 513–523

Rzeznicka K, Walker CJ, Westergren T, Kannangara CG, von Wettstein D, Merchant S, Gough SP and Hansson M (2005) Xantha-l encodes a membrane subunit of the aerobic Mg-protoporphyrin IX monomethyl ester cyclase involved in chlorophyll biosynthesis. Proc Natl Acad Sci USA 102: 5886–5891

Sakuraba Y, Yamasato A, Tanaka R and Tanaka A (2007) Functional analysis of N-terminal domains of Arabidopsis chlorophyllide *a* oxygenase. Plant Physiol Biochem 45: 740–749

Sakuraba Y, Yokono M, Akimoto S, Tanaka R, Tanaka A. (2010) Deregulated chlorophyll *b* synthesis reduces the energy transfer rate between photosynthetic pigments and induces photodamage in *Arabidopsis thaliana*. Plant Cell Physiol 51: 1055–1065

Sangwan I and O'Brian MR (1993) Expression of the soybean (*Glycine max*) glutamate 1-semialdehyde aminotransferase gene in symbiotic root nodules. Plant Physiol 102: 829–834

Santel HJ and Apel K (1981) The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). The effect of light on the NADPH: protochlorophyllide oxidoreductase. Eur J Biochem 120: 95–103

Sato Y, Morita R, Katsuma S, Nishimura M, Tanaka A and Kusaba M (2009) Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll b and light-harvesting complex II degradation during senescence in rice. Plant J 57: 120–131

Satoh S, Ikeuchi M, Mimuro M and Tanaka A (2001) Chlorophyll *b* expressed in cyanobacteria functions as a light-harvesting antenna in photosystem I through flexibility of the proteins. J Biol Chem 276: 4293–4297

Sawers RJ, Farmer PR, Moffett P and Brutnell TP (2006) In planta transient expression as a system for genetic and biochemical analyses of chlorophyll biosynthesis. Plant Methods 2:15

Schaumburg A, Schneider-Poetsch HA and Eckerskorn C (1992) characterization of plastid 5-aminolevulinate dehydratase (ALAD; EC 4.2.1.24) from spinach (*Spinacia oleracea* L.) by sequencing and comparison with non-plant ALAD enzymes. Z Naturforsch C 47: 77–84

Scheumann V, Ito H, Tanaka A, Schoch S and Rüdiger W (1996) Substrate specificity of chlorophyll(ide) *b* reductase in etioplasts of barley (*Hordeum vulgare* L.). Eur J Biochem 242: *163–170*

Scheumann V, Klement H, Helfrich M, Oster U, Schoch S and Rüdiger W (1999) Protochlorophyllide *b* does not occur in barley etioplasts. FEBS Lett 445: 445–448

Schmid HC, Oster U, Kögel J, Lenz S and Rüdiger W (2001) Cloning and characterisation of chlorophyll synthase from *Avena sativa*. J Biol Chem 382: 903–911

Schmid HC, Rassadina V, Oster U, Schoch S and Rudiger W (2002) Pre-loading of chlorophyll synthase with tetraprenyl diphosphate is an obligatory step in chlorophyll biosynthesis. J Biol Chem 383: 1769–1778

Schoefs B and Franck F (1993) Photoreduction of protochlorophyllide to chlorophyllide in 2-d-old dark-grown bean (*Phaseolus vulgaris* cv. Commodore) leaves. Comparison with 10-d-old dark-grown (etiolated) leaves. J Expt Bot 44: 1053–1057

Schoefs B and Bertrand M (2000) The formation of chlorophyll from chlorophyllide in leaves containing proplastids is a four-step process. FEBS lett 486: 243–246

Schoefs B and Franck F (2003) Protochlorophyllide reduction: mechanism and evolution. Photochem Photobiol 78: 543–557

Schoefs B, Garnir HP and Bertrand M (1994) Comparison of the photoreduction protochlorophyllide to chlorophyllide in leaves and cotyledons from dark-grown beans as a function of age. Photosynth Res 41: 405–417

Schoefs B, Bertrand M and Franck F (2000a) Spectroscopic properties of protochlorophyllide analyzed in situ in the course of etiolation and in illuminated leaves. Photochem Photobiol 72: 85–93

Schoefs B, Bertrand M and Franck F (2000b) Photoactive protochlorophyllide regeneration in cotyledons and leaves from higher plants. Photochem Photobiol 72: 660–668

Schon A, Krupp G, Gough S, Berry-Lowe S, Kannangara CG and Soll D (1986) The RNA required in the first step of chlorophyll biosynthesis is a chloroplast glutamate tRNA. Nature 322: 281–284

Schon A, Kannangara CG, Gough S and Soll D (1988) Protein biosynthesis in organelles requires misaminoacylation of tRNA. Nature 331: 187–190

Schulz R, Steinmuller K, Klaas M, Forreiter C, Rasmussen S, Hiller C and Apel K (1989) Nucleotide sequence of a cDNA coding for the NADPH–protochlorophyllide oxidoreductase (PCR) of barley (*Hordeum vulgare* L.) and its expression in *Escherichia coli*. Mol Gen Genet 217: 355–361

Schunmann PH and Ougham HJ (1996) Identification of three cDNA clones expressed in the leaf extension zone and with altered patterns of expression in the slender mutant of barley: a tonoplast intrinsic protein, a putative structural protein and protochlorophyllide oxidoreductase. Plant Mol Biol 31: 529–537

Scolnik P and Bartley GE (1996) A table of some cloned plant genes involved in isoprenoid biosynthesis. Plant Mol Biol Rep 14: 305–319

Seyedi M, Selstam E, Timko MP and Sundqvist C (2001a) The cytokinin 2-isopentenyladenine causes partial reversion to skotomorphogenesis and induces formation of prolamellar bodies and protochlorophyllide657 in the *lip1* mutant of pea. Physiol Plan. 112: 261–272

Seyedi M, Timko MP and Sundqvist C (2001b) The distribution of protochlorophyllide and chlorophyll within seedlings of the *lip1* mutant of pea. Plant Cell Physiol 42: 931–941

Shalygo NV, Mock HP, Averina NG and Grimm B (1998) Photodynamic action of uroporphyrin and protochlorophyllide in greening barley leaves treated with cesium chloride. J Photochem Photobiol B 42: 151–158

Shalygo N, Czarnecki O, Peter E, Grimm B (2009) Expression of chlorophyll synthase is also involved in feedback-control of chlorophyll biosynthesis. Plant Mol Biol 71: 425–436

Shashidhara LS and Smith AG (1991) Expression and subcellular location of the tetrapyrrole synthesis enzyme porphobilinogen deaminase in light-grown *Euglena gracilis* and three nonchlorophyllous cell lines. Proc Natl Acad Sci USA 88: 63–67

Shemin D (1976) 5-Aminolevulinic acid dehydratase: Structure, function and mechanism. Philos Trans R Soc London 273: 109–115

Shetty AS and Miller GW (1969) Purification and general properties of 5-aminolevulinate dehydratase from *Nicotiana tabaccum* L. Biochem J 114: 331–337

Shibata K (1957) Spectroscopic studies of chlorophyll formation in intact leaves. J Biochem Tokyo, Japan 44: 147–173

Shibata H and Ochiai H (1977) Purification and properties of δ-aminolevulinic acid dehydratase from radish cotyledons. Plant Cell Physiol 18: 420–429

Smith AG (1988) Subcellular localization of two porphyrin-synthesis enzymes in *Pisum sativum* (pea) and Arum (cuckoo-pint) species. Biochem J 249: 423–428

Smith AG and Francis JE (1981) Investigations of rat liver uroporphyrinogen decarboxylase. comparisons of porphyrinogens I and III as substrate nad inhibition by porphyrins. Biochem J 195: 241–250

Soll J, Schultz G, Rüdiger W and Benz J (1983) Hydrogenation of geranylgeraniol. Two pathways exist in spinach chloroplasts. Plant Physiol 71: 849–854

Spano AJ and Timko MP (1991) Isolation, characterization and partial amino acid sequence of a chloroplast-localized porphobilinogen deaminase from pea (*Pisum sativum* L.). Biochim Biophys Acta 1076: 29–36

Spano AJ, He Z, Michel H, Hunt DF and Timko MP (1992) Molecular cloning, nuclear gene structure, and developmental expression of NADPH:protochlorophyllide oxidoreductase in pea (*Pisum sativum* L.). Plant Mol Biol 18: 967–972

Spencer P and Jordan PM (1994) 5-Aminolevulinic acid dehydratase: Characterization of the α and ß metal-binding sites of the *Escherichia coli* enzyme. In: Chadwick DJ and Ackrill K (eds) The Biosynthesis of Tetrapyrrole Pigments, Ciba Foundation Symposium 180, pp 50–64. John Weily and Sons, Chichester

Spencer P and Jordan PM (1995) Characterization of the two 5-aminolevulinic acd binding sites of 5-aminolevulinic acid dehydratase from *Escheichia coli*. Biochem J 305: 151–158

Sperling U, Van Cleve B, Frick G, Apel K and Armstrong GA (1997) Overexpression of light-dependent PORA or PORB in plants depleted of endogenous POR by far-red light enhances seedling survival in white light and protects against photooxidative damage. Plant J 12: 649–658

Sperling U, Franck F, Cleve BV, Frick G, Apel K and Armstrong GA (1998) Etioplast differentiation in *Arabidopsis*: Both PORA and PORB restore the prolamellar body and photoactive protochlorophyllide-F655 to the cop1 photomorphogenic mutant. Plant Cell 10: 283–296

Stenbaek A and Jensen PE. (2010) Redox regulation of chlorophyll biosynthesis. Phytochem 71: 853–859

Stobart AK and Ameen-Bukhari I (1984) Regulation of δ-aminolaevulinic acid synthesis and protochlorophyllide regeneration in the leaves of dark-grown barley (*Hordeum vulgare*) seedlings. Biochem J 222: 419-426

Stobart AK and Ameen-Bukhari I (1986) Photoreduction of protochlorophyllide and its relationship to δ-aminolaevulinic acid synthesis in the leaves of dark-grown barley (*Hordeum vulgare*) seedlings. Biochem J 236: 741-748

Su Q, Frick G, Armstrong G and Apel K (2001) POR C of *Arabidopsis thaliana*: a third light- and NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by light. Plant Mol Biol 47: 805–813

Sytina OA, Heyes DJ, Hunter CN, Alexandre MT, Van Stokkum IH, Van Grondelle R and Groot ML (2008) Conformational changes in an ultrafast light-driven enzyme determine catalytic activity. Nature 456: 1001–1004

Tan FC, Cheng Q, Saha K, Heinemann IU, Jahn M, Jahn D and Smith AG (2008) Identification and characterization of the *Arabidopsis* gene encoding the tetrapyrrole biosynthesis enzyme uroporphyrinogen III synthase. Biochem J 410: 291–299

Tanaka R and Tanaka A (2005) Effects of chlorophyllide *a* oxygenase overexpression on light acclimation in *Arabidopsis thaliana*. Photosynth Res 85: 327–340

Tanaka R and Tanaka A (2007) Tetrapyrrole biosynthesis in higher plants. Annu Rev Plant Biol 58: 321–46

Tanaka R, Yoshida K, Nakayashiki T, Masuda T, Tsuji H, Inokuchi H and Tanaka A (1996) Differential expression of two *hemA* mRNAs encoding glutamyl-tRNA reductase proteins in greening cucumber seedlings. Plant Physiol 110: 1223–1230

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K and Okada K (1998) Chlorophyll *a* oxygenase (CAO) is involved in chlorophyll *b* formation from chlorophyll *a*. Proc Natl Acad Sci USA 95: 12719–12723

Tanaka R, Oster U, Kruse E, Rudiger W and Grimm B (1999) Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. Plant Physiol 120: 695–704

Tanaka R, Koshino Y, Sawa S, Ishiguro S, Okada K and Tanaka A (2001) Overexpression of chlorophyllide *a* oxygenase (CAO) enlarges the antenna size of photosystem II in *Arabidopsis thaliana*. Plant J 26: 365–373

Tchuinmogue SJ, Bruyant P and Balange AP (1992) Immunological characterization of two 5-aminolevulinate dehydratase in radish leaves. Plant Physiol Biochem 30: 255–261

Teakle GR and Griffiths WT (1993) Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*). Biochem J 296: 225–230

Tewari AK and Tripathy BC (1998) Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat. Plant Physiol 117: 851–858

Tewari AK and Tripathy BC (1999) Acclimation of chlorophyll biosynthetic reactions to temperature stress in cucumber (*Cucumis sativus* L.). Planta 208: 431–437

Tottey S, Block MA, Allen M, Westergren T, Albrieux C, Scheller HV, Merchant S and Jensen PE (2003) *Arabidopsis* CHL27, located in both envelope and thylakoid membranes, is required for the synthesis of protochlorophyllide. Proc Natl Acad Sci USA 100: 16119–16124

Townley HE, Sessions RB, Clarke AR, Dafforn TR and Griffiths WT (2001) Protochlorophyllide oxidoreductase: a homology model examined by site-directed mutagenesis. Proteins 44: 329–335

Tripathy BC and Rebeiz CA (1985) Chloroplast biogenesis: quantitative determination of monovinyl and divinyl Mg-protoporphyrins and protochlorophyll(ides) by spectrofluorometry. Anal Biochem 149: 43–61

Tripathy BC and Rebeiz CA (1986) Chloroplast biogenesis. Demonstration of the monovinyl and divinyl monocarboxylic routes of chlorophyll biosynthesis in higher plants. J Biol Chem 261: 13556–13564

Tripathy BC and Rebeiz CA (1988) Chloroplast biogenesis 60: Conversion of divinyl protochlorophyllide to monovinyl protochlorophyllide in green(ing) barley, a dark monovinyl/light divinyl plant species. Plant Physiol 87: 89–94

Tripathy BC, Mohapatra A and Gupta I (2007) Impairment of the photosynthetic apparatus by oxidative stress induced by photosensitization reaction of protoporphyrin IX. Biochim Biophys Acta 1767: 860–868

Tsai S, Bishop DF and Desnick RJ (1987) Purification and properties of uroporphyrinogen III synthase from human erythrocytes. J Biol Chem 262: 1268–1273

Tsang EW, Yang J, Chang Q, Nowak G, Kolenovsky A, McGregor DI and Keller WA (2003) Chlorophyll reduction in the seed of *Brassica napus* with a glutamate 1-semialdehyde aminotransferase antisense gene. Plant Mol Biol 51: 191–201

von Wettstein D, Gough S and Kannangara CG (1995) Chlorophyll biosynthesis. Plant Cell 7: 1039–1057

Walker CJ and Weinstein JD (1994) The magnesium-insertion step of chlorophyll biosynthesis is a two-stage reaction. Biochem J 299: 277–284

Walker CJ and Willows RD (1997) Mechanism and Regulation of Mg-chelatase. Biochem J 327: 321–333

Walker CJ, Mansfield KE, Smith KM and Castelfranco PA (1989) Incorporation of atmospheric oxygen into the carbonyl functionality of the protochlorophyllide isocyclic ring. Biochem J 257: 599–602

Walker CJ, Castelfranco PA and Whyte BJ (1991) Synthesis of divinyl protochlorophyllide. Enzymological properties of the Mg-protoporphyrin IX monomethyl ester oxidative cyclase system. Biochem J 276: 691–697

Warabi E, Usui K, Tanaka Y and Matsumoto H (2001) Resistance of a soybean cell line to oxifluorfen by overproduction of mitochondrial protoporphyrinogen oxidase. Pest Manag Sci 57: 743–748

Watanabe N, Che F-S, Iwano M, Takayama S, Yoshida S and Isogai A (2001) Dual targeting of spinach protoporphyrinogen oxidase II to mitochondria and chloroplast by alternative use in-frame initiation codons. J Biol Chem 276: 20474–20481

Whyte BJ and Castelfranco PA (1993) Further observations on the Mg-protoporphryin IX monomethyl ester (oxidative) cyclase system. Biochem J 290: 355–359

Wiktorsson B, Engdahl S, Zhong LB, Böddi B, Ryberg M and Sundqvist C (1993) The effect of cross-liking of the subunits of NADPH-protochlorophyllide oxidoreductase of the aggregational state of protochlorophyllide. Photosynthtica 29: 205–218

Wilks HM and Timko MP (1995) A light-dependent complementation system for analysis of NADPH:protochlorophyllide oxidoreductase: Identification and mutagenesis of two conserved residues that are essential for enzyme activity. Proc Natl Acad Sci USA 92: 724–728

Williams P, Hardeman K, Fowler J and Rivin C (2006) Divergence of duplicated genes in maize: evolution of contrasting targeting information for enzymes in the porphyrin pathway. Plant J 45: 727–739

Wise RR and Hoober JK (eds) (2006) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23. Springer, Dordrecht

Witty M, Wallace-Cook AD, Albrecht H, Spano AJ, Michel H, Shabanowitz J, Hunt DF, Timko MP and Smith AG (1993) Structure and expression of chloroplast-localized porphobilinogen deaminase from pea (*Pisum sativum* L.) isolated by redundant polymerase chain reaction. Plant Physiol 103: 139–147

Wu Z, Zhang X, He B, Diao L, Sheng S, Wang J, Guo X, Su N, Wang L, Jiang L, Wang C, Zhai H and Wan J (2007) A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. Plant Physiol 145: 29–40

Xu H, Vavilin D and Vermaas W (2001) Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria. Proc Natl Acad Sci USA 98: 14168–14173

Yamasato A, Nagata N, Tanaka R and Tanaka A (2005) The N-terminal domain of chlorophyllide *a* oxygenase confers protein instability in response to chlorophyll *b* accumulation in *Arabidopsis*. Plant Cell 17: 1585–1597

Yamasato A, Tanaka R and Tanaka A (2008) Loss of the N-terminal domain of chlorophyllide *a* oxygenase induces photodamage during greening of *Arabidopsis* seedlings. BMC Plant Biol 8: 64

Yaronskaya E, Vershilovskaya I, Poers Y, Alawady AE, Averina N and Grimm B (2006) Cytokinin effects on tetrapyrrole biosynthesis and photosynthetic activity in barley seedlings. Planta 224: 700–709

Zhang H, Li J, Yoo JH, Yoo SC, Cho SH, Koh HJ, Seo HS and Paek NC (2006) Rice *Chlorina-1* and *Chlorina-9* encode ChlD and ChlI subunits of Mg-chelatase, a key enzyme for chlorophyll synthesis and chloroplast development. Plant Mol Biol 62: 325–337

Chapter 4

Biosynthesis, Localization and Concentration of Carotenoids in Plants and Algae

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Summary

The biosynthesis of the tetraterpenoid carotenoids (carotenes and xanthophylls) in plants via the plastidic 1-deoxy-D-xylulose-4-phosphate/2-C-methylerythritol 5-phosphate pathway of isopentenyl and isoprenoid formation is described together with the different enzymatic steps (and genes) that lead from the active isoprenoid C_5 diphosphates dimethylallyl diphosphate and isopentenyl diphosphate via phytofluene dehydrogenation and introduction of hydroxy and epoxy groups to the final leaf and chloroplast carotenoids. Molecular cloning of genes has given detailed information on the biosynthetic pathways. The localization and concentration of carotenoids in the chloroplasts (thylakoids, envelope, but only traces in plastoglobuli) and their possible function is reviewed in this chapter. In addition, the differential carotenoid composition of sun and high-light leaves as compared to shade and low-light leaves is presented

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underlining the high adaptation capacity of the photosynthetic apparatus to the incident light. The de novo accumulation of β-carotene and zeaxanthin at high photon flux densities as well as evolutionary aspects of carotenoid biosynthesis are also, but briefly, discussed.

I. Introduction to Carotenoids

Carotenoids represent a class of natural fat-soluble, yellow, orange or sometimes red, isoprenoid pigments found principally in plants, algae, and photosynthetic bacteria (e.g., Frank et al., 1999). In the photosynthetic process they play an essential function in light absorption (accessory pigments) or in the protection of the chlorophyll *a* molecules in the photosynthetic reaction centers against photo-oxidation. Carotenoids are also formed in several non-green and non-photosynthetic organisms, such as yeasts, bacteria and molds, where they seem to possess a protective function against damage by light and oxygen. For an overview see Britton (1995), Britton et al. (1995), and Goodwin (1980). In photosynthetic organisms the different carotenoids are bound in particular ways to several chlorophyll-carotenoid-protein complexes of the photochemically active thylakoids (Lichtenthaler et al., 1982a, b; Lichtenthaler and Babani, 2004). Thylakoids are known to perform the two photosynthetic light reactions and associated electron transport reactions that lead to the photolysis of water and to oxygen evolution and the formation of ATP and NADPH. These functional carotenoids of the photosynthetic apparatus have been termed *primary carotenoids* (Lichtenthaler, 1969a, b; Lichtenthaler and Pfister, 1977).

In contrast to plants, photosynthetic bacteria, certain fungi and animals do not have the capacity of synthesizing carotenoids. However, many fishes, insects, birds and crustaceans take up carotenoids from their plant or algae diet providing them with a bright coloration as seen in the feathers of flamingos or in the pink color of salmon (Pfander, 1992). Such *secondary carotenoids* are mostly oxidized carotenoid forms with hydroxy and epoxy groups, and often represent keto-carotenoids (Fig. 4.1), e.g., canthaxanthin (a 4,4′-diketo β-carotene) found in shrimps, krills, flamingo feathers and in chanterelle mushrooms, as well as astaxanthin (a 3,3′-dihydroxy, 4,4′-diketo-β-carotene) formed in the microalga *Haematococcus pluvialis* and applied for coloring of salmon trouts. Secondary carotenoids also occur in non-green plant tissues of fruits and flowers (e.g., see Lichtenthaler, 1969b, 1970), such as in fruit scales of citrus fruits (in oranges, it is β-citraurin) or in red paprika/bell pepper fruits (*Capsicum annuum*) (capsanthin and capsorubin). These secondary carotenoids are often present as fatty acid esters. The orange carrot roots are colored with pure β-carotene (provitamin A), which, in green leaf tissue, is considered a primary carotenoid. The red tomato fruit is exceptionally colored by lycopene, a biosynthetic intermediate in carotenoid formation, which possesses an acyclic C_{40} hydrocarbon polyene chain with 13 double bonds (Fig. 4.1). Yellow daffodils (*Narcissus*), in turn, possess the two xanthophylls lutein and violaxanthin and some minor amounts of β-carotene and neoxanthin (Lichtenthaler and Pfister, 1977), all of which would represent primary carotenoids in green leaves.

In green plants and algae there occur simultaneously several carotenoids as genuine constituents of the photosynthetic apparatus that differ in their chemical structure. These carotenoids represent isoprenoid C_{40}-carbon compounds; they are tetra-terpenoids that are synthesized from 8 isoprene units (Fig. 4.1). Their isoprenoid carbon chain is terminated at both their ends by cyclic ring structures, the ionone rings, which contain a double bond and in addition oxygen-containing functional groups (e.g., hydroxy groups).

Abbreviations: $(a+b)/(x+c)$ – Weight ratio of chlorophylls to carotenoids; $a+b$ – Total chlorophylls; a/b – Ratio of chlorophyll *a* to *b*; c – Carotenes; DMAPP – Dimethylallyl diphosphate; DOXP – 1-deoxy-D-xylulose 5-phosphate; DOXP/MEP pathway – Plastidic 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate pathway; DXR – DOXP-reductoisomerase; GGPP – Geranylgeranyl diphosphate; GPP – Geranyl diphosphate; HMBPP – 1-hydroxy-2-methyl-2-(E)-butenyl diphosphate; HMGR – 3-hydroxy-3-methylglutaryl coenzyme A reductase; IPP – isopentenyl diphosphate; MEP – 2-C-methyl-D-erythritol 4-phosphate; PS I – Photosystem I; PS II – Photosystem II; x – Xanthophyll; x+c – Total carotenoids

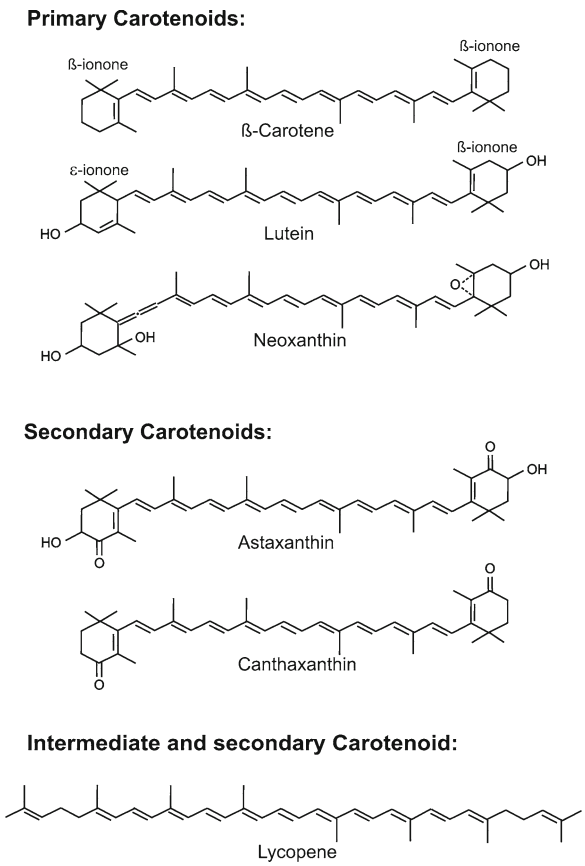

Fig. 4.1. Chemical structure of three carotenoids in chloroplasts of green leaves (primary carotenoids), of two ketocarotenoids (secondary carotenoids) found in chlorophyll-free plant tissues and of the acyclic carotenoid lycopene present in ripe red tomato fruits. β-carotene possesses two β-ionone rings one at each end, and α-carotene as well as its derivative lutein exhibit one ε-ionone ring and one β-ionone ring as indicated. Xanthophylls, such as lutein (3,3′-dihydroxy-α-carotene) and neoxanthin (a 3,3′5-trihydroxy-5′6′-epoxy-β-carotene derivative with an allene double bond structure) contain hydroxy and/or epoxy groups in their ionone rings. Astaxanthin is a 3,3′-dihydroxy-4,4′-diketo-β-carotene, and canthaxanthin a 4,4′keto-β-carotene.

The pure hydrocarbon carotenoids are known as carotenes, with β-carotene as the main compound that makes up about 25–33% of the total leaf carotenoids (Lichtenthaler and Calvin, 1964; Lichtenthaler, 2007). The oxygen containing carotenoids have been termed xanthophylls (which means ‘leaf yellow’) with lutein as their major compound, characterized by a hydroxy group in each ionone ring (Fig. 4.1). Lutein is bound to the light-harvesting chlorophyll *a*/*b* xanthophyll protein complexes (LHCPs), also termed LHCII, of photosystem II (PS II) of thylakoids (Lichtenthaler et al., 1982a, b; Lichtenthaler and Babani, 2004), and it is the most abundant carotenoid in green plant leaves and amounts to 40–55% of the total leaf carotenoids in all higher plants, whereby the percentage inversely depends on the degree of light exposure of leaves (Lichtenthaler and Park, 1963; Schindler et al., 1994; Lichtenthaler, 2007; Sarijeva et al., 2007). Within thylakoids, carotenoids and chlorophylls are bound in differential ways to specific chlorophyll-carotenoid-protein complexes which can be separated by gel electrophoresis from digitonin-digested chloroplast preparations. These are: (1) the two β-carotene containing Photosystem I (PS I) pigment-protein complexes (CPI and CPIa); (2) the light-harvesting chlorophyll *a*/*b* complexes, LHCPs (or LHCII) with lutein and neoxanthin, and (3) the chlorophyll-binding protein complex CPa of PS II that primarily contains chlorophyll *a* and β-carotene (Thornber, 1975; Lichtenthaler et al., 1982a; Bennett, 1983) and includes the PS II reaction center and the two later detected chlorophyll-binding proteins CP47 and CP43 known to contain primarily chlorophyll *a* and β-carotene as pigments (e.g., Bricker, 1990).

The different carotenoids of green leaves are derived either from β-*carotene* (the xanthophylls zeaxanthin, antheraxanthin, violaxanthin and neoxanthin) or from *α-carotene* (lutein). The two carotenes differ in the structure of their ionone rings. β-Carotene possesses two β-ionone rings where the double bond in each of the two ionone rings is conjugated to the 9 double bonds of the linear isoprenoid chain (Fig. 4.1). In contrast, α-carotene exhibits only one β-ionone ring, whereas the second is an ε-ionone ring with the double bond being allylic to the conjugated double bonds of the linear isoprenoid backbone chain (see lutein an α-carotene derivative in Fig. 4.1). Hence, with respect to their ionone ring the carotenoids of green leaf tissues represent either α-carotene or β-carotene derivatives.

II. The Compartmentation of Plant Isoprenoid Formation

Plant cells contain various isoprenoid compounds as regular constituents that are localized in different compartments. Chloroplasts contain the

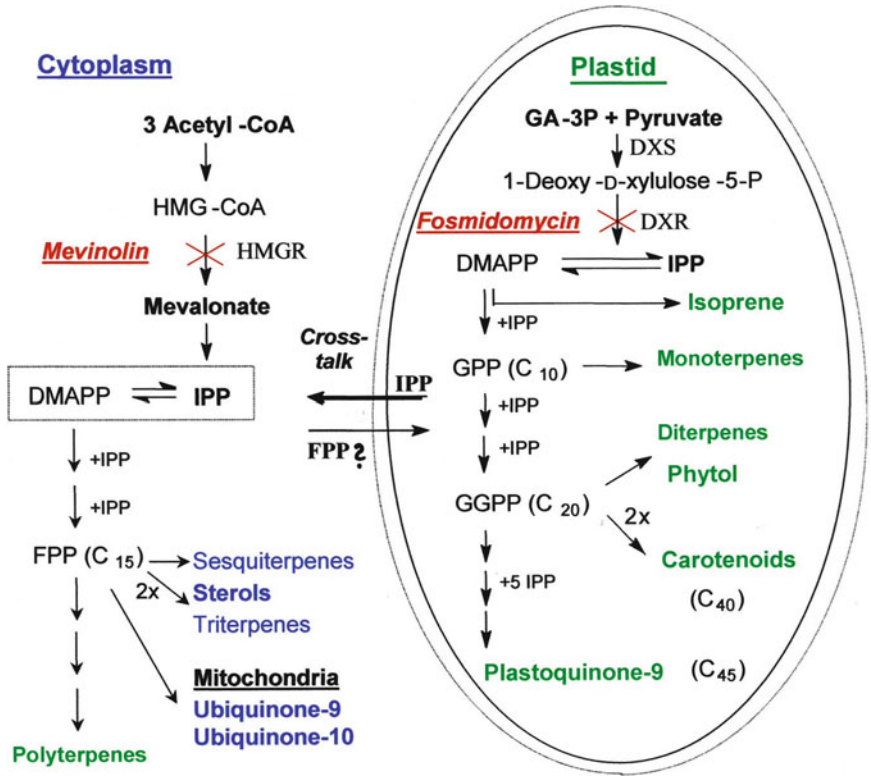

Fig. 4.2. The two cellular compartments of plant isoprenoid biosynthesis with two separate biosynthetic pathways: (1) the plastidic DOXP/MEP pathway (also termed MEP pathway) for the biosynthesis of the active C_5 unit (IPP) for chlorophylls (phytyl side-chain), carotenoids and prenylquinones (isoprenoid side-chains), and monoterpenes, and (2) the cytosolic acetate/mevalonate pathway of IPP biosynthesis for the formation of sterols, triterpenoids, the prenyl side-chain of the mitochondrial ubiquinones (Q-9, Q-10) and polyterpenes. The specific inhibition of the DOXP/MEP pathway by *fosmidomycin* (target: DOXP-reductoisomerase, *DXR*) and the cytosolic acetate/mevalonate pathway by *mevinolin* and other statins (target: HMG-CoA reductase) is indicated. FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; HMGR, hydroxymethylglutaryl-CoA reductase (Based on Lichtenthaler et al., 1997a, b; Zeidler et al., 1998; Lichtenthaler, 1999, 2000).

tetraterpenoid carotenoids composed of 8 isoprenic C_5 units, the chlorophylls with a diterpenic phytyl side chain and plastoquinone-9, with a nonaprenyl side chain and phytyl chains in phylloquinone K1 and α-tocopherol. Plant mitochondria contain the ubiquinones Q-9 and Q-10 with isoprenoid chains composed of either 9 or 10 isoprene C_5 units, respectively. The cytosol, in turn, contains the sterols representing triterpenoid derivates that are bound to several cytosolic biomembranes. These regular functional isoprenoids or terpenoids of the plant cell have been termed *prenyl lipids* (Goodwin, 1977; Lichtenthaler, 1977). In addition to these, several other isoprenoids e.g., monoterpenes, sesquiterpenes and polyterpenes, many of which are considered secondary plant products, are present in certain plant species.

All isoprenoids are synthesized from the active C_5 units isopentenyl diphoshate (IPP) and dimethylallyl diphosphate (DMAPP). Until the mid 1990s it was generally accepted that all plant isoprenoid chains including carotenoids would be synthesized from the isoprenoid C_5 units IPP and DMAPP, being formed by the cytosolic acetate/mevalonate pathway. Then a novel, non-mevalonate pathway for plant isoprenoid biosynthesis was detected in chloroplasts by Hartmut Lichtenthaler and Michel Rohmer, and their coworkers, in a joint scientific effort (Lichtenthaler et al., 1995, 1997a, b; Schwender et al., 1995, 1996, 1997; Zeidler et al., 1997, 1998) as reviewed in detail by Lichtenthaler (1998, 1999).

In plants, there exist two cellular compartments for isoprenoid biosynthesis: (1) the plastidic

1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway (also simply the MEP pathway) for carotenoid and prenyl side chain biosynthesis, and (2) the classical acetate/mevalonate pathway for sterol biosynthesis that also delivers the prenyl side chains of the mitochondrial ubiquinones. This compartmentation of isoprenoid biosynthesis in the plant cell is shown in Fig. 4.2. The plastidic DOXP/MEP pathway starts from glyceraldehyde-3-phosphate (GA-3-P) and pyruvate with 1-deoxy-D-xylulose 5-phosphate (DOXP) as the first product, which is transferred in 6 further enzymatic steps to DMAPP and IPP. By head-to-tail condensation, the latter form geranyl diphosphate (GPP) to which more C_5 carbon skeletons of IPP are condensed to eventually form the isoprenoid chains of the different carotenoids and plastidic prenyl lipids, mono- and diterpenes, etc. In contrast, the cytosolic acetate/mevalonate pathway starts from 3 acetyl CoA and provides IPP and DMAPP for cytosolic sterol biosynthesis, sesquiterpenes and polyterpernes and the side chains of ubiquinones Q-9 and Q-10. Both cellular isoprenoid pathways can specifically be inhibited: the acetate/mevalonate pathway by *mevinolin* and other statins (target: hydroxymethylglutaryl-CoA reductase) (Bach and Lichtenthaler, 1982, 1983a, b; Schindler et al., 1985), and the DOXP/MEP pathway by *fosmidomycin* (target: DOXP-reductoisomerase, DXR) as shown in Fig. 4.2 (Lichtenthaler, 2000a, 2004; Müller et al., 2000; Zeidler et al., 1998, 2000).

There exists some cross-talk between the two cellular isoprenoid forming pathways that can be demonstrated using e.g., the two inhibitors mentioned above. However, an adequate compensation for the missing activity of one cellular isoprenoid pathway by the second isoprenoid pathway of the other cell compartment apparently does not occur under standard physiological conditions of growth. In contrast, from the currently available data (see a review by Lichtenthaler, 2007), it is evident that under photosynthetic conditions, there occurs a unidirectional, relatively high flow of plastidic C_5 isoprenoid units into cytosolic sterol and sesquiterpene or polyterpene biosynthesis. This export and flow of isoprenoid C_5 units into cytosolic isoprenoid biosynthesis is presumably mediated by the plastidial unidirectional proton symport system (Bick and Lange, 2003) that can transport IPP and geranyl diphosphate (GPP) in a plastid-to-cytosol direction.

III. The Plastidic 1-Deoxy-D-Xylulose 5-Phosphate/2-C-Methyl-D-Erythritol 4-Phosphate (DOXP/MEP) Pathway of Isopentenyl Diphosphate Biosynthesis and its Inhibition

The plastidic DOXP/MEP pathway of IPP biosynthesis involves 7 enzymes and requires 3 ATP equivalents (ATP or CTP) and 3 NADPH (Fig. 4.3). These seven enzymes have been isolated and their genes (*DXS, DXR, IspD to IspH*) have been cloned in plants, algae and bacteria (Lichtenthaler, 2000, 2004; Eisenreich et al., 2004). The first enzyme DOXP synthase is involved in the condensation of (hydroxyethyl) thiamin derived from pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate to produce DOXP. The next step consists of an intramolecular rearrangement and reduction of DOXP yielding 2C-methyl-D-erythritol 4-phosphate (MEP). The latter is converted by the third enzyme into 4-diphosphocytidyl-2C-methyl-D-erythritol. Further steps lead to the formation of 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, whereby CMP is split off. The sixth enzyme yields 1-hydroxy-2-methyl-2-(E)-butenyl diphosphate (HMBPP). The final enzymatic step, catalyzed by HMBPP reductase (*LytB* or *IspH*), yields both active isoprenoid C_5-substrates required for isoprenoid biosynthesis: IPP and its isomer DMAPP (usually in a ratio of 5:1 or 3:1). DMAPP can be converted to IPP by the action of IPP isomerase and vice versa. Evidence for the final steps of this DOXP/MEP pathway came from several laboratories (see reviews by Lichtenthaler, 2000, 2004; Rodríguez-Concepción and Boronat, 2002; Eisenreich et al., 2004).

The enzyme DOXP synthase can specifically be inhibited by 5-ketoclomazone (Lichtenthaler, 2000; Müller et al., 2000; Zeidler et al., 2000). Thus, the light–induced biosynthesis and accumulation of carotenoids in etiolated seedlings can efficiently be blocked using 5-ketoclomazone (Zeidler et al., 2000). The DOXP reductoisomerase is a regulatory enzyme of the DOXP/MEP pathway, and it can be efficiently blocked by fosmidomycin and its derivative FR-900098, as has independently been shown for plants (Zeidler et al., 1998; Schwender et al., 1999), and for bacteria (Kuzuyama et al., 1998). Fosmidomycin is a structural analogue to 2-C-methylerythrose

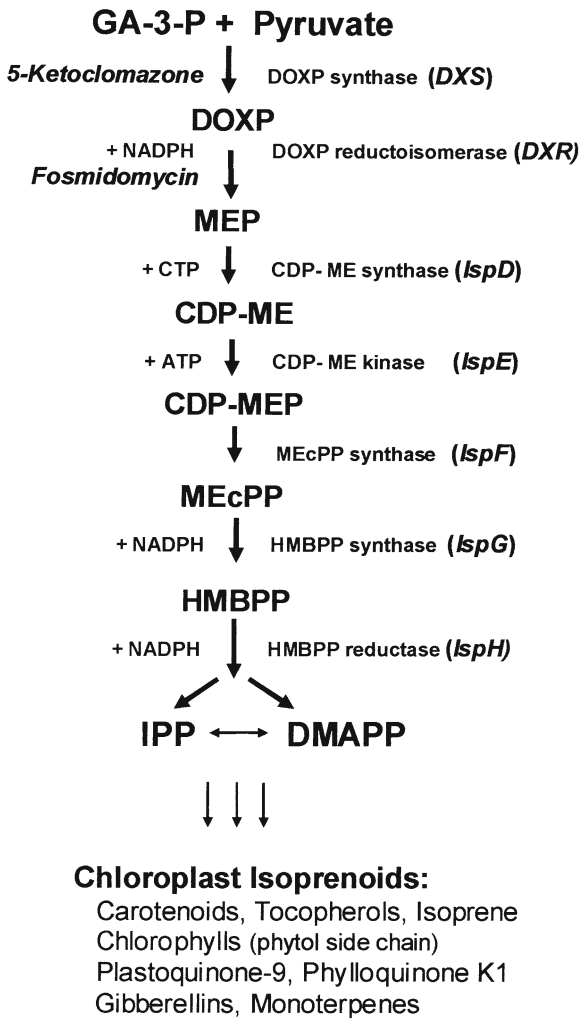

Fig. 4.3. Biochemical sequence of the DOXP/MEP pathway of plastidic isoprenoid biosynthesis in plants yielding the active isoprenic C_5-units IPP and DMAPP used for biosynthesis of carotenoids and several other plastidic isoprenoids. The pathway consists of seven enzymes and consumes 3 NADPH and 3 ATP equivalents to form one active C_5-diphosphate (IPP or DMAPP). The first two enzymatic steps can specifically be inhibited by 5-ketoclomazone and fosmidomycin, respectively, as shown by Zeidler et al. (1998), Lichtenthaler (2000) and Müller et al. (2000). The genes of the seven enzymes have been cloned, their names are shown in italics in parentheses following the enzyme names. IPP and DMAPP can be converted into each other via the enzyme IPP isomerase whose gene (*IDI*) is known as well. GA-3-P, D-glyceraldehyde 3-phosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; MEcPP, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; HBMPP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate.

4-phosphate, an intermediate in the enzymic reaction of DXR. Also, fosmidomycin blocks the light-induced carotenoid and chlorophyll formation in the etiolated seedling test-system (Zeidler et al., 1998).

IV. Biosynthesis of Carotenoids from Dimethylallyl Diphosphate and Isopenenyl Diphosphate

Carotenoid biosynthesis starts from the two 'active' isoprenoid C_5 units DMAPP and IPP by a head-to-tail addition of the two carbon skeletons. The first C_{10} product GPP, a monoterpene diphosphate, is converted to the sesquiterpene farnesyl diphosphate (FPP) by addition of another IPP. Using a third IPP, GGPPsynthase forms the diterpene diphosphate geranylgeranyl diphosphate, GGPP (Fig. 4.4). By tail-to-tail addition of two GGPP molecules the enzyme phytoene synthase produces the first tetraterpenoid *phytoene*, a colorless compound that only possesses three conjugated double bonds and therefore exclusively absorbs in the UV. Under the action of phytoene desaturase various conjugated double bonds are introduced in several dehydrogenation steps yielding ζ-carotene which is further dehydrogenated by ζ-carotene desaturase (via neurosporene as an intermediate) to the red-colored acyclic carotene *lycopene* (Fig. 4.1) exhibiting 11 conjugated double bonds. From lycopene on, the biosynthetic pathway of plant carotenoids splits into two branches (β-carotene and α-carotene).

1. Under the influence of lycopene β-cyclase, two β-ionone rings are introduced to the lycopene molecule at each end yielding the orange-colored β-carotene; γ-carotene is an intermediate in this reaction. A β-carotene specific hydroxylase converts β-carotene in two steps (via β-cryptoxanthin as an intermediate) to *zeaxanthin*, its 3,3′-dihydroxy derivative. The enzyme zeaxanthin epoxidase introduces in each β-ionone ring, at the site of the double bond, an epoxy group to yield *violaxnthin*; *antheraxanthin*, with only one epoxy group, is an intermediate. Violaxanthin, in turn, can be further oxidized by neoxanthin synthase to *neoxanthin* which exhibits one epoxy group and three hydroxy groups (see Fig. 4.1) and a particular double bond.

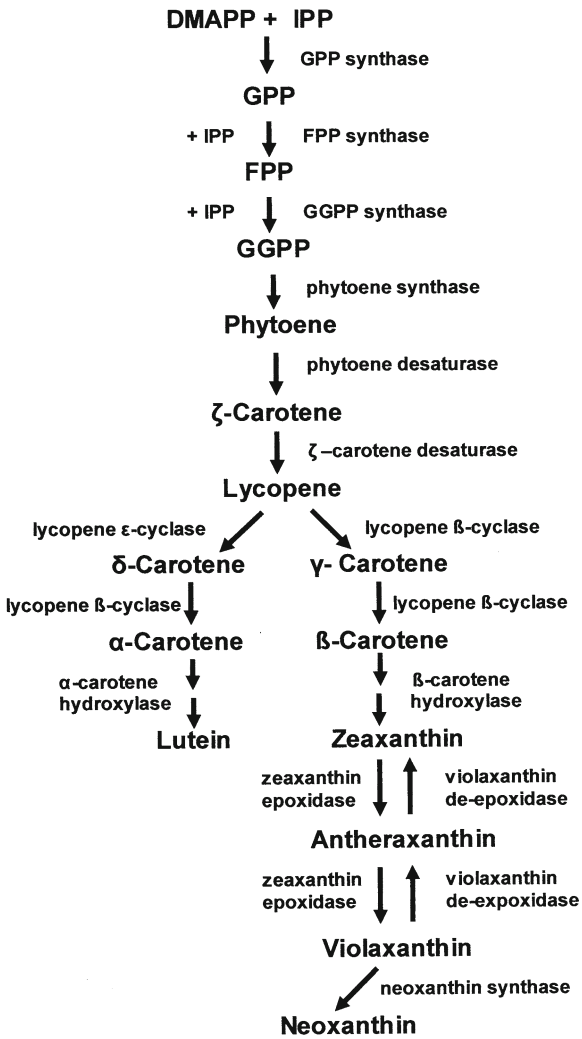

Fig. 4.4. Enzymatic steps in carotenoid biosynthesis in plants and algae proceeding in chloroplasts starting from the two C_5 prenylphosphates DMAPP (= dimethylallyl diphosphate) and IPP (= isopentenyl diphosphate). FPP, farnesyl diphosphate, GPP, geranyl diphosphate, GGPP, geranylgeranyl diphosphate. α-Carotene contains one ε-ionone and one β-ionone and β-carotene two β-ionone rings.

2. Through the action of lycopene ε-cyclase an ε-ionone ring is formed at one end of the linear lycopene molecule (yielding δ-carotene), whereas at the other end a β-ionone ring is established by lycopene β-cyclase, and the compound formed this way is *α-carotene*. From gene cloning results it is clear that this enzymic reaction of the transformation of lycopene to α-carotene is catalyzed by an ε-cyclase and the β-cyclase in a combined action (Cunningham and Gantt, 1998, 2007). α-Carotene is oxidized by a specific hydroxylase in two steps via α-cryptoxanthin as intermediate to *lutein*, a 3,3′-dihydroxy α-carotene derivative, which is the major leaf xanthophyll. Lutein is an isomer to zeaxanthin, where both differ in the position of the double bond in one of the two ionone rings. The genes of all enzymes of carotenoid biosynthesis shown in Fig. 4.4 have been cloned (Hirschberg et al., 1997; Hirschberg, 2001; Cunningham and Gantt, 1998, 2007), except for the neoxanthin synthase. An additional xanthophyll lutein-epoxide (a 5,6 monoepoxide lutein) has been found in green leaf extracts of several plants (Matsubara et al., 2005), which can be formed by a lutein specific epoxidase. However, it has not yet been characterized or cloned.

Theoretically, one can assume that lutein might successfully be oxidized in several steps to neoxanthin as well. In neoxanthin, known as an oxidation product of β-carotene and zeaxanthin, the double bonds of the two former β-ionone rings are no longer present. Thus, neoxanthin could not only be the final oxidation product of zeaxanthin, but also that of lutein. In thin layer chromatograms of green leaf pigment extracts on silicagel plates, obtained in the late 1960s and the 1970s, a second, small band next to neoxanthin was routinely found, which we termed neoxanthin-A. This could theoretically be an oxidation product of lutein. This concept needs, however, a careful scientific investigation including stereochemical research to prove if it is true or not.

V. Localization of Carotenoids and other Isoprenoids in Chloroplasts

In chloroplasts of plants and algae there are three sites for the localization of carotenoids, other isoprenoid lipids as well as prenylquinones: (1) thylakoids, (2) the chloroplast envelope and (3) the osmiophilic plastoglobuli of the plastid stroma (as is indicated in Fig. 4.5). The general structure and function of chloroplasts is described in more detail in the book of Wise and Hoober (2006) and in the review article of Lichtenthaler (2007).

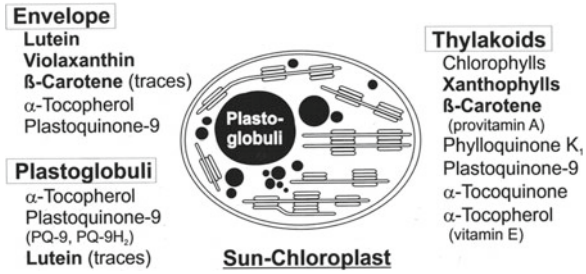

Fig. 4.5. Section through a chloroplast of a sun-exposed leaf with indication of the three sites of isoprenoid and carotenoid localization: (1) the chlorophyll-free envelope, (2) the chlorophyll-containing, photochemically active thylakoids, and (3) the chlorophyll-free osmiophilic plastoglobuli of the plastid stroma. The two plastidic vitamins α-tocopherol (vitamin E) and β-carotene (provitamin A) which are essential for human nutrition are listed as well (Modified after Lichtenthaler, 2007).

A. Thylakoids

Thylakoids contain, besides several carotenoids, the chlorophylls (isoprenoid phytyl chain), the prenylquinones plastoquinone-9, phylloquinone K1 and α-tocoquinone, as well as low amounts of the lipophilic antioxidant α-tocopherol (Lichtenthaler and Park, 1963; Lichtenthaler and Calvin, 1964). The carotenoids include β-carotene (and small amounts of α-carotene), and the xanthophylls lutein, neoxanthin, zeaxanthin, violaxanthin and antheraxanthin. The latter have been termed xanthophyll cycle carotenoids since they are interchangeable with the light-triggered xanthophyll cycle that is particularly active at high irradiance levels and de-epoxidizes violaxanthin via antheraxanthin to zeaxanthin (Schindler et al., 1994; Demmig-Adams and Adams, 1996; Schindler and Lichtenthaler, 1996; Demmig-Adams et al., 1996). Within thylakoids, carotenoids and the two chlorophylls *a* and *b* are bound in differential ways to particular chlorophyll-carotenoid-protein complexes which are obtained by gel electrophoresis (Thornber, 1975; Lichtenthaler et al., 1982a; Bennett, 1983; Green and Durnford, 1996). These are the (1) *light-harvesting chlorophyll a/b complexes*, LHCPs with lutein and neoxanthin, (2) the *chlorophyll a/β-carotene protein complex* CPa (including CP47, CP43 and the PS II reaction center complex), and (3) the *β-carotene containing pigment-protein complexes* (CPI and CPIa) which also comprise the reaction centers of PS I. All of these chlorophyll-carotenoid proteins are functionally integrated into the thylakoid membrane, together with the reaction centers of PS I and PS II (Nelson and Yocum, 2006).

Violaxanthin and zeaxanthin are present in the thylakoids as well, but in contrast to the other carotenoids they are not strictly bound to specific protein complexes. The chlorophyll-carotenoid protein complexes can be isolated from chloroplasts and thylakoids by gel electrophoresis with detergents such as digitonin. Even when applying mild detergents, violaxanthin and zeaxanthin show up in the free pigment fraction indicating that they are rather loosely bound to the chlorophyll-carotenoid-protein complexes in the thylakoids. This is required for their enzymatic oxidation or reduction in the light-driven xanthophyll cycle of thylakoids that de-epoxidizes violaxanthin via antheraxanthin to zeaxanthin, and which epoxidizes zeaxanthin to violaxanthin in the dark (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992, 1996; Lichtenthaler and Schindler, 1992; Schindler and Lichtenthaler, 1996).

B. Envelopes

Chloroplast envelopes consist of the outer and inner chloroplast membranes, preferentially contain the xanthophylls violaxanthin and lutein (Douce et al., 1973; Jeffrey et al., 1974; Lichtenthaler et al., 1981a), and minor amounts of other leaf carotenoids. Chloroplast envelopes also contain α-tocopherol and the prenylquinone plastoquinone-9, together with small amounts of phylloquinone K1 and α-tocoquinone (Lichtenthaler et al., 1981a), whereby the relative levels of prenylquinones and carotenoids are distinctly different from those of the thylakoids. Thylakoids possess a weight ratio of plastoquinone-9 to α-tocopherol of 4.0, and envelopes, in turn, with α-tocopherol as their major component, a plastoquinone-9/α-tocopherol ratio of only 0.4. In isolated spinach envelopes the three major carotenoids were violaxanthin (41%), lutein (28%) and zeaxanthin (12%), whereas β-carotene contributed only 10% and antheraxanthin and neoxanthin less than 5% each to the total carotenoid composition (Lichtenthaler et al., 1981a). In contrast, spinach thylakoids isolated from spinach leaves in the early morning contained primarily β-carotene (33%), lutein (40%) and violaxanthin (16%) and as minor components neoxanthin (7%) and zeaxanthin + antheraxanthin (ca. 4%).

Carotenoids have also been found in the envelopes of non-green plastids, together with glycerolipids, such as mono- and di-galactolipids and phosphatidylcholine as well as the phosphate translocator (Alban et al., 1988). It is now well established that the envelope is the site for the final steps of carotenoid and prenylquinone biosynthesis and also for the synthesis of galactolipids, sulfolipids, and certain phospholipids (Joyard et al., 1998) that are later deposited in the thylakoids. In addition to its biosynthetic competence, the envelope has also multiple functions in the export and import of metabolites from and into chloroplasts (Heber and Heldt, 1981).

C. Plastoglobuli

Plastoglobuli are regular globular, osmiophilic plastid structures that primarily function as a reservoir for excess amounts of plastoquinone-9 (predominantly the reduced form plastohydroquinone-9, PQ-9H_2) and α-tocopherol (Lichtenthaler and Sprey, 1966; Lichtenthaler, 1968, 1969a, c; Lichtenthaler and Weinert, 1970; Lichtenthaler, 2007) that cannot be stored in the photochemically active thylakoids. The osmiophilic plastoglobuli of chloroplasts contain carotenoids (e.g., lutein) only in trace amounts, and do not contain any chlorophylls. In contrast, the thylakoid-free chromoplasts of fruits and flower petals contain various carotenoids and in particular secondary carotenoids, the latter often in the form of fatty acid esters. The carotenoids found in thylakoid-free etioplasts of etiolated seedlings (mainly lutein, but only traces of β-carotene) are located in the plastoglobuli as well (a small portion is also in the envelope) that, together with the prolamellar bodies, are regular structures of etioplasts. During the light-induced thylakoid and chlorophyll biosynthesis the lutein accumulated in plastoglobuli in the dark is used for thylakoid formation, whereas β-carotene, present only in trace amounts in the dark, is synthesized de novo at high rates (Sprey and Lichtenthaler, 1966; Lichtenthaler, 1969d).

VI. The Concentration of Carotenoids in Green Leaves and Chloroplasts

In this section, we present information on the concentration of carotenoids (and of chlorophylls) on leaf area and leaf weight basis; the ratio of carotenoids to chlorophylls; and differences in carotenoids of sun and shade leaves.

A. The Ratio of Carotenoids to Chlorophylls

Within leaves, chlorophylls and carotenoids are exclusively found in chloroplasts. The relative levels of carotenoids in comparison to chlorophylls, prenylquinones and glycerolipids of chloroplasts and thylakoids were first determined in spinach by Lichtenthaler and Park (1963) and Lichtenthaler and Calvin (1964). Chlorophylls comprise ~ 21–24%, carotenoids ~ 4–5%, and the three prenylquinones (plastoquinone-9, α-tocoquinone and phylloquinone K1), together with α-tocopherol, ~ 3–5% of the total thylakoid lipids, whereas phospholipids make up ~ 11%, galactolipids ~ 50%, and sulfolipids 5–6% of the thylakoid lipids. Thus, isoprenoid pigments and prenylquinones make up ~ 33%, and diacylglycerol lipids forming the basic bilayer structure of thylakoids constitute the remaining two thirds of the thylakoid lipids. The relative amounts of chlorophylls ($a+b$) and carotenoids (xanthophylls (x) + carotenes (c)) in leaves and green plant tissues are characterized by the weight ratio of total chlorophyls to total carotenoids (x + c), a ratio that has been termed $(a+b)/(x+c)$ (Willstätter and Stoll, 1918; Seybold and Egle, 1937, 1938). In fact, the values of the ratio $(a+b)/(x+c)$ are an indicator of the greenness of plants and their leaves. In spinach the values of this weight ratio were found within a range of 4.5–6.5, depending on the incident photon flux density during leaf growth and on the age of the leaves. A general rule valid for all plants is: shaded leaves possess fewer carotenoids on a chlorophyll basis and as a consequence higher values for $(a+b)/(x+c)$ of 5.3–7.0, and they appear greener as compared to sun-exposed leaves. In contrast, sun leaves and sun-exposed leaves exhibit lower ratio values of only 4.2–5.2 and show a different green color that has a slight touch of a yellowish-green (see a review Lichtenthaler and Babani, 2004). Values of $(a+b)/(x+c)$ lower than 4.0 are an indicator of senescence, stress, and damage to the plant and its photosynthetic apparatus. The lowering of this pigment ratio, indicating a breakdown of chlorophylls faster than that of the total carotenoids, is well known from the successive autumnal discoloration of green leaves of trees, and also occurs

under various stress constraints. As senescence progresses, the values of $(a+b)/(x+c)$ decrease continuously and at the final step of autumnal discoloration they reach values lower even than 1.0 (Lichtenthaler and Babani, 2004) in yellowish leaves.

B. Differences in Chlorophyll and Carotenoid Composition of Sun and Shade Chloroplasts

Plant leaves and their photosynthetic pigment apparatus possess a great capacity for light adaptation (Lichtenthaler et al., 1981b, 1982c) as indicated by differences in leaf anatomy, chloroplast ultrastructure and the content of individual carotenoids. Thus, sun and shade leaves of trees as well as leaves of high-light (HL) and low-light (LL) plants differ considerably in their quantitative composition of photosynthetic pigments, electron carriers, thylakoid frequency, width of grana stacks and photosynthetic rates (Boardman, 1977; Lichtenthaler et al., 1981b; Meier and Lichtenthaler, 1981; Wild et al., 1986; Givnish, 1988; Anderson et al., 1995). Leaves that develop under high irradiance (sun leaves and HL leaves) possess sun-type chloroplasts that are adapted to higher photosynthetic CO_2 assimilation rates on a leaf area basis and on a chlorophyll basis as compared to shade leaves or leaves from LL plants as reviewed by Lichtenthaler and Babani (2004) and Sarijeva et al. (2007). Sun and HL chloroplasts possess a lower degree of stacked thylakoids (lower amounts of appressed membranes) than shade leaves and LL plants. They are characterized by a higher chlorophyll $a+b$ content per leaf area unit, and a much lower level of light-harvesting chlorophyll a/b proteins (i.e., LHCPs) as compared to low-irradiance shade-type chloroplasts (Lichtenthaler et al., 1981b, 1982b, 1984). The ultrastructure of sun-type chloroplasts with lower and narrower grana thylakoid stacks as well as rather large osmiophilic plastoglobuli is quite different from shade-type chloroplasts that contain much higher and broader grana stacks and only few small plastoglobuli (Fig. 4.6).

C. Chlorophyll a/b ratio

The chlorophyll *a*/chlorophyll *b* ratio, known as the chlorophyll a/b ratio, is an easily determined indicator for the functional light adaptation of

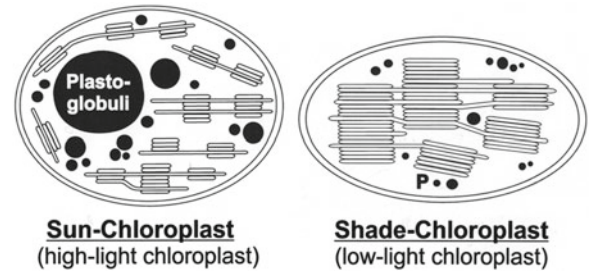

Fig. 4.6. Differences in fine structure, thylakoid frequency, plastoglobuli content as well as width and stacking degree of thylakoids between sun-chloroplasts and shade-chloroplasts in sun-exposed or shaded leaves of trees and herbaceous plants. P = Plastoglobuli (Based on Lichtenthaler, 1981; Lichtenthaler et al., 1981, 1982b, c; Meier and Lichtenthaler, 1981; also see Lichtenthaler and Babani, 2004).

leaves; it is in the range of 2.9–3.8 in sun and HL leaves of various trees and crop plants, whereas in shade and LL leaves the values range from 2.3 to 2.8. Table 4.1 shows the data for for sun and shade leaves of hornbeam and HL and LL leaves of radish. Further examples for such differences in the chlorophyll a/b ratio induced by HL or LL growth conditions are found in the literature (e.g., Lichtenthaler and Babani, 2004; Sarijeva et al., 2007).

D. Carotenoid Levels

Sun leaves and HL leaves show a higher total carotenoid content on a chlorophyll basis as compared to shade and LL Leaves. Sun and HL leaves exhibit a higher percentage of β-carotene (37 and 36%) and xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin) (18 and 19%, respectively), and a lower percentage of lutein and neoxanthin (38 and 37%) as compared to shade leaves and LL leaves. In contrast, the latter possess only 12% and 11% of the xanthophyll cycle carotenoids (Table 4.1). The higher level of β-carotene in sun and HL leaves is also visible in lower values for the ratio *a*/c (chlorophyll *a* to β-carotene) of 9.9 and 10.9, and in the ratio xanthophylls/β-carotene (x/c) of 1.7 and 1.8 as compared to shade and LL leaves (Table 4.1). The lower proportion of the two xanthophylls lutein and neoxanthin in sun and HL leaves is due to the fact that they are bound to the light-harvesting chlorophyll a/b proteins, (i.e., LHCPs) (Lichtenthaler et al., 1982a), which are less frequent in chloroplasts of sun leaves and HL leaves (Lichtenthaler et al., 1982b). In contrast, β-carotene is

Table 4.1. Differences in pigment levels (of chlorophylls and carotenoids) and in pigment weight ratios between sun and shade leaves of hornbeam (*Carpinus betulus* L.): and in cotyledons of radish seedlings (*Raphanus sativus* L.) grown at either high-light (HL) or low-light (LL) conditions

	Hornbeam		Radish	
	Sun	Shade	HL	LL
Pigment levels (mg m^{-2})				
Chlorophyll *a*+*b*	571	431	345	296
Carotenoids x+c	117	71	70	54
β-Carotene (c)	43	18	25	15
Xanthophylls (x)	77	47	45	39
Pigment ratios				
Chlorophyll *a*/*b*	**3.2**	2.5	**3.8**	2.8
Chlorophyll *a*/β-carotene, *a*/c	**9.9**	16.1	**10.9**	14.5
x/*c*	**1.7**	2.7	**1.8**	2.8
(*a*+*b*)/(x+c)	**4.9**	6.1	**4.9**	5.5
% Composition of carotenoids				
β-Carotene	**37**	27	**36**	26
Lutein	**38**	50	**37**	54
Neoxanthin	**7**	11	**8**	11
v+an+z	**18**	12	**19**	11
Zeaxanthin (z)	10	0	11	0
Antheraxanthin (an)	2	1	1	1
Violaxanthin (v)	6	11	7	10

The pigment levels of fully developed leaves are given in mg m^{-2} leaf area. For better comparison the pigment ratios and percentage carotenoid composition of sun and HL leaves are shown in bold print. Mean values of 10 determinations from three hornbeam trees on a sunny day in June of two different years, and six determinations from two repetitions of 8-day-old radish seedlings. Standard deviation < 7% (pigment levels) and < 4% (pigment ratios). The differences are highly significant $p < 0.01$. v+an+z is the sum of the xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin, respectively), x = xanthophylls and c = β-carotene. The ratio (*a* + *b*)/(x + c) is the weight ratio of total chlorophylls *a* + *b* to total carotenoids x + c. The photosynthetic pigments, chlorophylls *a* and *b* as well as total carotenoids x + c, were determined spectrophotometrically (Lichtenthaler, 1987, Lichtenthaler and Buschmann, 2001) and the individual carotenoids via HPLC (Schindler and Lichtenthaler, 1996). The β-carotene fraction shown here contains minor amounts (usually < 5%) of α-carotene

localized in the reaction center pigment proteins of both PS I and PS II, which are more prevalent on a chlorophyll basis in sun-type chloroplasts. In the morning after the onset of illumination the level of zeaxanthin in sun and HL leaves is usually relatively low. At mid-day on sunny days (hornbeam) and several hours after the start of the illumination (radish seedlings) the major part of violaxanthin has, indeed, been de-epoxidated to zeaxanthin. In contrast, in shade and LL leaves a zeaxanthin accummulation did not occur.

E. Zeaxanthin Function

The de-epoxidation of violaxanthin to zeaxanthin under HL conditions at noon, as well as considerably higher levels of the xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin) in sun leaves have also been described by different authors for various other plants (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992, 1996; Lichtenthaler and Schindler, 1992; Schindler and Lichtenthaler, 1996; Garcia-Plazaola et al., 1997; Lichtenthaler, 2007). The HL induced formation of zeaxanthin is a photoprotective mechanism for the photosynthetic pigment apparatus at excess light conditions, because zeaxanthin is positively involved in non-photochemical quenching of chlorophyll *a* fluorescence and in heat emission by dissipation of excessive excitation energy as well as by preventing photoinhibition of the photosynthetic reaction centers (Young, 1991; Adams and Demmig-Adams, 1994; Schindler and Lichtenthaler, 1996; Thiele et al., 1996; Brugnoli et al., 1998) as reviewed in detail in the book of Demmig-Adams et al. (1996, 2005).

VII. Differences in Chlorophyll and Carotenoid Levels on a Leaf Area and a Dry Weight Basis

Sun leaves of trees are smaller and thicker, possess thicker cell walls, a lower leaf water content (range: 50–65% of the leaf fresh weight) and a higher dry weight as compared to the thinner and larger shade leaves which exhibit a higher water content (62–80% of the leaf fresh weight) as found in a comparison of sun and shade leaves of 18 deciduous trees. As a consequence, sun leaves are characterized by a higher specific leaf weight, expressed as mg dry weight per cm^2 leaf area, and by lower values of specific leaf area expressed as cm^2 leaf area per gram dry weight. These special leaf parameters are listed in Table 4.2 for sun and shade leaves of three deciduous trees.

It was mentioned before that on a leaf area unit as a reference system (mg cm^{-2} leaf area) sun and HL leaves possess significantly higher chlorophyll and carotenoid levels as compared to shade and LL leaves (Table 4.1). Due to their particular differences in specific leaf weight and water content, sun leaves exhibit, however, considerably lower levels of chlorophylls and total carotenoids as compared to shade leaves when *dry weight* is used as the reference system. This is indicated in Table 4.2 for the sun and shade leaves of three tree species. When *fresh weight* is applied as the reference system the differences in chlorophyll content between both leaf types are much smaller but still significant. However, there are no differences in the carotenoid levels between sun and shade leaves on a fresh weight basis (Table 4.2). This demonstrates that fresh weight should never be applied as a reference system, neither in pigment analysis nor when determining biochemical parameters of leaves. Thus, when differences in pigment or biochemical parameters between sun-exposed and shaded leaves or between leaves of HL and LL plants are described one should always determine carotenoid and chlorophyll levels and other parameters on a leaf area basis and on a dry weight basis. This also applies to the comparison of stressed and non-stressed plants.

VIII. Accumulation of β-Carotene and Zeaxanthin at Sudden High Light Stress

The photosynthetic pigment apparatus of young and of fully developed younger leaves can respond to sudden HL conditions and an exposure to excess light by rapid de novo biosynthesis and accumulation of additional amounts of two leaf carotenoids. This applies to the two carotenoids β-carotene and zeaxanthin, whereas the other carotenoids and the level of chlorophyll are not affected. An example of such an adaptive response is shown for an 'aurea' mutant of tobacco (*Nicotiana tabacum* L. var. Su/su) in Table 4.3. This tobacco mutant is relatively poor in chlorophyll *b*, has lower total chlorophyll content as compared to regular green tobacco, and is characterized by higher values for the chlorophyll *a*/*b* ratio of around 7. Upon exposure to high irradiance of 2,200 μmol photons m^{-2} s^{-1} for 5 h, the level of β-carotene increased 1.33-fold (Table 4.3), whereas the total chlorophyll content changed little (increase of 7%). The accumulation of zeaxanthin occurred in two steps by different mechanisms: (1) there was a fast de-epoxidation of violaxanthin (90% transformation of violaxanthin into zeaxanthin), which was completed after 15–20 min. This was followed by (2) a continuous increase of the zeaxanthin pool through de novo biosynthesis. Within 5 h the zeaxanthin level doubled and with it the total violaxanthin + antheraxanthin + zeaxanthin pool (Table 4.3), whereas the levels of lutein and neoxanthin were not affected. The newly synthesized and accumulated zeaxanthin pool epoxidated to 92% violaxanthin after 16 h continuous darkness.

The changes in pigment levels induced by 5 h HL also changed the pigment weight ratios in the typical way known for an HL adaptation response of the photosynthetic apparatus. The chlorophyll *a*/*b* ratio, relatively high in this tobacco mutant, which is poor in chlorophyll *b*, increased from 7.0 to 7.7 under this condition, whereas the ratio of chlorophylls to carotenoids $(a+b)/(x+c)$ and chlorophyll *a* to β-carotene (*a*/c) decreased. Due to the doubling of the zeaxanthin levels the ratio of xanthophylls to β-carotene x/c increased from

Table 4.2. Differences in chlorophyll and carotenoid levels in sun and shade leaves of three deciduous tree species depending on the choice of the reference system

	Beech		Hornbeam		Poplar	
	Sun	Shade	Sun	Shade	Sun	Shade
Chlorophylls						
mg m^{-2} leaf area	**511**	450	**571**	431	**724**	568
mg g^{-1} dw	**6.3**	12	**8.2**	19.1	**8.0**	12.4
mg g^{-1} fw	**2.86**	4.08	**3.45**	4.97	**3.76**	4.46
Carotenoids						
mg m^{-2} leaf area	**126**	86	**117**	71	**162**	109
mg g^{-1} dw	**1.56**	2.29	**1.68**	3.13	**1.80**	2.39
mg g^{-1} fw	**0.70**	0.78	**0.71**	0.82	**0.85**	0.86
Pigment ratios						
Chlorophyll *a*/*b*	**3.22**	2.65	**3.20**	2.45	**3.30**	2.74
(*a*+b)/(x+c)	**4.04**	5.25	**4.88**	6.09	**4.44**	5.20
Leaf parameters						
Water content (% of fw)	**55**	66	**58**	74	**53**	64
Specific leaf area	**123**	267	**144**	443	**111**	218
Specific leaf weight	**8.13**	3.75	**6.94**	2.26	**9.01**	4.59

The pigment levels differ depending on the reference system as shown here for (1) leaf area, (2) dry weight (*dw*) and (3) for fresh weight (*fw*). The trees were beech (*Fagus sylvatica* L.), hornbeam (*Carpinus betulus* L.) and poplar (*Populus nigra* L.). The pigment levels and ratios of sun leaves are shown in bold print to better contrast the differences to shade leaves. Specific leaf area was expressed as cm^2 g^{-1} dry weight and the inverse specific leaf weight as mg cm^{-2} leaf area. Mean values from 5 leaves of two trees for each species. The ratio $(a+b)/(x+c)$ is the weight ratio of total chlorophylls $a+b$ to total carotenoids x+c. Standard deviation < 6% (pigment levels) and < 3% (pigment ratios) within one leaf type. The differences between both leaf types are highly significant ($p < 0.01$). Fresh weight (fw) and dry weight (dw) of 2 cm^2 leaf area were determined and the relative water content (% of fw) was calculated

Table 4.3. Increase in carotenoid levels of the low-chlorophyll tobacco 'aurea' mutant Su/su grown at medium irradiance at a sudden 5 h exposure to high irradiance (HL) stress[a]

	Control	+ 5 h HL	Increase
Pigment levels			
Chlorophyll *a*+*b*	108.7	116.7	1.07 x
Carotenoids x+c	27.9	41.2	1.48 x
β-Carotene (c)	7.8	10.4	1.33 x
Xanthophylls (x)	20.1	30.8	1.53 x
v+an+z	9.7	19.4	2.00 x
Pigment ratios			
Chlorophyll *a*/*b*	7.1	7.7	1.08 x
Chlorophyll *a*/β-carotene, *a*/c	2.3	9.9	0.80 x
x/c	2.6	3.0	1.15 x
(*a*+*b*)/(x+c)	3.9	2.8	0.72 x

[a] The aurea mutant possesses high values for the chlorophyll *a*/*b* ratio and was grown at a medium irradiance of 400 µmol photons m^{-2} s^{-1} and then exposed to a high irradiance of 2,200 µmol photons m^{-2} s^{-1}. The increase in carotenoid levels with doubling of the xanthophyll cycle carotenoids (indicated here in bold print) changed the pigment weight ratios, whereas the chlorophyll levels were not affected. Mean of three determinations, standard deviation < 6% (pigments) and < 4% (pigment ratios). The pigment levels are presented in mg m^{-2} leaf area. v+an+z is the sum of xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin), and $(a+b)/(x+c)$ is the weight ratio of totals chlorophylls $a+b$ to total carotenoids x+c, i.e., xanthophylls (x) + β-carotene (c). (Based on Schindler et al., 1992)

2.6 to 3.0 (Table 4.3). All these changes were typical high irradiance adaptation responses of the photosynthetic apparatus. The high increase of the zeaxanthin pool under HL conditions by de novo biosynthesis is understandable since zeaxanthin can function as a non-photochemical quencher of absorbed excess light energy. The increase in β-carotene, localized in the PS I and PS II reaction centers (Lichtenthaler et al., 1982a, b), is a clear indicator that under high photon flux density more photosynthetic reaction centers are formed. Further examples and references of the photosynthetic apparatus being fairly responsive and adaptive to excess light are found in the reviews of Lichtenthaler and Babani (2004) and Lichtenthaler (2007).

IX. Evolutionary Aspects of Carotenoid Biosynthesis

In all mosses, ferns, conifers and higher plants the carotenoids are synthesized in chloroplasts from the two C5 isomers IPP and DMAPP, which have been formed via the DOXP/MEP pathway of isoprenoid formation (Lichtenthaler et al., 1997b;

Lichtenthaler, 1999, 2004). This is also the case for the secondary carotenoids in fruits or flowers, e.g., for lycopene biosynthesis in chloroplasts of tomato fruits (Schwender and Lichtenthaler, 1998). Carotenoid biosynthesis via the DOXP/MEP pathway also occurs in the different algae groups, such as red algae (*Cyanidium*, Rhodophytes), Heterokonts such as *Ochromonas* (Lichtenthaler, 1999) and marine diatoms *Nitzschia* and *Phaeodactylum* (Cvejic and Rohmer, 2000), as well as in the different groups of 'green algae' (*Chlorophyta, Charales, Klebsomidiales, Zygnematales*) (Lichtenthaler, 1999; Schwender et al., 2001) and *cyanobacteria* (Disch et al., 1998; Proteau, 1998; Lichtenthaler, 1999, 2004). One branch of green algae, i.e., the chlorophytes, such as *Chlorella*, *Chlamydomonas, Scenedesmus* and *Trebouxia* have lost their cytosolic acetate/mevalonate pathway during evolution and are completely dependent on the plastidic isoprenoid pathway, not only for carotenoid biosynthesis but also for the biosynthesis of their cytosolic sterols (Schwender et al., 2001). The inverse situation and the only exception of all photosynthetic organisms is the genus *Euglena* (Euglenophytes), where the DOXP/MEP pathway of isoprenoid biosynthesis was lost during evolution, apparently in the course of the secondary endosymbiotic event. Thus, in the genus *Euglena* carotenoids are synthesized from isoprenoid C_5 units produced via the cytosolic acetate/mevalonate pathway (Disch et al., 1998; Lichtenthaler, 1999, 2004).

Certain Fungi and yeasts are also able to synthesize carotenoids (e.g., *Erwinia, Neurospora*) (Sandmann, 1994, 2002; Hirschberg et al., 1997). This carotenoid biosynthesis, however, proceeds via the acetate/mevalonate pathway because the DOXP/MEP pathway is not present in these heterotrophic organisms as has been shown for the fungal genus *Aschersonia* and the yeast genus *Rhodotorula* (Disch and Rohmer, 1998). In evolutionary terms the DOXP/MEP pathway shows up first in photosynthetic and heterotrophic Bacteria, whereas Archaea possess the acetate/mevalonate pathway for isoprenoid formation. The early anoxygenic *photosynthetic bacteria* (one photosynthetic light reaction) and the later *cyanobacteria* (two light reactions and oxygenic photosynthesis) that form a link to the endosymbiotic chloroplasts contain the DOXP/MEP pathway for isoprenoid and carotenoid biosynthesis (see overview, Lichtenthaler, 2004).

X. Concluding Remarks

Plants possess two biosynthetic pathways for the biosynthesis of IPP and isoprenoid formation: (1) in chloroplasts the DOXP/MEP pathway that starts from glyceraldehyde-3-P and pyruvate and (2) in the cytosol the acetate/mevalonate pathway. Both cellular isoprenoid pathways operate independently of each other and yield the active isoprenoid C_5 diphosphates IPP and DMAPP that are the building blocks to form all the different functional isoprenoid compounds of plant cells. In all photosynthetic organisms, carotenoids are solely produced via the plastidic DOXP/MEP pathway, except for the genus *Euglena* which has lost its DOXP/MEP pathway during evolution. The cytosolic acetate/mevalonate pathway, in turn, is responsible for the synthesis of sterols, the ubiquinone prenyl side chains and several other isoprenoids which can vary depending on the plant species. The cytosolic isoprenoid pathway does not provide IPP for the biosynthesis of carotenoids in chloroplasts and chromoplasts. In contrast, under photosynthetic conditions the plastidic DOXP/MEP pathway delivers and exports IPP for the cytosolic biosynthesis of sterols which means a flow of photosynthetic carbon into sterols (Lichtenthaler, 2007).

The formation of carotenoids from IPP and DMAPP by head-to-tail of the C_5 carbon skeletons, then by tail-to-tail condensation of two GGPP, and all the furthers steps of successive desaturation of the first linear C_{40} isoprenoid chain phytoene to the final carotenes and their transformation to the oxygen-bearing xanthophylls and secondary carotenoids proceed in cyanobacteria, algae and higher plants in the same manner and catalyzed by the same enzymes and gene products. In other photosynthetic bacteria and in non-photosynthetic fungi and yeasts, that are able to synthesize carotenoids, principally the same biochemical transformation of phytofluene occurs, yet the enzymes involved may have slightly different characteristics.

In photosynthetic organisms the final carotenoids of the photochemical active thylakoids have acquired different physiological functions during evolution. Carotenes function in the photosynthetic reaction centers as the protection of the reaction center chlorophylls, and lutein and neoxanthin are bound to special chlorophyll *a*/*b* proteins that are responsible for light harvesting. Zeaxanthin and the xanthophyll cycle carotenoids

provide protection against photoinibition and photo-oxidation of the photosynthetic apparatus by the dissipation of excess light energy. The photosynthetic pigment apparatus exhibits a highly flexible reactivity and adaptation capacity of its carotenoid content to either HL or LL conditions. This is documented by the spontaneous de novo biosynthesis and accumulation of carotenoids (β-carotene, zeaxanthin) at high photon flux density and the differential carotenoid compositions of sun and shade leaves as well as HL and LL leaves. Thus, carotenoids are essential and indispensable for the performance of photosynthesis.

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References

Adams WW III and Demmig-Adams B (1994) Carotenoid composition and down regulation of photosystem II in three conifer species during the winter. Physiol Plant 92: 451–458

Alban C, Joyard J and Douce R (1988) Preparation and characterization of envelope membranes from non green plastids. Plant Physiol 88: 709–711

Anderson JM, Chow WS and Park Y-I (1995) The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. Photosynth Res 46: 129–139

Bach TJ and Lichtenthaler HK (1982) Mevinolin, a highly specific inhibitor of microsomal 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase of radish plants. Z Naturforsch 37c: 46–50

Bach TJ and Lichtenthaler HK (1983a) Mechanisms of inhibition by mevinolin (MK 803) of microsome-bound radish and of partially purified yeast HMG-CoA reductase, (EC. 1.1.1.34). Z Naturforsch 37c: 212–219

Bach TJ and Lichtenthaler HK (1983b) Inhibition by mevinolin of plant growth, sterol formation

Bennett J (1983) Regulation of photosynthesis by reversible phosphorylation of the light harvesting chlorophyll *a*/*b* proteins. Biochem J 212: 1–13

Bick JA and Lange BM (2003) Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. Arch Biochem Biophys 415: 146–154

Boardman N (1977) Comparative photosynthesis of sun and shade plants. Annu Rev Plant Physiol 28: 355–377

Bricker TM (1990) The structure and function of CPa-1 and CPa-2 in photosystem II. Photosynth Res 24: 1–13

Britton G (1995). Structure and properties of carotenoids in relation to function. FASEB J9: 1551–1558

Britton G, Liaaen-Jensen S and Pfander H (1995) Carotenoids today and challenges for the future. In: Britton G, Liaaen-Jensen S and Pfander H (eds), Carotenoids vol 1A: Isolation and Analysis. Basel: Birkhäuser.

Brugnoli E, Scartazza A, De Tullio MC, Monterverdi MC, Lauteri M and Augusti A (1998) Zeaxanthin and non-photochemical quenching in sun and shade leaves of C3 and C4 Plants. Physiol Plant 104: 727–734

Cvejic J H and Rohmer M (2000) CO_2 as main carbon source for isoprenoid biosynthesis via the mevalonate-independent methylerythritol 4-phosphate route in the marine diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*. Phytochem 53**:** 21–28

Cunningham FX and Gantt E (1998) Genes and enzymes of carotenoid biosynthesis. Annual Rev. Plant Physiol Plant Molec Biol 49: 557–583

Cunningham FX and Gantt E (2007) A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. Photosynth Res 92: 245–259

Demmig-Adams B and Adams WW III (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol 43: 599–626

Demmig-Adams B and Adams WW III (1996) The role of the xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1: 21–26

Demmig-Adams B, Adams WW III and Mattoo AK (eds) (2005) Photoprotection, Photoinhibition, Gene Regulation and Environment, Advances in Photosynthesis and Respiration, Vol 21, Springer, Dordrecht

Disch A and Rohmer M (1998) On the absence of the glyceraldehyde 3-phosphate/pyruvate pathway for isoprenoid biosynthesis in fungi and yeasts. FEMS Lett 168: 201–208

Disch A, Schwender J, Müller C, Lichtenthaler HK and Rohmer M (1998). Distribution of the mevalonate and glyceraldehyde phosphate/pyruvate pathways for isoprenoid biosynthesis in unicellular algae and the cyanobacterium *Synechocystis* PCC 6714. Biochem J 333: 381–388

Douce R, Holtz B and Benson AA (1973) Isolation and properties of the envelope of spinach chloroplasts. J Biol Chem 248: 7215–7222

Eisenreich W, Bacher A, Arigoni D and Rohdich F (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell Mol Life Sci 61: 1401–1426

Frank HA, Young AJ, Britton G and Cogdell RJ (eds) (1999) Photochemistry of Carotenoids, Advances in Photosynthesis, Vol 8, Kluwer Academic Publishers, Dordrecht

Garcia-Plazaola JI, Faria T, Abadia J, Chavess MM and Pereira JS (1997) Seasonal changes in xanthophyll composition and photosynthesis of cork oak (*Quercus suber* L.) leaves under mediterranean climate. J Exp Bot 48: 1667–1674

Givnish TJ (1988) Adaptation to sun vs. shade: a whole plant perspective. Austr J Plant Physiol 15: 63–92

Goodwin T W (1977) The prenyllipids of the membranes of higher plants. In: Tevini M and Lichtenthaler HK (eds) Lipids and Lipid Polymers in Higher Plants, pp. 29–45. Springer, Berlin

Goodwin T W (1980) The Biochemistry of the Carotenoids, 2nd edition vol 1. Chapman and Hall, London

Green BR and Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 47: 685–715

Heber U and Heldt HW (1981) The chloroplast envelope: structure, function and role in leaf metabolism. Annu Rev Plant Physiol 32: 139–168

Hirschberg J. (2001) Carotenoid biosynthesis in flowering plants. Current Opinion Plant Biol 4: 210–218

Hirschberg J, Cohen M., Harker M, Loban T, Mann V and Pecker I (1997) Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. Pure Appl Chem 69: 2151–2158

Jeffrey SW, Douce R and Benson AA (1974) Carotenoid transformations in the chloroplast envelope. Proc Nat Acad Sci USA 71: 807–810

Joyard J, Teyssier E, Miège C, Berny-Seigneurin D, Maréchal E. Block MA, Dorne A-J, Rolland N, Ajlani G and Douce R (1998) The biochemical machinery of plastid envelope membranes. Plant Physiol 118: 715–723

Kuzuyama T, Shimizu T and Seto H (1998) Fosmidomycin, a specific inhibitor of 1-deoxy-d-xylulose 5-phosphate reductoisomerase in the non-mevalonate pathway for terpenoid biosynthesis. Tetrahedron Lett 39: 7913–7916

Lichtenthaler HK (1968) Plastoglobuli and the fine structure of plastids. Endeavour vol XXVII: 144–149

Lichtenthaler HK (1969a) Localization and functional concentrations of lipoquinones in chloroplasts. In: Metzner H (ed) Photosynth Research vol I, pp 304–314. Tübingen

Lichtenthaler HK (1969b) Zur Synthese der lipophilen Plastidenchinone und Sekundär-carotinoide während der Chromoplastenentwicklung. Ber Dtsch Bot Ges 82: 483–497

Lichtenthaler HK (1969c) Die Bildung überschüssiger Plastidenchinone in den Blättern von *Ficus elasticus* Roxb Z Naturforsch 24b: 1461–1466

Lichtenthaler H K (1969d) Light-stimulated synthesis of plastid quinones and pigments in etiolated barley seedlings. Biochim Biophys Acta 184: 164–172

Lichtenthaler HK (1970) Die Lokalisation der Plastidenchinone und Carotinoide in den Chromoplasten der Petalen von *Sarothamnus scoparius* (L.) Wimm ex Koch. Planta 90: 142–152

Lichtenthaler HK (1977) Regulation of prenylquinone synthesis in higher plants. In: Tevini M and Lichtenthaler HK (eds) Lipids and Lipid Polymers in Higher Plants, pp. 231–258. Springer, Berlin

Lichtenthaler HK (1981) Adaptation of leaves and chloroplasts to high quanta fluence rates. In: Akoyunoglou G (ed) Photosynthesis VI, pp 273–287. Balaban Internat Science Service, Philadelphia

Lichtenthaler HK (1987) Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: Douce R and Packer L (eds) Methods Enzymol 148, pp 350–382. Academic Press Inc, New York

Lichtenthaler HK (1998) The plants' 1-deoxy-D-xylulose-5-phospate pathway for biosynthesis of isoprenoids. *Fett/Lipid* 100: 128–138

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50: 47–65

Lichtenthaler HK (2000) The non-mevalonate isoprenoid biosynthesis: enzymes, genes and inhibitors. Biochem Soc Transactions 28: 787–792

Lichtenthaler HK (2004) Evolution of carotenoid and sterol biosynthesis in photosynthetic and non photosynthetic organisms. In: Biacs P and Gerely P (eds) Proceedings of the 16th Plant Lipid Symposium, pp 11–24. Mete Publisher, Budapest (www.mete.mtesz.hu/pls/proceedings/index.htm)

Lichtenthaler HK (2007) Biosynthesis, accumulation and emission of carotenoids, -tocopherol. Plastoquinone, and isoprene in leaves under high irradiance. Photosynth Res 92: 163–179

Lichtenthaler HK and Buschmann C (2001) Chlorophylls and carotenoids – Measurement and characterisation by UV-VIS. Current Protocols in Food Analytical Chemistry (CPFA), (Supplement 1) F4.3.1 - F 4.3.8. John Wiley, New York

Lichtenthaler HK and Babani F (2004) Light adaptation and senescence of the photosynthetic apparatus. Changes in pigment composition, chlorophyll fluorescence parameters and photosynthetic activity. In: Papageorgiou GC and Govindjee (eds) Chlorophyll Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 713—736. Springer, Dordrecht

Lichtenthaler HK and Calvin M (1964) Quinone and pigment composition of chloroplasts and quantasome aggregates from *Spinacia oleracea*. Biochim Biophys Acta 79: 30–40

Lichtenthaler HK and Park RB (1963) Chemical composition of chloroplast lamellae from spinach. Nature 198: 1070–1072

Lichtenthaler HK and Pfister K (1977) Praktikum der Photosynthese (Practical course of photosynthesis) pp 1–184. Quelle and Meyer, Wiebaden

Lichtenthaler HK and Schindler C (1992) Studies on the photoprotective function of zeaxanthin at high-light conditions. In: N. Murata N (ed) Research in Photosynthesis, Vol IV, pp 517–520. Kluwer Academic Publishers, Dordrecht

Lichtenthaler HK and Sprey B (1966) Über die osmiophilen globulären Lipideinschlüsse der Chloroplasten. Z Naturforsch 21b: 690–697

Lichtenthaler HK and Weinert H (1970) Die Beziehungen zwischen Lipochinonsynthese und Plastoglobulibildung in den Chloroplasten von *Ficus elastica* Roxb. Z Naturforsch 25b: 619–623

Lichtenthaler HK, Prenzel U, Douce R and Joyard J (1981a) Localization of prenylquinones in the envelope of spinach chloroplasts. Biochim Biophys Acta 641: 99–105

Lichtenthaler HK, Buschmann C, Döll M, Fietz H-J, Bach T, Kozel U, Meier D and Rahmsdorf U (1981b) Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. Photosynth Res 2: 115–141

Lichtenthaler HK, Prenzel U and Kuhn G (1982a) Carotenoid composition of chlorophyll-carotenoid-proteins from radish chloroplasts. Z Naturforsch 37c: 10–12

Lichtenthaler HK, Kuhn G, Prenzel U, Buschmann C and Meier D (1982b) Adaptation of chloroplast-ultrastructure and of chlorophyll-protein levels to high-light and low-light growth conditions. Z Naturforsch 37c: 464–475

Lichtenthaler H K, Kuhn G, Prenzel U and Meier D (1982c) Chlorophyll-protein levels and stacking degree of thylakoids in radish chloroplasts from high-light, low-light and bentazon-treated plants. Physiol Plant 56: 183–188

Lichtenthaler HK, Meier D and Buschmann C (1984) Development of chloroplasts at high and low light quanta fluence rates. Israel J Botany 33: 185–194

Lichtenthaler H K, Schwender J, Seemann M and Rohmer M (1995) Carotenoid biosynthesis in green algae proceeds via a novel biosynthetic pathway. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, pp 115–118. Kluwer Academic Publishers, Amsterdam

Lichtenthaler HK, Schwender J, Disch A and Rohmer M (1997a) Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate independent pathway. FEBS Lett 400: 271–274

Lichtenthaler HK, Rohmer M and Schwender J (1997b) Two independent biochemical pathways for isopentenyl diphosphate (IPP) and isoprenoid biosynthesis in higher plants. Physiol Plant 101: 643–652

Matsubara S, Naumann M, Martin R, Nichol C Rascher U, Tomas Morosinotto T, Roberto Bassi R and Osmond B (2005) Slowly reversible de-epoxidation of lutein-epoxide in deep shade leaves of a tropical tree legume may 'lock-in' lutein-based photoprotection during acclimation to strong light. J Exp Bot 56: 461–468

Meier D and Lichtenthaler HK (1981) Ultrastructural development of chloroplasts in radish seedlings grown at high and low light conditions and in the presence of the herbicide bentazon. Protoplasma 107: 195–207

Müller C, Schwender J, Zeidler J and Lichtenthaler HK (2000) Properties and inhibition of the first two enzymes of the non-mevalonate Pathway of isoprenoid biosynthesis. Biochem Soc Transactions 28: 794–795

Nelson N and Yocum CF (2006) Structure and function of photosystems I and II. Annu Rev Plant Biol 57: 521–565

Pfander H (1992) Carotenoids: an overview. Meth Enzymol 213: 3–13

Proteau PJ (1998) Biosynthesis of phytol in the cyanobacterium *Synechocystis* spec. UTEX 2470: utilization of the non-mevalonate pathway. J Nat Prod 61: 841–843

Rodríguez-Concepción M, and Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130: 1079–1089

Sandmann G (1994) Carotenoid biosynthesis in microorganisms and plants. Eur J Biochem 223: 7–24

Sandmann G (2002) Molecular evolution of carotenoid biosynthesis from bacteria to higher plants. Physiol Plant 116: 431–440

Sarijeva G, Knapp M and Lichtenthaler HK (2007) Differences in photosynthetic activity, chlorophyll and carotenoid levels, and in chlorophyll fluorescence parameters in green sun and shade leaves of *Ginkgo* and *Fagus*. J Plant Physiol 164: 950–955

Schindler C and Lichtenthaler HK (1996) Photosynthetic CO_2 assimilation, chlorophyll fluorescence and zeaxanthin accumulation in field-grown maple trees in the course of a sunny and a cloudy day. J Plant Physiol 148: 399–412

Schindler S, Bach TJ and Lichtenthaler HK (1985) Differential inhibition by mevinolin of prenyllipid accumulation in radish seedlings. Z Naturforsch 40c: 208–214

Schindler C, Burkart S and Lichtenthaler HK (1992) High-light induced accumulation of zeaxanthin in aurea-tobacco: a biphasic process. In: Cherif A, Miled-Daoud DB, Marzouk B and Zarrouk M (eds) Metabolism, Structure and Utilisation of Plant Lipids, pp 269–274. Centre National Pedagogique, Tunis

Schindler C, Reith P and Lichtenthaler HK (1994) Differential levels of carotenoids and decrease of zeaxanthin cycle performance during leaf development in a green and an aurea variety of tobacco. J Plant Physiol 143: 500–507

Schwender J and Lichtenthaler HK (1998) Biosynthesis of lycopene in tomato fruits proceeds via the non-mevalonate isoprenoid pathway. In: Sanchez J, Cerda-Olmedo E and Martinez-Force E (eds) Advances in Plant Lipid Research, pp 429–432. Univ Sevilla: Secretaria do Publ

Schwender J, Lichtenthaler HK, Seemann M and Rohmer M (1995) Biosynthesis of isoprenoid chains of chlorophylls and plastoquinone in *Scenedesmus* by a novel pathway. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, pp 1001–1004. Kluwer Academic Publishers, Amsterdam

Schwender J, Seeman M, Lichtenthaler HK and Rohmer M (1996) Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophyll and plastoquinone) via a novel pyruvate/glycero-aldehyde-3-phosphate non-mevalonate pathway in the green alga *Scenedesmus*. Biochem J 316: 73–80

Schwender J, Zeidler J, Gröner R, Müller C, Focke M, Braun S, Lichtenthaler FW and Lichtenthaler HK (1997) Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae. FEBS Lett 414: 129–134

Schwender J, Müller C, Zeidler J and Lichtenthaler HK (1999) Cloning and heterologous expression of a cDNA encoding

1-deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana.* FEBS Lett 455: 140–144

Schwender J, Gemünden HK and Lichtenthaler HK (2001) Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. Planta 212: 416–423

Seybold A and Egle K (1937) Lichtfeld und Blattfarbstoffe I. Planta 26: 491–515

Seybold A and Egle K (1938) Lichtfeld und Blattfarbstoffe II. Planta 28: 87–123

Sprey B and Lichtenthaler HK (1966) Zur Frage der Beziehungen zwischen Plastoglobuli und Thylakoidgenese in Gerstenkeimlingen. Z Naturforsch 21b: 697–699

Thayer SS and Björkman O (1990) Leaf xanthophyll content and composition in sun and shade determined by HPLC. Photosynth Res 23: 331–343

Thiele A, Schirwitz K, Winter K and Krause GH (1996) Increased xanthophyll cycle activity and reduced D1 protein inactivation in two plant systems acclimated to excess light. Plant Sci 115: 237–250

Thornber JP (1975) Chlorophyll-proteins: light-harvesting and reaction center components of plants. Annu Rev Plant Physiol 26: 127–158

Wild A, Höpfner M, Rühle W and Richter M (1986) Changes in the stochiometry of photosystem II components as an adaptive response to high-light and low-light conditions during growth. Z Naturforsch C 41: 597–603

Willstätter R and Stoll A (1918) Untersuchungen über Chlorophyll. Springer, Berlin

Wise RR and Hoober JK (eds) (2006) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23. Springer, Dordrecht

Young AJ (1991) The photoprotective role of carotenoids in higher plants. Physiol Plant 83: 702–708

Zeidler JG, Lichtenthaler HK, May HU and Lichtenthaler FW (1997) Is isoprene emitted by plants synthesized via the novel isopentenyl pyrophosphate pathway? Z Naturforsch 52c: 15–23

Zeidler JG, Schwender J, Müller C, Wiesner J, Weidemeyer C, Beck E, Jomaa H and Lichtenthaler HK (1998) Inhibition of the non-mevalonate 1-deoxy-D-xylulose-5-phosphate pathway of plant isoprenoid biosynthesis by fosmidomycin. Z Naturforsch 53c: 980–986

Zeidler J, Schwender J, Müller C and Lichtenthaler HK (2000) The isoprenoid biosynthesis of plants as test-system for drugs against malaria and pathogenic bacteria. Biochem Soc Transactions 28: 798–800

Chapter 5

Assembly of Light Harvesting Pigment-Protein Complexes in Photosynthetic Eukaryotes

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Summary

In photosynthetic eukaryotes, the antenna system includes members of a protein family of Light-harvesting complexes encoded by the Lhc genes. These proteins bind 8–14 chlorophylls (Chls) and 2–4 carotenoid molecules per 22–28 kDa polypeptide; further, the pigments are needed for the assembly of monomeric proteins. Some members form dimers (LHCI) or trimers (LHCII). Chl *a* is needed for the assembly of all the Lhc proteins, while two members, Lhca1 and Lhca3, can refold in vitro without Chl *b*. Among carotenoids, lutein is bound to site L1 in all the Lhc proteins, whose occupancy is essential for protein assembly. Violaxanthin and zeaxanthin can also drive protein folding, although with a lower efficiency with respect to lutein. Current knowledge on the assembly mechanisms is also reviewed in this chapter: in vitro experiments have shown how Lhc folding is triggered by the binding of Chl *a* and of lutein; in contrast Chl *b* is only bound in a second phase and functions to stabilize the pigment-protein complexes. Together with the reaction centers, antenna complexes are organized as supercomplexes in the thylakoid membranes. The structural organization of the antenna of Photosystem I (PS I) is quite different from that of Photosystem II (PS II): the PS II antenna system is flexible and its size is modulated according to environmental conditions, while in PS I antenna protein content is maintained constant with respect to the reaction center.

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I. Introduction

Light required to power photosynthesis is absorbed by chlorophyll (Chl) and carotenoid molecules bound to thylakoid membrane proteins. These pigment-binding proteins are organized in two supramolecular complexes: Photosystem (PS) I and II. In turn, each Photosystem is composed of two moieties: (1) a core complex, responsible for charge separation and the first steps of electron transport, and (2) a peripheral antenna system, which plays a role in light harvesting and transfer of excitation energy to the reaction center. Reaction centers are widely conserved among organisms and only small differences are found between higher plants and cyanobacteria while antenna systems, in contrast, are more variable (Green and Durnford, 1996; Green, 2003; Nelson and Ben Shem, 2005; Alboresi et al., 2008). In organisms belonging to Viridiplantae (higher plants and green algae) the antenna system is composed of polypeptides encoded by a multigene family designated as the Lhc genes (for "Light-Harvesting Complexes" (Green and Durnford, 1996)). Two sub-classes of Lhc proteins are preferentially associated with PS I or PS II and are respectively defined as Lhca and Lhcb (Jansson, 1999). In *Arabidopsis thaliana*, six different polypeptides were identified for PS I (Lhca1-6) and a total of eight for PS II (Lhcb1-8, (Jansson, 1999; Klimmek et al., 2006)).

Lhca and Lhcb polypeptides share a common evolutionary origin and have a conserved structural organization: they all have three transmembrane and one amphipathic helix, as summarized in Fig. 5.1 (Van Amerongen and Dekker, 2003). This hypothesis, driven from sequence analysis, has found experimental evidence in structural data on PS I and PS II antenna complexes, which allowed for the first time a direct comparison (Ben Shem et al., 2003; Liu et al., 2004; Amunts et al., 2007, 2010). All these polypeptides coordinate Chl molecules and their binding sites are largely conserved in the different members of the family and at least eight of them are consistently found in all Lhc complexes analyzed in detail

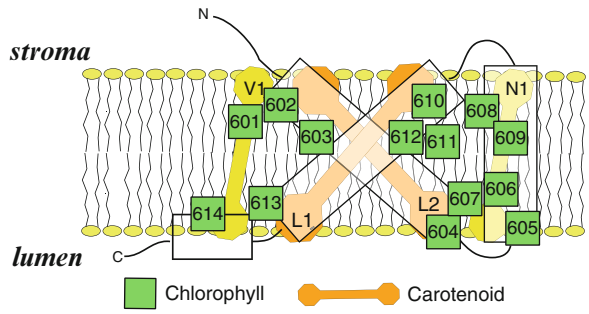

Fig. 5.1. Schematic representation of Lhcb1 structure. A scheme of the structure of Lhcb1 (Liu et al., 2004) underlining the overall organization in transmembrane helices which is conserved in all Lhc proteins. Chlorophyll (Chl) and carotenoid binding sites are also indicated. Not all the pigment-binding sites reported here have been identified in each member of the Lhc multigene family. Only eight of them are generally conserved (Chl 602, 603, 606, 609, 610, 612, 613 and 614) in all Lhc polypeptides.

thus far (Bassi et al., 1999; Remelli et al., 1999; Morosinotto et al., 2002b; Ben Shem et al., 2003; Liu et al., 2004; Morosinotto et al., 2005b; Croce et al., 2006; Pan et al., 2011). Additional binding sites have been identified in specific antenna polypeptides and the maximum total number is represented by the 14 Chls bound to Lhcb1, which is shown in Fig. 5.1 (Liu et al., 2004).[1] Antenna complexes bind both Chl *a* and *b*: different binding sites can be specific for one or the other Chl type but can also have mixed occupancy (Bassi et al., 1999; Remelli et al., 1999) due to lack of selectivity. Binding affinity of a particular binding site can also vary in different complexes; for example, the Chl 606 binding site is occupied by only Chl *b* in Lhcb1 (Remelli et al., 1999; Liu et al., 2004) while it is a mixed site in other antenna proteins like Lhcb 4 and Lhca1 (Bassi et al., 1999; Morosinotto et al., 2002b; Pan et al., 2011). All mutational and high resolution structural data available, however, showed that central Chl binding sites (Chl 610, 612, 602 and 603) are always occupied by Chl *a* (Bassi et al., 1999;

Abbreviations: Chl – Chlorophyll; Lhca (b) – Light-harvesting complex of Photosystem I (II); LHCI (II) – Antenna complex of Photosystem I (II); PQ – Plastoquinone; PS I (II) – Photosystem I (II)

[1] Chl nomenclature has changed with the availability of protein structures with higher resolution. Previously the most widely used nomenclature was from (Kühlbrandt et al., 1994); more recently new binding sites were identified in the structure from (Liu et al., 2004). Nowadays the latter is preferable also because it does not suggest the occupancy of the sites by Chl *a* or *b* which might be misleading for proteins different from Lhcb1. The correspondence between the two nomenclatures is reported in (Liu et al., 2004).

Remelli et al., 1999; Morosinotto et al., 2002b; Liu et al., 2004; Standfuss et al., 2005; Pan et al., 2011).

In addition to Chls, Lhc complexes also bind carotenoid molecules: specifically xanthophylls (Hiller, 1996). The number of carotenoids is variable between a minimum of two and a maximum of four (Ruban et al., 1999; Morosinotto et al., 2003). Two carotenoid binding sites, named L1 and L2 (or Lut620 – Lut621 as in (Liu et al., 2004)), are present in all the Lhc complexes and are found to be parallel to the two transmembrane helices, named respectively A and B (Fig. 5.1). Site N1 has been localized in the domain between helix C and the helix A/B cross by mutational and structural analysis of Lhcb1 protein (Croce et al., 1999a; Van Amerongen and Dekker, 2003; Liu et al., 2004; Pan et al., 2011) and it was proposed to be present also in Lhca1 (Croce et al., 2002b). An additional binding site, named V1, was described in LHCII (Ruban et al., 1999; Caffarri et al., 2001; Liu et al., 2004) and it is possibly present in Lhca3 as well (Castelletti et al., 2003). The comprehensive picture of carotenoid binding sites in all Lhcs (Morosinotto et al., 2003) allows an understanding of the conserved properties and differences between different polypeptides. The most conserved feature is the binding of a lutein molecule in site L1: this site, in fact, shows a much higher binding affinity for lutein with respect to any other carotenoid species. Nevertheless, when lutein is absent, this binding site can also accommodate other carotenoids like violaxanthin or zeaxanthin, but not neoxanthin (Croce et al., 1999b). The occupancy of site L2 is the most variable: both lutein and violaxanthin are generally found in different proportions in all Lhcs. A higher violaxanthin content is in fact found in monomeric antenna complexes (Lhcb4, Lhcb5 and Lhcb6) with respect to LHCII components, consistent with their larger ability to participate in the xanthophyll cycle and the capacity for binding zeaxanthin following de-epoxidation (Jahns et al., 2001; Morosinotto et al., 2002a; Betterle et al., 2010).

In Lhcb4 and Lhcb5, small amounts of neoxanthin are also found bound to the site L2. Nevertheless, in Lhcb4-5 as well, as in Lhcb1-3, the largest amount of neoxanthin is found in site N1 which is specific for this xanthophyll species (Croce et al., 1999a; Caffarri et al., 2007). Due to this specificity, it is possible to obtain complexes reconstituted in vitro with site N1 empty, when neoxanthin is absent from the refolding pigment mix (Croce et al., 1999a). In addition to neoxanthin, only violaxanthin was ever found bound to this binding site both in vivo in mutants missing neoxanthin synthase (Dall'Osto et al., 2007a) and in Lhcb1 refolded in vitro with a low stringency protocol which allows filling of binding sites with lower stability (Hobe et al., 2000).

The fourth carotenoid binding site, V1, loosely binds violaxanthin and lower proportions of lutein (Ruban et al., 1999; Caffarri et al., 2001). Site V1 is proposed to be present in Lhca3, but its occupancy in this case is not yet clear (Castelletti et al., 2003).

II. Pigments are Required for Antenna Complex Folding

Antenna proteins are very peculiar since, as shown in Fig. 5.1, they bind a remarkable high number of cofactors. As an example, a Lhcb1 monomer can coordinate up to 14 Chls and 4 carotenoid molecules, to a 25 kDa polypeptide. Since the molecular weight of each Chl and carotenoid is 900 and 600 Da, respectively, all cofactors account for 15 kDa, approx 40% of the total molecular mass of the native protein. Considering this huge content in cofactors, it is not surprising to observe that pigments deeply influence not only spectroscopic or functional properties but also biochemical characteristics. A clear demonstration is that the assembly of Lhc proteins is largely dependent on pigments, whose presence is absolutely required for their folding, differently from other pigment binding proteins, which are usually stable in their "apo" form. This difference is probably due to the high number of pigments bound by antenna proteins. In addition, since both carotenoids and Chls are hydrophobic molecules, they probably contribute to the formation of the hydrophobic protein core driving folding.

The role of pigments is so relevant that their addition to the denatured apoprotein can promote the folding of pigment-binding complexes in vitro, in the absence of any other component besides the polypeptide and pigments. These complexes,

reconstituted in vitro, have been shown to be completely indistinguishable from purified complexes (Pascal et al., 2001). The only known difference is that, while most of the pigment-binding sites are stable upon refolding, others, like the loose external site V1 in Lhcb1, are stabilized by protein-protein interactions, and might remain empty in monomeric refolded complexes.

In vitro reconstitution of Chl – protein complexes is a powerful system to study pigment requirement for the assembly, stability and the final composition of the complex. As already mentioned, these proteins bind both Chl *a* and *b* and some binding sites are specific for one or the other molecule, while others can have mixed occupancy. The folding of most antenna proteins depends on the presence of both Chl species and if one of them is missing, many binding sites are empty and the protein becomes unstable. There are, however, a few exceptions: e.g., Lhca1 and Lhca3 are able to fold in vitro even if Chl *b* is completely absent (Castelletti et al., 2003; Havaux et al., 2007). This is consistent with the fact that these complexes have a high Chl *a*/*b* ratio and, according to mutational analyses, do not have any Chl binding site specific for Chl *b*; therefore, all binding sites can be filled by Chl *a* (Morosinotto et al., 2002b; Mozzo et al., 2006). This result, obtained in vitro, is consistent with in vivo evidence: in Chl *b*-less mutants the number of antenna polypeptides is considerably reduced, due to the large de-stabilization of these pigment-protein complexes. However, Lhca1 and Lhca3 are detectable and shown to be properly folded, as demonstrated by low temperature fluorescence and CD spectra reproducing the fingerprints of these complexes (Havaux et al., 2007).

In comparison, no antenna complex is able to fold in the absence of Chl *a*: even in the presence of a large excess of Chl *b*, a few Chl *a* molecules must be bound in order to obtain a stable folded complex, in agreement with the observation that some binding sites are specific for Chl *a* in all Lhc complexes (Croce et al., 2002a). In some cases, complexes with extremely low Chl *a* content were obtained in vitro but never with only Chl *b* (Kleima et al., 1999).

Several Lhc complexes have been analyzed by mutational analysis, by selectively exchanging the amino acid residue coordinating the Chl in a specific binding site. This work has shown that Lhc proteins are generally stable enough to survive purification even with a decreased number of Chl molecules. The only exception is Chl 610: when the residue coordinating this Chl is mutated, in fact, Lhc complexes are not stable enough to yield a stable refolded complex. The only exception is Lhcb1, which is the most stable among antenna proteins, where it is still possible to purify a pigment-protein complex depleted in Chl 610 (Remelli et al., 1999). However, even in Lhcb1, the Chl 610 mutant is very unstable and has a low refolding yield, confirming that the presence of this peculiar Chl binding site is fundamental for the folding of all Lhc complexes.

Carotenoids also play a basic role for Lhc folding and in their absence proteins are completely unstable. As shown in Fig. 5.1, carotenoids in site L1 and L2 are bound close to the central transmembrane α-helices: these binding sites are occupied in all the Lhc complexes and they are particularly relevant for folding. Site L1, in particular, plays a major role in complex folding: this is exemplified by Lhcb4 where stable mutants with only one xanthophyll molecule bound to site L1 have been purified (Pesaresi et al., 1997). Moreover, antenna complexes are known to be able to exchange xanthophylls in vivo, during the operation of the xanthophyll cycle. During this process, violaxanthin bound to site L2 is freed in the membrane and exchanges with zeaxanthin (Morosinotto et al., 2002a). Of course this exchange requires antenna subunits to remain stable even if the L2 is transitorily empty and L1 occupancy is thus sufficient for sustaining the stability of the holocomplex.

Mutational analyses on Lhca1 also confirm the role of L1 in inducing antenna folding: here, in fact, the carotenoid in site L2 has been proven to be important for protein stability (Morosinotto et al., 2002b), differently from what is observed in other Lhcb proteins (Formaggio et al., 2001). Despite this difference, however, the occupancy of L1 still is the most important factor for protein folding. This is clarified by the comparison of the phenotype of the mutants in Chl 613 and 603: mutation on Chl 613 causes a partial loss of carotenoid in site L1 and the complex has a very low refolding yield. Chl 603 depletion, instead, causes a destabilization of xanthophyll binding in site L2 but the complex has a refolding yield

close to wild type. When the thermal stability of the same mutants is considered, however, mutant 603 is shown to be very unstable, while mutation on Chl 613 has a small effect on thermal stability. In the case of this protein, while L2 has a peculiar role in stability, it is still the L1 which drives the first step of protein folding (Morosinotto et al., 2002b).

Additional binding sites in Lhc complexes, N1 and V1, are generally less relevant when protein folding and stability are considered (Croce et al., 1999a; Caffarri et al., 2001). The case of Lhcb1, where both N1 and V1 binding sites are present, is clear in this respect. In fact, it has been possible to obtain Lhcb1 complexes refolded in vitro (Formaggio et al., 2001) or purified from carotenoid biosynthesis mutants (Dall'Osto et al., 2007a) with one or both these sites empty without observing any effects on protein folding and stability. This is in agreement with the fact that these binding sites are not present in all Lhc subunits, suggesting they have a specialized role in some members of the multigene family.

In higher plants, lutein, violaxanthin, neoxanthin, beta-carotene and, only after high light treatment, zeaxanthin are the carotenoid species bound to Lhc complexes. These molecules show different affinity for each of the four binding sites in Lhc proteins and their efficiency in promoting Lhc protein folding is also different. Lutein, violaxanthin and zeaxanthin have been shown to be able, even alone, to drive Lhc protein folding in vitro, in agreement with data coming from mutants with defects in carotenoid biosynthesis. In the absence of lutein, in fact, additional violaxanthin or zeaxanthin molecules were found bound to antenna complexes (Pogson et al., 1998). On the contrary, neoxanthin and β-carotene are not sufficient for Lhc folding (Phillip et al., 2002), most likely because these two carotenoid species cannot bind to the L1 site (Croce et al., 1999b; Formaggio et al., 2001) and, as mentioned, un-occupancy of this site impaired protein folding. It is worth mentioning in this respect a report which clarifies the structural requirements within carotenoid structure which determine the capacity to drive Lhc refolding in vitro. By testing a very large collection of different carotenoid molecules, it was shown that a 3-hydroxy-β-end group (Phillip et al., 2002) was needed for Lhcb1 folding.

Lutein, violaxanthin and zeaxanthin are thus all sufficient to drive protein folding, but not equivalent: in fact, a lower stability of PS II antenna complexes was shown in vivo in mutants accumulating zeaxanthin (Polle et al., 2001; Havaux et al., 2004).

III. Assembly Mechanisms of Light Harvesting Complexes

As discussed above, Lhc folding is highly dependent on the presence of pigments and much information is available on the requirement of different polypeptides for different pigment species. Less is known, however, on the mechanisms of this folding. Again, in vitro systems were exploited to obtain such information, by analyzing the folding kinetics of Lhcb1 apoproteins when exposed to pigments in stopped flow experiments. Energy transfer between Chl *b* and Chl *a* does not occur in solution; only when these molecules are bound together into a pigment-binding complex does one observe energy transfer from Chl *b* to Chl *a*. Thus, establishment of this energy transfer can be exploited to monitor Lhc pigment-protein complex assembly. By this method it was shown that Lhcb1 is folded in two temporal steps: the first one occurs in the range of 10 s^{-1} while a slower step develops in the range of several minutes (Booth and Paulsen, 1996; Reinsberg et al., 2000). In order to know whether Chl *a* or Chl *b* was bound first to the antenna polypeptide, fluorescent dyes which are either donating or receiving energy from Chls were also inserted in the protein backbone; results showed that the binding of Chl *a* occurs in the faster phase of folding and preceded Chl *b* involvement in the slower phase (Horn and Paulsen, 2004; Horn et al., 2007). It was also shown that Chl *a* is able to bind to Lhcb1 in the absence of Chl *b*, but the latter was necessary to stabilize pigment-protein complexes. These experiments thus provided confirmation and further detail to what was previously suggested by mutation analysis: Chl *a* is first bound to central binding sites, the most relevant for the protein folding. Chl *b* is bound later and its presence allows reaching the natural stability of the pigment-protein complex. This hypothesis is also in agreement with the observation that some Lhcs, like Lhcb4, are indeed able to fold with

only Chl *a* but in this case lack of Chl *b* makes the complex unstable preventing it to survive purification (Giuffra et al., 1996).

These kinetic studies also confirmed the relevance of lutein for the folding of Lhc complexes, consistent with its constant presence in the L1 binding site. In fact, while different carotenoids were shown to promote Lhc folding, the presence of lutein was shown to increase the rate of the process (Booth and Paulsen, 1996; Reinsberg et al., 2000).

IV. Assembly In Vivo

Relatively little is known about the mechanism of assembly of these proteins in vivo. Lhc polypeptides are encoded by the nuclear genome, translated in the cytoplasm and then translocated into the chloroplast. The largest part of these polypeptides are translocated by the cpSRP pathway (Schuenemann et al., 1998; Hutin et al., 2002); interestingly, however, an alternative mechanism also is likely to be active since the mutation of all the components in this pathway does not completely abolish the import of Lhc polypeptides (Hutin et al., 2002; Tzvetkova-Chevolleau et al., 2007b).

After translocation, light-harvesting complexes are assembled within the thylakoid membranes: this process needs to be highly coordinated with Chl biosynthesis because free Chl molecules are efficient reactive oxygen producers and thus very dangerous for the cell. One proposed mechanism suggests that, upon import, Lhc polypeptides first bind Chl molecules in a conserved motif located in between the first and third transmembrane helices, EXXHXR or EXXNXR, where the binding sites of Chl 610, 612, 602 and 603 are located (Eggink and Hoober, 2000; Hoober and Eggink, 2001). These motifs were shown to be able to stably interact with Chl molecules even when contained in oligopeptides, suggesting they might be the first binding sites occupied during folding (Eggink and Hoober, 2000). Consistent with this hypothesis, is the observation that plants carrying Lhcb1 mutated at conserved residues, E, N or H, fail to accumulate the assembled polypeptide in the thylakoid membranes (Flachmann and Kuhlbrandt, 1996), while most of the very same mutants were shown to be able to refold in vitro similar to wild type Lhcb1 (Remelli et al., 1999). This observation suggests that without the interaction of these motifs with Chls the insertion of Lhc proteins in thylakoid membranes is impaired and the polypeptide is degraded. This motif alone, however, is not sufficient to drive efficient accumulation of Lhc polypeptides in the thylakoids: in fact, without its interaction with Chl molecules, the polypeptide is not imported and undergoes degradation (Kuttkat et al., 1997; Park and Hoober, 1997). The relevance of this motif for assembly is also confirmed by the observation that it is highly conserved in all the proteins encoded by the multigene family, even in proteins with a lower similarity such as PsbS or the one-helix Lhc-like proteins in cyanobacteria which most likely do not stably bind any Chl molecule (Funk et al., 1995; Funk and Vermaas, 1999; Dominici et al., 2002), or ELIPs (Early Light Induced Proteins), which are suggested to interact with Chl precursors (Tzvetkova-Chevolleau et al., 2007a).

As mentioned earlier, carotenoid molecules are also known to be necessary for correct folding of these proteins in vitro as well as in vivo. They are probably involved from the very first steps of folding: in fact, considering their position in Lhc structure, they probably are involved in the generation of the hydrophobic core driving protein folding. Moreover, their presence is likely to provide protection from reactive oxygen species potentially formed by Chl molecules during the earliest stages of protein folding. A detailed model of their association to Lhc polypeptides in vivo is still missing.

V. Determinants for Oligomerization

Different proteins encoded by the Lhc multigene family are found in different oligomeric states: Lhcb4, 5 and 6 are usually found as monomers upon membrane solubilization, while Lhcb1, Lhcb2 and Lhcb3 are found as trimers, in the complex called LHCII (Jansson, 1999). Moreover, PS I antenna proteins are found as heterodimers of Lhca1/4 and Lhca2/3 (Fig. 5.2, (Croce et al., 2002b)).

In the case of LHCII trimers, it was shown that trimerization requires the presence of a particular

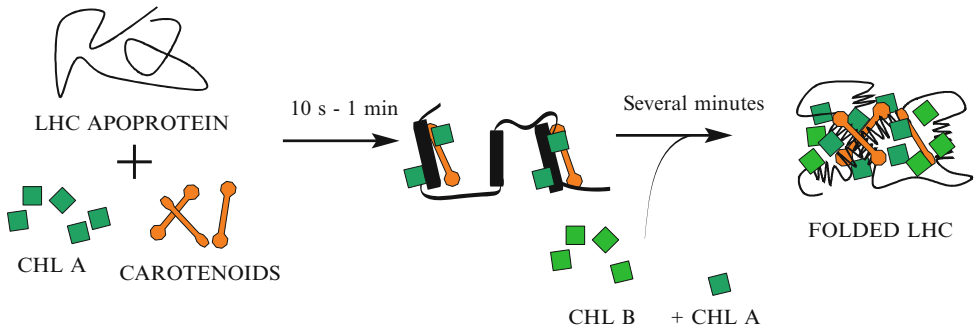

Fig. 5.2. Different steps of Lhc folding. A scheme of the proposed mechanism for protein folding of Lhc complexes. The first step is represented by the binding of Chl *a* molecules to the central binding sites, located near the transmembrane helices. Later, binding of additional Chl *a* and *b* molecules stabilize the protein structure (This figure is based on concepts presented by Horn et al. (2007)).

lipid molecule (Phosphatidylglycerol, (PG)), which in fact was found in the LHCII structure at the interface between monomers (Liu et al., 2004). In fact, a treatment with a phospholipase which degrades specifically this lipid molecule was shown to induce monomerization of LHCII complexes (Nussberger et al., 1994).

Some amino acid residues were also shown to be specifically involved in the trimerization in mutational studies: the presence of three residues in the N terminus (W, Y, and R) as well as another (W) on the C terminus were necessary for trimer formation (Hobe et al., 1995; Kuttkat et al., 1996). Interestingly, the presence of trimers was also shown to be dependent on the carotenoid composition. In fact, mutant plants depleted in lutein were shown to be unable to form trimers (Lokstein et al., 2002). However, further analyses showed that trimers without lutein are formed in vivo but they are unstable and do not survive purification (Dall'Osto et al., 2006).

Concerning LHCI, Lhca1-4 dimerization was also shown to be dependent on the presence of PG. In fact, Lhca1-4 were shown to be able to form dimers also in vitro, provided that PG was present in the reaction mixture (Schmid et al., 1998). Also in the case of Lhca polypeptides, oligomerization was shown to depend on the presence of specific amino acids: dimerization is dependent on sequence determinants located in the N terminal domain in the case of Lhca1 and on the second transmembrane helix in the case of Lhca4 (Schmid et al., 2002; Ben Shem et al., 2003; Corbet et al., 2007).

VI. Assembly of Supercomplexes

Antenna proteins are organized as supercomplexes with their reaction centers, whose assembly is still largely obscure. From available knowledge, however, it clearly appears that PS I and PS II behave differently. In the case of PS II, a basic structure composed by Lhcb4, Lhcb5 and one LHCII trimer (called S, Fig. 5.3) is organized around a PS II core dimer. Larger supercomplexes are formed by the binding of additional trimers, called M and L, and of Lhcb6 as shown in Fig. 5.3 (Boekema et al., 1999b). Even if each antenna polypeptide is bound to a specific position within the supercomplex, PS II antenna have a certain level of flexibility: in plants depleted in Lhcb1 and Lhcb2, in fact, Lhcb3 and Lchb5 were overexpressed and shown to be able to build trimers substituting for the LHCII component (Ruban et al., 2003). Such flexibility, however, is not present in the case of Lhcb4 and Lhcb5, which are not substituted by any other Lhc polypeptide (Yakushevska et al., 2003).

In the case of PS I, instead, when one Lhca is absent no compensatory replacement by other polypeptide(s) was observed. Lhca1-4, thus, bind to specific sites whose occupancy is very selective. In addition, a substantial de-stabilization of the whole antenna system was observed in these mutants, suggesting a binding cooperativity (Klimmek et al., 2005; Morosinotto et al., 2005a). Thus, despite the similarity between the antenna polypeptides, antenna systems in PS I and PS II are probably assembled differently.

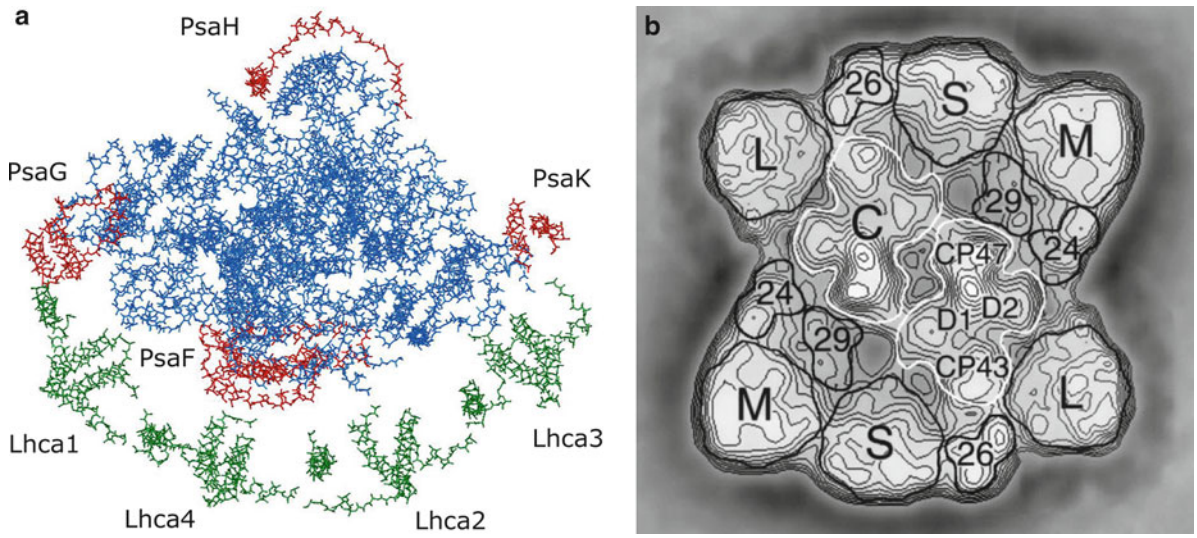

Fig. 5.3. Structural organization of PS I-LHCI and PS II-LHCII supercomplexes. (**a**) Polypeptide organization of the PSI-LHCI complex: the four Lhca subunits are aligned into a crescent-like structure on the lower part of the figure. Subunits exclusive of eukaryotic PS I complex (G, H) or relevant in the interaction with the antenna moiety (F, K) are also indicated. The coordinates of the polyA structure of PSI-LHCI were kindly provided by A. Ben Shem and N. Nelson from The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. (**b**) Organization of a dimeric complex of PS II together with its antenna. C, PS II core complex; S, strongly bound LHCII; M, moderately bound LHCII; L, loosely bound LHCII; 29, CP29; 26, CP26; 24, CP24. The right-hand PS II core complex shows the locations of its four largest subunits (Reproduced from Boekema et al. (1999b)).

The above hypothesis is confirmed by the observation that the PS I antenna system shows stronger interactions with the core complex thus making the PS I-LHCI supercomplex stable to detergent treatments. PS II-LHCII complexes, instead, are only partially retained even in the mildest solubilization conditions. This difference in stability could be related to the presence in PS I, but not in PS II, of gap and linker pigments bound at the interface between core and antenna moieties, which provide additional interactions between protein subunits (Ballottari et al., 2004).

While the PS I core complex polypeptides are largely conserved during evolution in their sequence and structure, a large modification occurred in supercomplex organization (Ben Shem et al., 2003). In fact, whereas plant PS I is a monomer, its cyanobacterial counterpart assembles in vivo into trimers (Chitnis, 2001). Structures available from different organisms showed that PS I cannot form trimers because its PsaH subunit, not present in cyanobacteria, hinders the formation of contacts between monomers. As PsaH probably forms part of the docking site for LHCII (Lunde et al., 2000), it implies that trimerization was lost in plants to facilitate re-allocation of phosphorylated LHCII to PS I under light conditions favoring PS II excitation. Furthermore, the C terminus of PsaL, which protrudes in the cyanobacterial reaction center and facilitates trimer formation, is lost in plants (Ben Shem et al., 2003).

VII. Modulation of Antenna Size by Assembly Regulation

As discussed thus far, antenna proteins are characterized by a significant similarity and they also show conserved assembly properties. Nevertheless, they also show some peculiarities, especially concerning their association to reaction centers.

A possible reason for these differences is found by considering that antenna systems of the two photosystems are regulated differently in response to environmental conditions. As already mentioned, PS I antenna is characterized by high

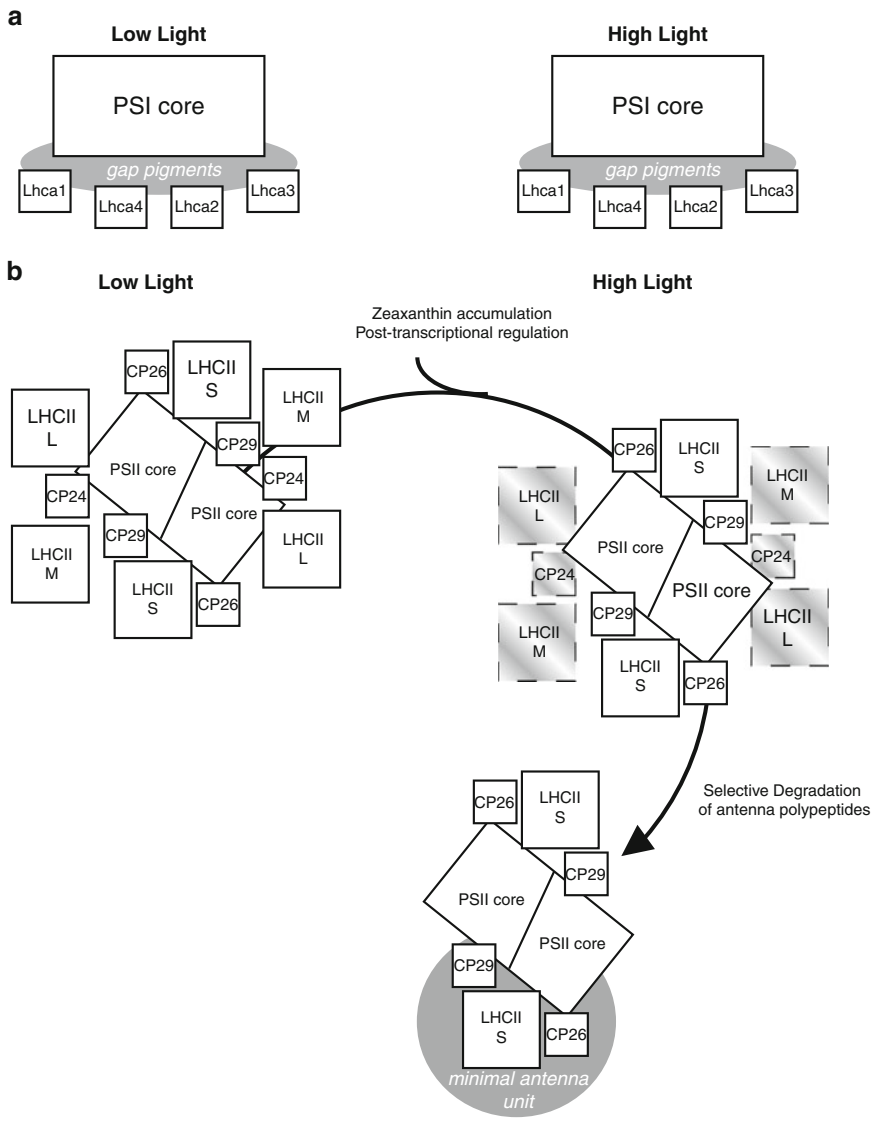

Fig. 5.4. Schematic model of antenna size regulation in PS I and PS II. The stoichiometry of antenna polypeptides associated with reaction centers is regulated according to environmental conditions. Data in the literature suggests that this regulation occurs only in PS II, while PS I antenna (**a**) is maintained unchanged. This stability is correlated with the presence of gap pigments bound at the interface between the antenna and core polypeptides, which stabilize the supercomplex and, at the same time, make it more rigid. (**b**) In the case of PS II, instead, the number of antenna polypeptides is higher in low light, increasing the light-harvesting capacity. Three monomeric antenna polypeptides (CP24, CP26, CP29) and at least three different LHCII trimers (S, M, L) are bound to PS II supercomplexes. In excess light conditions the number of antenna polypeptides is reduced. Regulation mechanisms involved have been shown to be post-transcriptional: activation of selective proteolysis was suggested to play a major role. Accumulation of zeaxanthin, which occurs in high light conditions, was shown to increase antenna susceptibility to proteases, thus contributing to antenna size reduction. Reduction of antenna size has limitations: a minimal antenna unit, composed of a CP26, CP29 monomer and one (S-type) LHCII trimer, was shown to be present even in very high light, probably serving in photoprotection.

stability: each polypeptide has a specific binding site that cannot be occupied by other proteins. This lack of flexibility in PS I antenna is correlated with the observation that the antenna size in PS I is not regulated in response to environmental conditions and the same number of antenna polypeptides is always found associated to the reaction center (Ballottari et al., 2007), and see Fig. 5.4.

On the contrary, PS II is more flexible in its structure and several supercomplexes with different size of antenna are assembled (Boekema et al., 1999a; Boekema et al., 1999b). This difference is likely to have a functional meaning since it allows for the regulation of antenna size in response to environmental conditions for this photosystem. In fact, extensive changes in the number of bound LHCII subunits are experienced by PS II (Walters and Horton, 1994; Bailey et al., 2001; Betterle et al., 2009) while only State I – State II transitions play a major role in PS I antenna size regulation (Wollman, 2001; Ballottari et al., 2007).

Depending on the growth condition, PS II supercomplexes with different sizes of antenna systems can be observed. A core antenna system is composed of a Lhcb4, Lhcb5 and LHCII trimer (S). These subunits are always present regardless of the intensity of growth light (Morosinotto et al., 2006). Larger supercomplexes are, instead, found in plants grown in lower light conditions: here additional trimers (M and L) and Lhcb6 are found to be bound to PS II reaction centers, as shown in Figs. 5.3b and 5.4 (Boekema et al., 1999a; Boekema et al., 1999b). Lhcb6 has been suggested to play a key role in the regulation of stability of these supercomplexes of different sizes. This polypeptide, in fact, is under-accumulated in high light conditions and virtually absent in extreme high light (Morosinotto et al., 2006; Ballottari et al., 2007). Since high light conditions also lead to decrease of L and M type LHCII trimers, it is suggested that their binding to the PS II reaction center is destabilized by the absence of CP24 leading to a decrease in the antenna size of supercomplexes. Consistent with this hypothesis, in mutants depleted in Lhcb6, only S trimers are stably associated with the PS II core: additional LHCII trimers are indeed present in grana membranes, but they are not associated with supercomplexes and they segregate in LHCII-rich domains, resulting in a lower efficiency of energy transfer to reaction centers (Kovacs et al., 2006; Betterle et al., 2009; Johnson et al., 2011).

The assembly of smaller versus larger PS II antenna systems is regulated not only by Lhcb6 content but also by the presence of different carotenoid species. Zeaxanthin bound to Lhcb proteins induces strong reduction in antenna size (Havaux et al., 2004). A functional explanation for this effect can be found considering that zeaxanthin is synthesized from violaxanthin under high light conditions. Upon binding to Lhcb antenna, zeaxanthin induces their destabilization and therefore an increased turnover rate: this causes a decrease of protein content under the precise conditions when the antenna size needs to be reduced in order to decrease photon harvesting.

According to the above model, zeaxanthin accumulation would thus act as a signal for the regulation of PS I antenna size during acclimation thus preventing over-reduction of the plastoquinone (PQ) pool. Several observations support this hypothesis: (a) zeaxanthin was shown not to affect the abundance of Lhcas (Havaux et al., 2004); (b) Lhca proteins, contrary to Lhcb, are highly flexible in binding different carotenoid species without affecting protein stability in vitro (Ballottari et al., 2007); (c) mutants affected in several steps of xanthophyll biosynthesis undergo large effects on Lhcb antenna proteins, while PS I-LHCI was unaffected (Dall'Osto et al., 2007b), similar to the acclimation of wild type to different light intensities (Ballottari et al., 2007).

Support for this model is provided by the observation that antenna size acclimation is mostly due to post-transcriptional events (Frigerio et al., 2007), rather than to transcriptional regulation (Escoubas et al., 1995). In particular, it was suggested that the regulation of protein degradation is responsible for antenna size decrease in high light conditions, in agreement with the identification of proteases active against Lhc proteins (Zelisko et al., 2005). All together, these observations strongly suggest that the accumulation of different carotenoid species in response to different light conditions modulates Lhcb assembly and relative abundance with respect to Lhcb, thus contributing to the regulation of PS II antenna size ensuring that the capacity for light harvesting of PS II does not exceed that of PS I, a condition that would determine over-reduction of PQ and photoinhibition.

VIII. Conclusions

Assembly of light-harvesting complexes is realized at several levels: the folding of individual pigment-protein units, their oligomerization, the formation of supercomplexes with the

corresponding core complex moieties and the step-wise increase/degradation of photosystem antenna size (for PS II only). Different Lhc proteins, despite their homology, behave in a specific way in all these processes. These differences have functional consequences in terms of the overall regulation of PS I vs. PS I antenna size. Under different light conditions, the availability of different pigment ligands to Lhc proteins is modified, thus affecting the stability of specific Lhc complexes.

We have now gathered knowledge on the different Lhc complexes and we know which components are most critical for their assembly and stability. There are, however, many open questions on how Lhc assembly is achieved in vivo. In fact, the observation that their folding is so much dependent on pigment availability strongly suggests that the regulation of pigment-protein interactions during folding in the membrane is crucial. However, the presence of free Chl is dangerous for the cell because it is a source of reactive oxygen species. Thus, pigment biosynthesis and protein folding must be strongly coordinated. Interestingly, some Lhc-like proteins, the ELIPs, have been suggested to be associated with Chl precursors during pigment-binding complex assembly and might turn out to have a central role in the biogenesis of the photosynthetic apparatus and determination of overall level of photosynthetic complexes in the thylakoids (Tzvetkova-Chevolleau et al., 2007a).

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References

Alboresi A, Caffarri S, Nogue F, Bassi R and Morosinotto T (2008) In silico and biochemical analysis of *Physcomitrella patens* photosynthetic antenna: identification of subunits which evolved upon land adaptation. PLoS ONE 3:e2033

Amunts A, Drory O and Nelson N (2007) The structure of a plant photosystem I supercomplex at 3.4 Å resolution. Nature 447: 58–63

Amunts A, Toporik H, Borovikova A and Nelson N (2010) Structure determination and improved model of plant photosystem I. J Biol Chem 285: 3478–3486

Bailey S, Walters RG, Jansson S and Horton P (2001) Acclimation of *Arabidopsis thaliana* to the light environment: the existence of separate low light and high light responses. Planta 213: 794–801

Ballottari M, Dall'Osto L, Morosinotto T and Bassi R (2007) Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. J Biol Chem 282: 8947–8958

Ballottari M, Govoni C, Caffarri S and Morosinotto T (2004) Stoichiometry of LHCI antenna polypeptides and characterisation of gap and linker pigments in higher plants photosystem I. Eur J Biochem 271: 4659–4665

Bassi R, Croce R, Cugini D and Sandona D (1999) Mutational analysis of a higher plant antenna protein provides identification of chromophores bound into multiple sites. Proc Natl Acad Sci USA 96: 10056–10061

Betterle N, Ballottari M, Hienerwadel R, Dall'Osto L and Bassi R (2010) Dynamics of zeaxanthin binding to the photosystem II monomeric antenna protein Lhcb6 (CP24) and modulation of its photoprotection properties. Arch Biochem Biophys 504: 67–77

Betterle N, Ballottari M, Zorzan S, De Bianchi S, Cazzaniga S, Dall'Osto L, Morosinotto T and Bassi R (2009) Light-induced dissociation of an antenna hetero-oligomer is needed for non-photochemical quenching induction. J Biol Chem 284: 15255–15266

Ben Shem A, Frolow F and Nelson N (2003) Crystal structure of plant photosystem I. Nature 426: 630–635

Boekema EJ, Van Roon H, Calkoen F, Bassi R and Dekker JP (1999a) Multiple types of association of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. Biochemistry 38: 2233–2239

Boekema EJ, Van Roon H, Van Breemen JF and Dekker JP (1999b) Supramolecular organization of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. Eur J Biochem 266: 444–452

Booth PJ and Paulsen H (1996) Assembly of light-harvesting chlorophyll *a*/*b* complex in vitro. Time- resolved fluorescence measurements. Biochemistry 35: 5103–5108

Caffarri S, Croce R, Breton J and Bassi R (2001) The major antenna complex of photosystem II has a xanthophyll binding site not involved in light harvesting. J Biol Chem 276: 35924–35933

Caffarri S, Passarini F, Bassi R and Croce R (2007) A specific binding site for neoxanthin in the monomeric antenna proteins CP26 and CP29 of photosystem II. FEBS Lett 581: 4704–4710

Castelletti S, Morosinotto T, Robert B, Caffarri S, Bassi R and Croce R (2003) Recombinant Lhca2 and Lhca3 subunits of the photosystem I antenna system. Biochemistry 42: 4226–4234

Chitnis PR (2001) PHOTOSYSTEM I: Function and physiology. Annu Rev Plant Physiol Plant Mol Biol 52: 593–626

Corbet D, Schweikardt T, Paulsen H and Schmid VH (2007) Amino acids in the second transmembrane helix of the Lhca4 subunit are important for formation of stable heterodimeric light-harvesting complex LHCI-730. J Mol Biol 370: 170–182

Croce R, Canino g, Ros F and Bassi R (2002a) Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry 41: 7334–7343

Croce R, Morosinotto T and Bassi,R (2006) LHCI: The antenna complex of photosystem I in plants and green algae. In: Golbeck JH (ed) Photosystem I The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24, pp 119–137. Springer, Dordrecht

Croce R, Morosinotto T, Castelletti S, Breton J and Bassi R (2002b) The Lhca antenna complexes of higher plants photosystem I. Biochim Biophys Acta 1556: 29–40

Croce R, Remelli R, Varotto C, Breton J and Bassi R (1999a) The neoxanthin binding site of the major light-harvesting complex (LHC II) from higher plants. FEBS Lett 456: 1–6

Croce R, Weiss S and Bassi R (1999b) Carotenoid-binding sites of the major light-harvesting complex II of higher plants. J Biol Chem 274: 29613–29623

Dall'Osto L, Cazzaniga S, North H, Marion-Poll A and Bassi R (2007a) The *Arabidopsis aba4-1* mutant reveals a specific function for neoxanthin in protection against photooxidative stress. Plant Cell 19: 1048–1064

Dall'Osto L, Fiore A, Cazzaniga S, Giuliano G and Bassi R (2007b) Different roles of α- and β-branch xanthophylls in photosystem assembly and photoprotection. J Biol Chem 282: 35056–35068

Dall'Osto L, Lico C, Alric J, Giuliano G, Havaux M and Bassi R (2006) Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection in vivo under strong light. BMC Plant Biol 6: 32

Dominici P, Caffarri S, Armenante F, Ceoldo S, Crimi M and Bassi R (2002) Biochemical properties of the PsbS subunit of photosystem II either purified from chloroplast or recombinant. J Biol Chem 277: 22750–22758

Eggink LL and Hoober JK (2000) Chlorophyll binding to peptide maquettes containing a retention motif. J Biol Chem 275: 9087–9090

Escoubas JM, Lomas M, LaRoche J and Falkowski PG (1995) Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci USA 92: 10237–10241

Flachmann R and Kuhlbrandt W (1996) Crystallization and identification of an assembly defect of recombinant antenna complexes produced in transgenic tobacco plants. Proc Natl Acad Sci USA 93: 14966–14971

Formaggio E, Cinque G and Bassi R (2001) Functional architecture of the major light-harvesting complex from higher plants. J Mol Biol 314: 1157–1166

Frigerio S, Campoli C, Zorzan S, Fantoni LI, Crosatti C, Drepper F, Haehnel W, Cattivelli L, Morosinotto T and Bassi R (2007) Photosynthetic antenna size in higher plants is controlled by the plastoquinone redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 282: 29457–29469

Funk C, Adamska I, Green BR, Andersson B and Renger G (1995) The nuclear-encoded chlorophyll-binding photosystem II-S protein is stable in the absence of pigments. J Biol Chem 270: 30141–30147

Funk C and Vermaas W (1999) A cyanobacterial gene family coding for single-helix proteins resembling part of the light-harvesting proteins from higher plants. Biochemistry 38: 9397–9404

Giuffra E, Cugini D, Croce R and Bassi R (1996) Reconstitution and pigment-binding properties of recombinant CP29. Eur J Biochem 238: 112–120

Green BR (2003) The evolution of light harvesting antennas. In: Green BR and Parson WW (eds) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 129–168. Kluwer Academic Publishers, Dordrecht

Green BR and Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 47: 685–714

Havaux M, Dall'Osto L and Bassi R (2007) Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in *Arabidopsis* leaves and functions independent of binding to PSII antennae. Plant Physiol 145: 1506–1520

Havaux M, Dall'Osto L, Cuine S, Giuliano G and Bassi R (2004) The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana.* J Biol Chem 279: 13878–13888

Hiller RG (1996) Carotenoids as components of the light-harvesting proteins of green algae. In: Frank HA, Young DA, Britton G and Cogdell RJ (eds) The Photochemistry of Carotenoids, Advances in Photosynthesis, Vol 8, pp 81–98. Kluwer Academic Publishers, Dordrecht

Hobe S, Förster R, Klingler J and Paulsen H (1995) N-proximal sequence motif in light-harvesting chlorophyll *a*/*b*-binding protein is essential for the trimerization of light- harvesting chlorophyll *a*/*b* complex. Biochemistry 34: 10224–10228

Hobe S, Niemeier H, Bender A and Paulsen H (2000) Carotenoid binding sites in LHCIIb – Relative affinities towards major xanthophylls of higher plants. Eur J Biochem 267: 616–624

Hoober JK and Eggink LL (2001) A potential role of chlorophylls *b* and *c* in assembly of light-harvesting complexes. FEBS Lett 489: 1–3

Horn R, Grundmann G and Paulsen H (2007) Consecutive binding of chlorophylls *a* and *b* during the assembly in vitro

of light-harvesting chlorophyll-*a*/*b* protein (LHCIIb). J Mol Biol 366: 1045–1054

Horn R and Paulsen H (2004) Early steps in the assembly of light-harvesting chlorophyll *a*/*b* complex: Time-resolved fluorescence measurements. J Biol Chem 279: 44400–44406

Hutin C, Havaux M, Carde JP, Kloppstech K, Meiherhoff K, Hoffman N and Nussaume L (2002) Double mutation cpSRP43--/cpSRP54-- is necessary to abolish the cpSRP pathway required for thylakoid targeting of the light-harvesting chlorophyll proteins. Plant J 29: 531–543

Jahns P, Wehner A, Paulsen H and Hobe S (2001) De-epoxidation of violaxanthin after reconstitution into different carotenoid binding sites of light-harvesting complex II. J Biol Chem 276: 22154–22159

Jansson S (1999) A guide to the Lhc genes and their relatives in *Arabidopsis.* Trends Plant Sci 4: 236–240

Johnson MP, Goral TK, Duffy CD, Brain AP, Mullineaux CW, and Ruban AV (2011) Photoprotective energy dissipation involves the reorganization of photosystem II light-harvesting complexes in the grana membranes of spinach chloroplasts. Plant Cell 23: 1468–1479

Kleima FJ, Hobe S, Calkoen F, Urbanus ML, Peterman EJG, Van Grondelle R, Paulsen H and Van Amerongen H (1999) Decreasing the chlorophyll *a*/*b* ratio in reconstituted LHCII: Structural and functional consequences. Biochemistry 38: 6587–6596

Klimmek F, Ganeteg U, Ihalainen JA, Van Roon H, Jensen PE, Scheller HV, Dekker JP and Jansson S (2005) Structure of the higher plant light-harvesting complex I: in vivo characterization and structural interdependence of the Lhca proteins. Biochemistry 44: 3065–3073

Klimmek F, Sjodin A, Noutsos C, Leister D and Jansson S (2006) Abundantly and rarely expressed Lhc protein genes exhibit distinct regulation patterns in plants. Plant Physiol 140: 793–804

Kovacs L, Damkjaer J, Kereiche S, Ilioaia C, Ruban AV, Boekema EJ, Jansson S and Horton P (2006) Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts. Plant Cell 18: 3106–3120

Kühlbrandt W, Wang DN and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Kuttkat A, Edhofer I, Eichacker LA and Paulsen H (1997) Light-harvesting chlorophyll *a*/*b*-binding protein stably inserts into etioplast membranes supplemented with Zn-pheophytin *a*/*b.* J Biol Chem 272: 20451–20455

Kuttkat A, Hartmann A, Hobe S and Paulsen H (1996) The C-terminal domain of light-harvesting chlorophyll-*a*/*b*-binding protein is involved in the stabilisation of trimeric light- harvesting complex. Eur J Biochem 242: 288–292

Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X and Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. Nature 428: 287–292

Lokstein H, Tian L, Polle JE and DellaPenna D (2002) Xanthophyll biosynthetic mutants of *Arabidopsis thaliana*: altered nonphotochemical quenching of chlorophyll fluorescence is due to changes in photosystem II antenna size and stability. Biochim Biophys Acta 1553: 309–319

Lunde C, Jensen PE, Haldrup A, Knoetzel J and Scheller HV (2000) The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis. Nature 408: 613–615

Morosinotto T, Ballottari M, Klimmek F, Jansson S and Bassi R (2005a) The association of the antenna system to photosystem I in higher plants. Cooperative interactions stabilize the supramolecular complex and enhance red-shifted spectral forms. J Biol Chem 280: 3105–31058

Morosinotto T, Baronio R and Bassi R (2002a) Dynamics of chromophore binding to Lhc proteins in vivo and in vitro during operation of the xanthophyll cycle. J Biol Chem 277: 36913–36920

Morosinotto T, Bassi R, Frigerio S, Finazzi G, Morris E and Barber J (2006) Biochemical and structural analyses of a higher plant photosystem II supercomplex of a photosystem I-less mutant of barley. FEBS J 273: 4616–4630

Morosinotto T, Caffarri S, Dall'Osto L and Bassi R (2003) Mechanistic aspects of the xanthophyll dynamics in higher plant thylakoids. Physiol Plant 119: 347–354

Morosinotto T, Castelletti S, Breton J, Bassi R and Croce R (2002b) Mutation analysis of Lhca1 antenna complex. Low energy absorption forms originate from pigment-pigment interactions. J Biol Chem 277: 36253–36261

Morosinotto T, Mozzo M, Bassi R and Croce R (2005b) Pigment-pigment interactions in Lhca4 antenna complex of higher plants photosystem I. J Biol Chem 280: 20612–20619

Mozzo M, Morosinotto T, Bassi R and Croce R (2006) Probing the structure of Lhca3 by mutation analysis. Biochim Biophys Acta 1757: 1607–1613

Nelson N and Ben Shem A (2005) The structure of photosystem I and evolution of photosynthesis. Bioessays 27: 914–922

Nussberger S, Dekker JP, Kühlbrandt W, Van Bolhuis BM, Van Grondelle R and Van Amerongen H (1994) Spectroscopic characterization of three different monomeric forms of the main chlorophyll *a*/*b*-binding protein from chloroplast membranes. Biochemistry 33: 14775–14783

Pan X, Li M, Wan T, Wang L, Jia C, Hou Z, Zhao X, Zhang J, and Chang W (2011) Structural insights into energy regulation of light-harvesting complex CP29 from spinach. Nat Struct Mol Biol 18: 309–315

Park H and Hoober JK (1997) Chlorophyll synthesis modulates retention of apoproteins of light-harvesting complex II by the chloroplast in *Chlamydomonas reinhardtii.* Physiol Plant 101: 135–142

Pascal A, Gastaldelli M, Ceoldo S, Bassi R and Robert B (2001) Pigment conformation and pigment-protein

interactions in the reconstituted Lhcb4 antenna protein. FEBS Lett 492: 54–57

Pesaresi P, Sandona D, Giuffra E and Bassi R (1997) A single point mutation (E166Q) prevents dicyclohexylcarbodiimide binding to the photosystem II subunit CP29. FEBS Lett 402: 151–156

Phillip D, Hobe S, Paulsen H, Molnar P, Hashimoto H and Young AJ (2002) The binding of xanthophylls to the bulk light-harvesting complex of photosystem II of higher plants. A specific requirement for carotenoids with a 3-hydroxy-beta-end group. J Biol Chem 277: 25160–25169

Pogson BJ, Niyogi KK, Björkman O, and DellaPenna D (1998) Altered xanthophyll compositions adversely affect chlorophyll accumulation and nonphotochemical quenching in *Arabidopsis* mutants. Proc Natl Acad Sci USA 95: 13324–13329

Polle JE, Niyogi KK and Melis A (2001) Absence of lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of photosystem-II but not that of photosystem- I in the green alga *Chlamydomonas reinhardtii.* Plant Cell Physiol 42: 482–491

Reinsberg D, Booth PJ, Jegerschold C, Khoo BJ and Paulsen H (2000) Folding, assembly, and stability of the major light-harvesting complex of higher plants, LHCII, in the presence of native lipids. Biochemistry 39: 14305–14313

Remelli R, Varotto C, Sandona D, Croce R and Bassi R (1999) Chlorophyll binding to monomeric light-harvesting complex. A mutation analysis of chromophore-binding residues. J Biol Chem 274: 33510–33521

Ruban AV, Lee PJ, Wentworth M, Young AJ and Horton P (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. J Biol Chem 274: 10458–10465

Ruban AV, Wentworth M, Yakushevska AE, Andersson J, Lee PJ, Keegstra W, Dekker JP, Boekema EJ, Jansson S and Horton P (2003) Plants lacking the main light-harvesting complex retain photosystem II macro-organization. Nature 421: 648–652

Schmid VHR, Beutelmann P, Schmidt G and Paulsen H (1998) Ligand requirement for LHCI reconstitution. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 425–428. Kluwer Academic Publishers, Dordrecht

Schmid VHR, Paulsen H and Rupprecht J (2002) Identification of N- and C-terminal amino acids of Lhca1 and Lhca4 required for formation of the heterodimeric peripheral photosystem I antenna LHCI-730. Biochemistry 41: 9126–9131

Schuenemann D, Gupta S, Persello-Cartieaux F, Klimyuk VI, VI, Jones JDG, Nussaume L and Hoffman NE (1998) A novel signal recognition particle targets light-harvesting proteins to the thylakoid membranes. Proc Natl Acad Sci USA 95: 10312–10316

Standfuss J, Terwisscha Van Scheltinga AC, Lamborghini M and Kuhlbrandt W (2005) Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. EMBO J 24: 919–928

Tzvetkova-Chevolleau T, Franck F, Alawady AE, Dall'Osto L, Carriere F, Bassi R, Grimm B, Nussaume L and Havaux M (2007a) The light stress-induced protein ELIP2 is a regulator of chlorophyll synthesis in *Arabidopsis thaliana.* Plant J 50: 795–809

Tzvetkova-Chevolleau T, Hutin C, Noel LD, Goforth R, Carde JP, Caffarri S, Sinning I, Groves M, Teulon JM, Hoffman NE, Henry R, Havaux M and Nussaume L (2007b) Canonical signal recognition particle components can be bypassed for posttranslational protein targeting in chloroplasts. Plant Cell 19: 1635–1648

Van Amerongen, H and Dekker, J.P. (2003) Light harvesting in photosystem II. In: Green BR and Parson WW (eds) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 219–251. Kluwer Academic Publishers, Dordrecht

Walters RG and Horton P (1994) Acclimation of *Arabidopsis thaliana* to the light environment: Changes in composition of the photosynthetic apparatus. Planta 195: 248–256

Wollman FA (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J 20: 3623–3630

Yakushevska AE, Keegstra W, Boekema EJ, Dekker JP, Andersson J, Jansson S, Ruban AV and Horton P (2003) The structure of photosystem II in *Arabidopsis*: localization of the CP26 and CP29 antenna complexes. Biochemistry 42: 608–613

Zelisko A, Garcia-Lorenzo M, Jackowski G, Jansson S and Funk C (2005) AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. Proc Natl Acad Sci USA 102: 13699–13704

Chapter 6

Chlorophyll-Binding Proteins of Higher Plants and Cyanobacteria

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Summary

This chapter focuses on the structural aspects and the organization of the chlorophyll-binding complexes of higher plants and cyanobacteria. We have included a discussion of both the proteins and the chromophores, with particular attention to the comparison of similar systems in different organisms and different members of a protein family. In the last few years the structures of many components of the photosynthetic apparatus have become available at high and intermediate resolution; comparison of these systems in organisms which are separated by a billion years of evolution has allowed the identification of the building blocks of the photosynthetic apparatus. Moreover, the differences between the complexes in different organisms are mainly related to the adaptation of the organism to specific environmental conditions. The appearance of the structures has also allowed us to organize in a "visual framework" the large amount of information obtained during the last 20 years by genetic, biochemical and biophysical methods and this represents now a new starting point for functional studies.

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DOI 10.1007/978-94-007-1579-0_6,

I. Introduction

The light-reactions of oxygenic photosynthesis in higher plants and cyanobacteria are catalyzed by four multiprotein complexes associated with the membrane. Two of these complexes, Photosystem I (PS I) and Photosystem II (PS II) contain a large number of chlorophylls (Chls), which absorb light and transfer excitation energy to the reaction center where it is used to perform charge separation (for PS I, see Golbeck, 2006; for PS II, see Wydrzynski and Satoh, 2005; for Chls, see Grimm et al., 2006). The electron moves along an electron-transport chain from PS II to PS I via the cytochrome b_6f complex and is finally used for the reduction of $NADP^+$ to NADPH. The primary electron donor of PS II (P680) obtains its electrons from water, which leads to the production of O_2. During electron transport a H^+ gradient is formed across the membrane, which is used by the ATPase for the production of ATP. The overall process, that involves both PS II and PS I, produces ATP and NADPH, which are used by the organism to drive all other reactions. All the complexes, which participate in the light reactions, are located in the thylakoid membrane. In higher plants this membrane is morphologically separated in two parts, a stack of thylakoid discs, called grana, which contains PS II and a lamella part called the stroma lamellae which contains PS I and ATPase, while the cytochrome b_6f complex is present in both parts. The basic functional units of PS I and PS II are called core complexes and they are present in both higher plants and cyanobacteria. They play a role in both light harvesting and electron transfer. In addition to the core complexes, plants and cyanobacteria are equipped with an antenna system, the principal role of which is to increase the absorption cross section of the system (for information on antennas, see Green and Parson, 2003). The light-harvesting systems of plants and cyanobacteria are very different. In higher plants, the outer antenna is composed of members of the light-harvesting complexes (Lhc) family, which are integral membrane proteins and bind Chl *a*, Chl *b* and carotenoids. In most of the cyanobacteria the outer antenna system is composed of phycobilisomes, a large assembly of different proteins located at the periphery of the membrane and containing phycobilins as chromophores. However, in certain species of cyanobacteria and under iron stress conditions a different antenna system has been observed: it is composed of integral membrane proteins which coordinate Chl *a* (see Section VI.C). Finally, one Chl *a* molecule is present in the structure of the cytochrome b_6f complex. In this chapter the principal aspects of the organization of the Chl-binding complexes are reviewed integrating the structural data with results obtained from genetics, biochemistry and spectroscopy.

II. Photosystem I

The PS I complex mediates light-driven electron transport from plastocyanin to the ferredoxin-NADP complex, providing energy for carbon dioxide assimilation (Golbeck, 2006). It is a multiprotein complex, composed of 12 subunits in cyanobacteria and of 19 in higher plants (10 of which are conserved between cyanobacteria and higher plants) embedded in the thylakoid membrane. It can be divided into two moieties: (1) the core complex, which is present in both plants and cyanobacteria containing all the cofactors of the electron transport chain together with an inner antenna system and (2) the outer antenna system, composed of *Lhc* gene products, which is present only in higher plants. In the following, the structure and subunit composition of the cyanobacterial system will be discussed first. Then, a comparison of the core of PS I from cyanobacteria and that of higher plants will be presented, focusing on the subunit composition and the pigment arrangement, to detect the changes shaped by the different environmental conditions to which these organisms have been exposed during evolution. The antenna system of higher plants is also discussed in this chapter.

Abbreviations: Chl – Chlorophyll; IsiA – Chlorophyll *a*-binding protein induced by Fe limitation; Lhc – Light-harvesting complex; Lhca/b – Gene encoding light-harvesting complex proteins from either photosystem I or photosystem II; LHCI – Antenna complex of photosystem I; LHCII – Antenna complex of photosystem II; P680 – The primary donor of photosystem II; P700 – The primary donor of photosystem I; Pcb – Prochlorophyte chlorophyll *a*/*b*-binding protein; PS I – Photosystem I; PS II – Photosystem II

A. Photosystem I: Structure of the Cyanobacteria Complex

The PS I structure of the cyanobacterial system from *Thermosynechococcus elongatus* was the first structure of the thylakoid membrane components that was obtained at high resolution (Jordan et al., 2001). It shows the location of 12 proteins, 96 chlorophylls and 22 β-carotene molecules, 4 lipids, 3 iron-sulfur clusters and 2 phylloquinone molecules per monomeric core complex. Most of the cofactors are coordinated by PsaA and PsaB, the two major subunits of the system. They both consist of 11 transmembrane helices: the 5 helices at the C-terminus of the two proteins form a shell, which contains the reaction center and part of the cofactors of the electron transport chain, namely P700 (Chl *a* and Chl *a'* molecules), A_0 (Chl *a*), A_1 (phylloquinone) and F_x (FeS center) (see Brettel (1997) for details on the electron transport chain in PS I). The other 6 helices at the N-terminus are located around this core and coordinate most of the core antenna Chl *a* molecules. These two proteins form a heterodimer with two-fold pseudosymmetry and more than 80% of the Chls that are associated with the core are related to this symmetry. In addition to PsaA and PsaB, the structure shows the location of 10 other subunits. Three of them, namely PsaC, PsaD and PsaE are peripheral membrane proteins, located at the cytoplasmic side of the membrane, i.e., the reducing (electron accepting) side of PS I. PsaC binds the terminal electron acceptors, F_A and F_B (FeS centers). PsaD provides the ferredoxin docking site and PsaE seems to play a role in stabilizing the cytoplasmic domain (Lelong et al., 1994; Xu et al., 1994a). PsaF, PsaI, PsaJ, PsaK, PsaL, PsaM and PsaX are small integral membrane proteins, mainly involved in the stabilization and correct assembly of the system (Table 6.1). Moreover, they are also Chl-binding proteins and they coordinate 11 Chl *a* molecules in total. Their role has been extensively studied by deletion or inactivation of specific genes (Xu et al. (2001a) and references therein). However, for most of the mutants it was not possible to recognize a specific phenotype and it seems that none of the small subunits affects photoautotrophic growth, thus leaving several questions open (for a description of the deletion mutants of the small subunits of cyanobacteria PS I see (Chitnis, 2001)). It is possible that some of these subunits can give advantage to the organization under particular environmental conditions, which have not been experimentally tested yet. The main characteristics of the subunits and their putative role are summarized in Table 6.1.

Table 6.1. Protein composition of Photosystem I core in cyanobacteria and higher plants

Subunit	Present in P and C	MW kDa	TMH	Chls (C/P)	Function
A	Y	83.2	11	40	Light harvesting, charge separation,
B	Y	82.5	11	39	electron transport
C	Y	8.9	–	–	Electron transport
D	Y	17.9/17.7	–	–	Binding of ferredoxin
E	Y	10.4/10.5	–	–	Binding of ferredoxin
F	Y	17.3	1	–	Binding of plastocianin, binding of Lhca4
G	Only P	11.0	2	–/1	Regulation, binding of the antenna
H	Only P	10.4	1	–/1	Binding of LHCII
I	Y	4.1	1	–	Stabilization
J	Y	5.0	1	3/2	Stabilization
K	Y	8.5	2	2	Binding antenna
L	Y	18	3	3	Stabilization
M	Only C	3.4	1	1/–	Trimerization
N	Only P	9.7	–	–	Docking of plastocyanin
X	Only C	3.0	1	1/–	
O	Only P	10.1	2	?	Binding of LHCII
P	Only P	?	?	?	?

P higher plants, *C* cyanobacteria, *MW* molecular weight, *TMH* transmembrane helices, *Y* yes

B. Cyanobacteria Versus Higher Plants: The Proteins

The structure of PS I from higher plants has now been resolved at 3.4 Å (Amunts et al., 2007). Compared to the previous structure at 4.4 Å (Ben-Shem et al., 2003), further details can be observed concerning the protein and pigment organization, especially for the central part of the structure, i.e., the core. This allows a direct comparison of the PS I characteristics of cyanobacteria and higher plants. The first striking difference is that cyanobacterial PS I is present as a trimer, while in higher plants it is a monomer with the outer antenna system located on one side of the core (Boekema et al., 2001b; Ben-Shem et al., 2003). Ben-Shem et al. (2004) have suggested that the fact that PS I from higher plants cannot form trimers is due to the presence of PsaH, a subunit which is not present in cyanobacteria and which is located in the putative trimerization center. This difference is probably related to the fact that the different antenna complexes are present in higher plants and cyanobacteria, i.e., Lhc versus phycobilisomes. For instance, it has been shown that PsaH is the docking site of the PS II antenna complex LHCII when this complex moves to PS I during state transitions (Lunde et al., 2000; Kouril et al., 2005) creating a larger antenna size of PS I (Allen, 1995). Mullineaux (2005) has proposed that the presence of trimeric PS I is more effective than the monomer in avoiding spillover, a problem that cannot occur in plants due to the spatial separation of the two photosystems.

Although the supramolecular organization of PS I in the two organisms is different, a close look at the level of the monomeric unit reveals high homology. In Fig. 6.1 the protein part of the structure of PS I from *T. elongatus* and pea is superimposed. Remarkably, a billion years of evolution has produced very few changes in the structural organization of the system. The two major subunits of the photosystem, PsaA and PsaB completely overlap in the two structures, as well as the three stromal subunits PsaC-E (except for the N-terminus of PsaD which is unique for the higher plant protein (Nelson and Yocum, 2006)) and most of the small transmembrane complexes (PsaI, J and L). The transmembrane moiety of PsaF is identical in higher plants and cyanobacteria, while the lumenal N-terminus, which is longer in higher plants, shows an additional

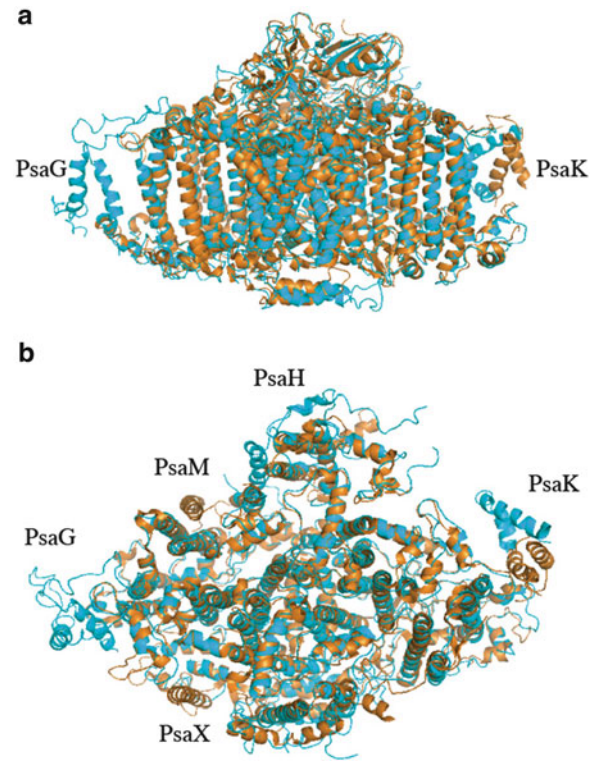

Fig. 6.1. Comparison of the protein moieties of the structure of PS I from Thermosynechococcus elongatus (*orange*, PDB 1JB0) and Pea (*blue*, PDB 2O01). (**a**) side view – perpendicular to the membrane (**b**) top view –from the stromal side. In (b) PsaC, PsaD and PsaE are omitted (The figure was created with Pymol).

helix-loop-helix motif. Hippler et al. (1998) have shown that this domain is involved in the docking of plastocyanin. The presence/absence of this domain seems to have a strong influence on the role of PsaF. Indeed, in eukaryotic cells the absence of PsaF drastically reduces electron transfer from plastocyanin to $P700^+$ (Farah et al., 1995; Hippler et al., 1997), clearly showing a strong involvement of PsaF in this process, while the absence of PsaF in cyanobacteria does not seem to have a major influence on the system (Chitnis et al., 1991; Xu et al., 1994b; Hippler et al., 1996), suggesting a different role for PsaF in these organisms. The largest difference between the conserved subunits of the core concerns PsaK that, although having the same structure in both organisms, has a somewhat different location. While the PsaK-less mutant of cyanobacteria did not show any significant phenotype (Nakamoto and Hasegawa, 1999; Tang and Chitnis, 2000),

analysis of the same mutant in higher plants indicates that PsaK is involved in the stabilization of the outer antenna system, in particular Lhca2 and Lhca3 (Jensen et al., 2000; Ihalainen et al., 2002; Varotto et al., 2002). Indeed, the structure shows that PsaK is located on the same side of the complex as these two antenna subunits, but it does not reveal any interaction between the subunits. PsaK in the cyanobacterial structure (Jordan et al., 2001) is located at a position that in higher plants would lead to a direct interaction with Lhca3. However, the sequence of PsaK could not be traced in the structure of higher plants due to lower resolution in this region (Amunts et al., 2007), similar to what was observed in the structure of cyanobacteria where the region of PsaK was less well resolved than the rest of the complex (Jordan et al., 2001). These observations suggest a high flexibility of this structural domain, where PsaK can possibly take different positions or organization. Moreover, it has also been shown that PsaK is not necessary for the assembly of PS I and in cyanobacteria it is incorporated in the system in the last step of assembly of the supercomplex (During et al., 2007). The additional subunits present in higher plants are probably related to the presence of a membrane-embedded antenna system. PsaG is located on one side of the core and it has been suggested to be the anchor for Lhca complexes (Nelson and Yocum, 2006), being the only subunit which interacts "strongly" with an antenna complex, namely Lhca1 (Ben-Shem et al., 2004). However, the knockout mutant of this complex affects the stability of the whole photosystem and in particular the binding dynamics of plastocyanin (Zygadlo et al., 2005), but it does not have a direct effect on the binding of the Lhca complexes (Jensen et al., 2002), suggesting that the interaction with PsaG is not the key factor for the connection of the outer antenna to the core. In the structure of PS I, the subunit PsaN, a soluble protein located in the lumen of the thylakoid (He and Malkin, 1992), was also modeled (Amunts et al., 2007). It is located in the outer ring of the complex, on the side of Lhca2 and Lhca3, with which it weakly interacts. The PsaN antisense mutant shows that the absence of this subunit influences the electron transfer from plastocyanin to P700 (Haldrup et al., 1999), suggesting an interaction of this subunit with PsaF, an interaction that is, however, not visible in the structure. Two other subunits, PsaO and PsaP (previously TMP14) are present in higher plants but not in cyanobacteria (Jensen et al., 2007). The structure does not show the location of these two proteins, but an analysis of the knockout mutants, and cross-linking experiments, indicate that they are both located in the proximity of PsaH and PsaL (Knoetzel et al., 2002; Jensen et al., 2004; Khrouchtchova et al., 2005), contributing to the stability of this domain and of the docking site of LHCII during state transitions.

We can conclude that the organization of the individual subunits of the core complex is highly conserved in higher plants and cyanobacteria and that most of the observed differences in subunit composition are evolved in higher plants in relation to the presence of an antenna system composed of membrane proteins.

C. Cyanobacteria Versus Higher Plants: The Pigments

The structure of *T. elongatus* reveals the position of 96 molecules of Chl *a*, of which 85 are coordinated by PsaA and PsaB; in addition, there are 22 carotenoids. Also several of the small subunits coordinate Chls (see Table 6.1). The new structure of pea PS I reveals the position of 168 Chls, and for 65 of these the phytol chain is also resolved (Amunts et al., 2007) revealing their orientation. The pigment organization of the core complex is very similar for higher plants and cyanobacteria. Most of the Chls perfectly overlap, especially the ones of, or close to, the reaction center. Nineteen additional Chls are present in higher plants, which are not part of the outer antenna complexes and are not present in the cyanobacterial system. A few of them are associated with higher plant-specific subunits, like PsaH and PsaG (2 Chls each). Several are located in between the core and the Lhca complexes (see below). Only 5 β-carotenes are resolved for higher plants, and their location and orientation are practically identical to those in the cyanobacterial system with the exception of BCR16 (nomenclature of Jordan et al., 2001), which is in a slightly different position, probably due to the absence of PsaX in higher plants (Amunts et al., 2007). Moreover, biochemical analysis reveals that only 14–18 β-carotenes are associated with the PS I core of higher plants (Siefermann-Harms, 1985; Damm et al., 1990;

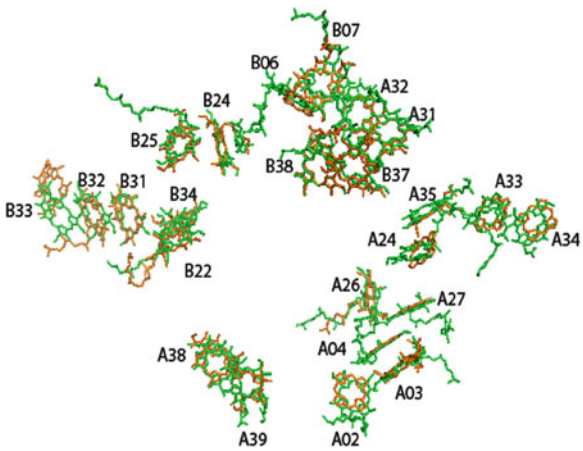

Fig. 6.2. Comparison of the putative red chlorophylls in the structure of PS I of Thermosynechococcus elongates (*green*) and pea (*only the core – orange*) (The figure was created with Pymol).

Croce and Bassi, 1998). Considering the high degree of conservation of both the protein and the Chl moieties in cyanobacteria and in higher plants, it is likely that the positions of the 9–13 β-carotene molecules that are not resolved in the structure of PS I of pea are also similar to those of PS I of *T. elongatus*.

The major spectroscopic characteristic of PS I is the presence of Chls which absorb at energies lower than those in the reaction center (Butler, 1978). Several modelling approaches based on the structure of *T. elongatus* have been used to elucidate the energy transfer process in PS I and to determine the location of the red Chl forms (Byrdin et al., 2002; Damjanovic et al., 2002; Sener et al., 2002; Gobets et al., 2003b; Bruggemann et al., 2004; Vaitekonis et al., 2005). Although experimental evidence for the location of the low-energy forms in the core complex is still lacking, the different models have restricted the number of possible candidates to only a few Chl clusters. In all cases the red forms are suggested to be associated with dimers or trimers of strongly interacting Chls. In Fig. 6.2 the geometrical arrangement of the putative red Chls in the core of the cyanobacteria is compared with the arrangement of the same Chls in the core complex of higher plants. The PS I core complexes of different cyanobacteria are known to differ in the number and spectroscopic properties of the red Chl forms (for a review, see Gobets and Van Grondelle, 2001). In *T. elongatus*, 2 (or 3) different red absorption forms were detected, with absorption maxima at 719 nm, (715 nm) and 708 nm (Palsson et al., 1996). The core complex of higher plants also contains red Chl forms, as can be concluded from the presence of the emission at 720 nm (Croce et al., 1998), but it does not contain the Chl form absorbing at 719 nm and emitting around 730 nm (Palsson et al., 1996). The comparison of the geometrical organization of the putative red Chls in *T. elongatus* and in pea reveals that in most cases the location and orientation of the pigments is maintained. One clear difference can be observed at the level of the Chl trimer associated with PsaB in positions B31-B32-B33 (nomenclature of Jordan et al., 2001). For higher plants only two of these chlorophylls overlap with those of cyanobacteria, while the third one (B33) is located further apart and shows a different orientation (Amunts et al., 2007). Because *T. elongatus* shows an absorption form at 719 nm, which is not present in higher plants it can be suggested that this forms originates from the B31-B32-B33 trimer.

Several studies are available about the energy transfer and trapping process in the core complex of several cyanobacteria and higher plants (see (Gobets and Van Grondelle, 2001; Melkozernov et al., 2006)). Two main models have been put forward; one describes the system as completely trap-limited with very fast excitation energy transfer from the antenna to the reaction center (Melkozernov et al., 2000; Muller et al., 2003). In the second model the migration time is shown to contribute significantly to the kinetics (Byrdin et al., 2002; Gobets et al., 2003a). This second model is more in line with the structure in which it can be seen that the reaction center Chls are partially isolated from the rest, thus suggesting a slow transfer step to the trap. However, both models can satisfactorily describe the experimental data. Additional experiments and the use of mutants partially impaired in the energy transfer process are thus necessary to clarify this point.

The comparison of the pigment organization in PS I of pea and *T. elongatus* reveals a very high degree of conservation similar to the homology in the protein part already discussed above. However, the spectroscopic properties of the PS I complexes in the different organisms differ considerably, due the high tunability of the properties of the pigments by the environment, meaning that

even very small changes in the environment can produce large differences in the energy levels of the pigments. This is clearly the case for the red Chl forms, the energy of which is quite different in PS I complexes from different organisms. The final result is that PS I complexes from different organisms, although showing a very high conservation of their structure, differ in their excitation energy transfer and trapping processes, which have been demonstrated to be strongly influenced by the red Chl forms (Gobets and Van Grondelle, 2001; Ihalainen et al., 2005).

III. Photosystem II

The PS II complex is a water/plastoquinone oxidoreductase located in the thylakoid membrane of plants and cyanobacteria (see Wydrzynski and Satoh, 2005). In higher plants it is composed of two moieties, the core complex, which coordinates all the cofactors of the electron transport chain and several Chls, and the outer antenna system, located around the core and composed of members of the Lhc family. In cyanobacteria only the core complex is present, while the antenna function is fulfilled by the phycobilisomes associated with the complex on the cytoplasmic side of the membrane. The core is a multiprotein complex composed of around 20 subunits and it is present in the membrane as a heterodimer both in higher plants and cyanobacteria. Several crystallographic models of PS II from cyanobacteria with resolution ranging from 3.8 to 2.9 Å are now available (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005; Guskov et al., 2009), while for the core complex from higher plants, only a 5.5 Å projection map is available determined by electron crystallography (Buchel and Kuhlbrandt, 2005). However, due to the high sequence similarity between the main proteins of the higher plant and cyanobacterial cores, it is believed that the model for the cyanobacterial core is also representative of the core complex of higher plants (see below).

A. Core Complex of Cyanobacteria

The structure of PS II from *Thermosynecococcus elongatus* at 3.0 Å resolution (Loll et al., 2005) shows the location of 35 Chl *a*, 2 pheophytin *a* (Pheo), 2 plastoquinones and 11 β-carotenes per monomer in addition to the Mn clusters and other cofactors. The core is composed of four large integral membrane proteins, the products of the *psbA-psbD* genes, which together contain 22 membrane-spanning helices and coordinate all the Chls present in the complex. A number of small subunits account for 14 additional transmembrane helices (see Table 6.2). Several excellent reviews that summarize the structural results of PS II have been published (Barber, 2002; Minagawa and Takahashi, 2004; Muh et al., 2008), and the reader may consult these reviews for details about the localization and the possible functions of the small subunits.

The products of the *psbA* and *psbD* genes (complexes D1 and D2, respectively) form a heterodimer which coordinates 6 Chls, 2 Pheos and 2 plastoquinones, most of which are participating in the electron transport chain. Both proteins are composed of 5 transmembrane helices. On the periplasmic side (lumenal side in higher plants) D1 provides most of the ligands for the Mn cluster which oxidizes water (Barber, 2006). The cofactors of the electron transport chain are organized in two symmetric branches, with the two Chls forming the "special pair" being coordinated with His 198 of D1 (P_{D1} Chl 1) and His 197 of D2 (P_{D2} Chl 2) near the lumenal side of the membrane. Two additional Chls (Chl $_{D1}$ or Chl 3 and Chl $_{D2}$ or Chl 4), probably coordinated to the protein via water molecules, are located in close proximity of the "special pair". It seems that in PS II all four of these Chls interact to form the reaction center (Durrant et al., 1995) and that Chl_{D1} is the primary electron donor and $Pheo_{D1}$ the primary electron acceptor as shown by experimental measurements and theoretical calculations (Prokhorenko and Holzwarth, 2000; Diner et al., 2001; Barter et al., 2003; Groot et al., 2005; Holzwarth et al., 2006). Two other Chls (ChlzD1 and Chlz D2) are coordinated by His 118 of D1 and His 117 of D2 and they are probably involved in energy transfer from the inner antenna chlorophyll *a*-binding complexes CP43 and CP47 (Ruffle et al., 2001; Wang et al., 2002). PsbE and PsbF (the two subunits of cytochrome b_{559}, which play a protective role against photoinduced damage of the reaction center) are tightly bound to the D1/D2 heterodimer with which they form the reaction center complex. The *psbB* and *psbC*

Table 6.2. Protein composition of Photosystem II core in cyanobacteria and higher plants

Subunits (Psb)	Name	P and C	MW kDa	TMH	Pigments	Function
A	D1	Y	38.3	5	3 Chl *a*, 1 Pheo *a*, β-car	RC
B	CP47	Y	56.6	6	16 Chl *a*, β-car	Light harvesting
C	CP43	Y	51.6	6	13 Chl *a*, β-car	Light harvesting
D	D2	Y	39.4	5	3 Chl *a*, 1 Pheo *a*, β-car	RC
E	Cyt*b*559a	Y	9.5	1	Heme	ET, photoprotection
F	Cyt*b*559b	Y	5.1	1	Heme, β-car	ET, photoprotection
H		Y	7.3	1	β-car	Assembly/stabilization
I		Y	4.4	1	β-car	Assembly/stabilization
J		Y	4.1	1	β-car	Assembly
K		Y	4.1	1	β-car	Stabilization
L		Y	4.3	1	β-car	Stabilization
M		Y	4.0	1	β-car	Stabilization
O	OEC 33 kDa	Y	30	–	–	Mn stabilization
P	OEC 23 kDa	Only P	23	–	–	Inding Ca and Cl
Q	OEC 16 kDa	Only P	17	–	–	O_2 evolving enhancer
R		Only P	10	–	–	?
PsbS		Only P	22	4	–	Photoprotection
PsbTn		Only P	5	1	–	PS II repair
PsbT		Y	3.9		–	Repair of photodamage
PsbU		Only C	11.6	–	–	Stabilization of Mn cluster
PsbV		Only C	15.1	–	–	Stabilization of Mn cluster
PsbW		Only P	6.1	1	–	Dimerization?
PsbX		Y	4.3	1	–	Stabilization of Chl z
PsbY		Y	–	1	–	?
PsbZ		Y	6.8	2	β-car	Stabilization supercomplex

P higher plants, *C* cyanobacteria, *MW* molecular weight, *TMH* transmembrane helices, *Y* yes, *RC* reaction center, *ET* energy transfer

encode the two inner antenna complexes known as CP47 and CP43 with the former being located on the side of D2 and the latter adjacent to D1. The crystal structure, published by Loll et al. (2005) shows that CP47 coordinates 16 Chls and CP43 coordinates 13. Most of the Chls in the two complexes are related by a twofold symmetry. Out of the 16 Chls of CP47, 13 are coordinated by a His residue, one by a lipid molecule, one by the backbone carbonyl group of a Trp and the last one possibly via a water molecule. In CP43, 10 His, 1 Ser, 1 Asn and one lipid molecule are involved in Chl coordination (Loll et al., 2005). In addition to the Chls, these two core antenna proteins accommodate 5 (CP47) and 4 (CP43) β-carotene molecules, respectively.

None of the small subunits of the core complex is directly involved in Chl binding, although one of the residues of PsbH and one of PsbI forms an H bond with a Chl coordinated to CP47 and D1, respectively (Muh et al., 2008). Most of the small subunits seem to be involved in the assembly and stabilization of the complex although the effect of their absence is sometimes different in cyanobacteria, higher plants and green algae (Minagawa and Takahashi, 2004) indicating their slightly different roles.

B. Core Complex: Cyanobacteria Versus Higher Plants

The main difference in the protein composition between cyanobacteria and higher plants is related to the extrinsic proteins of the periplasmic (lumenal in higher plants) side, which are involved in the stabilization of the water-splitting cluster. Although PsbO is conserved in all oxygenic photosynthetic organisms, the cyanobacterial core contains two proteins, PsbU and PsbV, which in higher plants are substituted by PsbP and PsbQ as

part of the oxygen-evolving complex (Nield et al., 2000; Roose et al., 2007). The comparison of the primary structure of the conserved proteins of cyanobacteria and higher plants indicates a very high degree of identity, thus suggesting that these structures are similar. The comparison of the X-ray structure of *T. elongatus* with the 5.5 Å projection map of spinach obtained by electron crystallography (see Buchel and Kuhlbrandt, 2005 for full details) largely supports this view. However, although the structure of the individual complexes seems to be highly conserved, differences are observed in their relative orientation. The major difference was detected in the position of cytochrome b_{559} that in spinach is 4 Å away from its position in cyanobacteria. The position of CP47 with respect to that of the D1/D2 heterodimer is also slightly changed and even the relative orientation of D1 and D2 seems to be different in higher plants as compared to cyanobacteria. Interestingly, the major variations in the organization are observed at the stromal side of the membrane, a region that in vivo has distinct functions in cyanobacteria and in higher plants, due to the binding of phycobilisomes in the former, while in higher plants this region is in close contact with another PS II complex of the adjacent layer of the membrane. On the other hand, a comparison of the pigment organization in the two systems shows that the relative distances between the cofactors involved in the electron transport chain seem to be conserved. However, it is very important to keep in mind that the system is finely tuned and that even very small differences in the environment, especially around the cofactors of the electron transport chain, can influence performance. A clear example of this is the presence of a glutamic acid or a glutamine at position 130 of the D1 protein which modulates the redox potential of pheophytin (Merry et al., 1998; Kos et al., 2008). This indicates that, although the overall structure is conserved, extrapolation of the results obtained on the cyanobacterial system to higher plants requires some caution. This is particularly important considering that most of the studies on energy transfer and charge separation in the system were carried out on the PS II core of cyanobacteria and on the reaction center complex of higher plants, which can be slightly different, especially in the free energy of the primary radical pair.

C. Photosystem I Versus Photosystem II

The comparison of the structures of monomeric PS I and PS II shows a clear difference in the peripheral subunits, which extend into the stroma in the case of PS I and into the lumen for PS II. However, looking at the transmembrane region, a high similarity can be observed between the organization of the D1/D2/CP47/CP43 proteins in PS II and the PsaA/PsaB core in PS I, which supports the view that they evolved from a common ancestor (Blankenship, 1992). The small membrane proteins of the two supercomplexes do not share structural similarity, suggesting that they have been added to the complexes later during evolution (Grotjohann et al., 2004). The organization of the ten central helices (belonging to D1 and D2 in PS II and to the C-terminal domain of PsaA and PsaB in PS I) is highly conserved. High similarity can be observed also between the 6 helices of CP47 and CP43 and the 6 helices of the N-terminal domain of PsaA and PsaB (Schubert et al., 1998). As far as the pigment organization is concerned, two major differences exist between the photosystems: PS I has the inner antenna "fused" to the reaction center, which means that most of the Chls of the core are coordinated with PsaA and PsaB, which also coordinate the reaction center Chls. In PS II the Chls responsible for light harvesting are coordinated by CP43 and CP47, while the cofactors of the electron transport chain are coordinated by D1 and D2. This difference is probably related to the need for higher flexibility for PS II when compared to PS I, especially concerning the photoprotective mechanisms and the requirement of a high turnover of the D1 protein, which gets damaged due to photoinhibition and needs to be repaired and re-integrated in the system (Aro et al., 1993). The fact that D1 coordinates only few Chls thus has an advantage for when the complex is disassembled because it reduces the risk of having too many free Chls in the membrane which can form triplets and react with molecular oxygen, thus producing oxidative species.

A second clear difference can be seen in Fig. 6.3 where the pigments of PS I and PS II are superimposed: the pigment density of PS I is far higher than that of PS II, since the two supercomplexes coordinate 100 and 35 Chl molecules, respectively, in a very similar volume. However, this

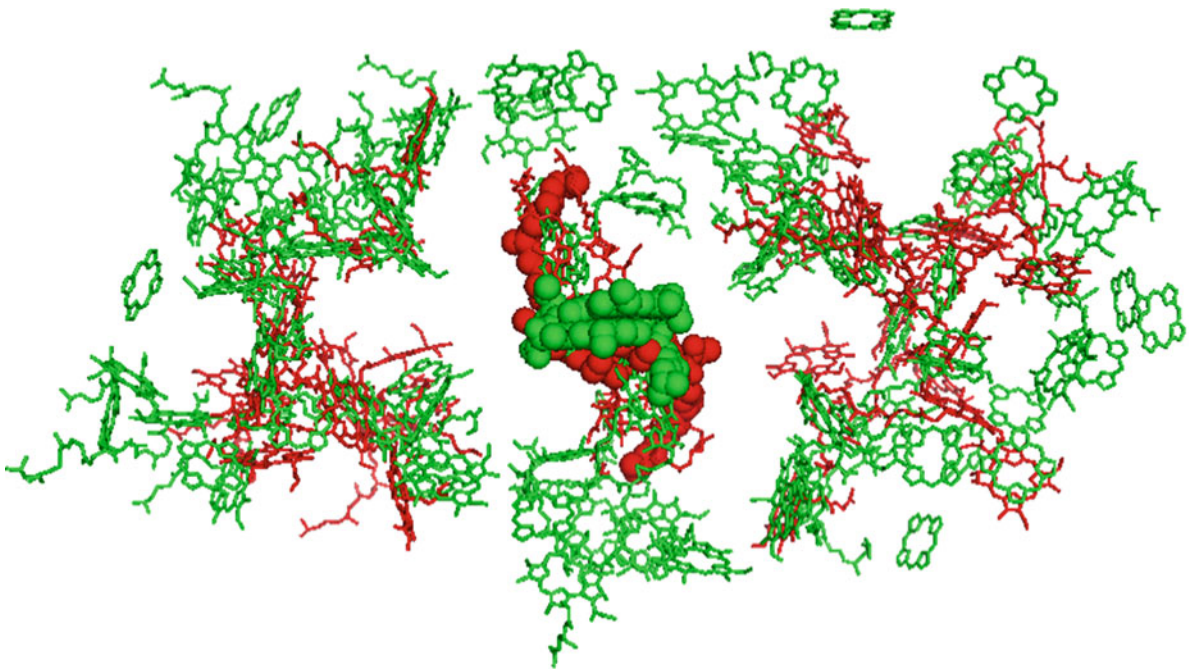

Fig. 6.3. Comparison of the Chl organization in PS I (*green*, PDB 1JB0) and PSII (*red*, PDB 2AXT) monomeric cores of cyanobacteria (The figure was created with Pymol).

difference is mainly related to the light-harvesting pigments, while the Chls, which make up the reaction center are arranged in a more similar way. Interestingly, in both cases the distance between the reaction center Chls and the antenna Chls is quite large and only a few pigments connect the two systems suggesting that in both cases there are preferential pathways for energy transfer from the antenna to the trap. The presence of a structural gap between the antenna and the reaction center Chls suggests that the transfer to the trap can be a limiting factor in the overall trapping time, although at the moment there is no agreement on this issue (see also below).

IV. The Outer Antenna Complexes

In both higher plants and cyanobacteria the core complexes of PS I and PS II are associated with outer antenna systems whose primary role is to enlarge the absorption cross section for light harvesting, increasing excitation energy transfer to the reaction center, where it can be used for charge separation (conversion of excitonic energy to chemical energy). A second role of these complexes is to protect the plant against high light damage via a series of photoprotective mechanisms (Horton and Ruban, 2005). In higher plants, the outer antenna system is composed of integral membrane Chl-binding proteins belonging to the *Lhc* multigene family (Jansson, 1999). In cyanobacteria, the outer antenna system is composed of giant soluble macromolecular structures called phycobilisomes, attached to the cytoplasmic surface of the membrane and containing phycobilins as chromophores (Mimuro and Kichchi, 2003). However, in several cyanobacterial species an additional antenna system composed of Chl *a*/*b*/*d*-binding membrane proteins is present, probably in response to a different quality of the available light (Stomp et al., 2007). In the following sections the Chl-binding outer antenna proteins will be discussed.

A. Outer Antenna of Higher Plants: The Lhc Multigene Family

The Lhc proteins of the outer antenna system of higher plants coordinate Chl *a*, Chl *b* and xanthophylls in different amounts. Six genes are responsible for encoding the antenna complexes

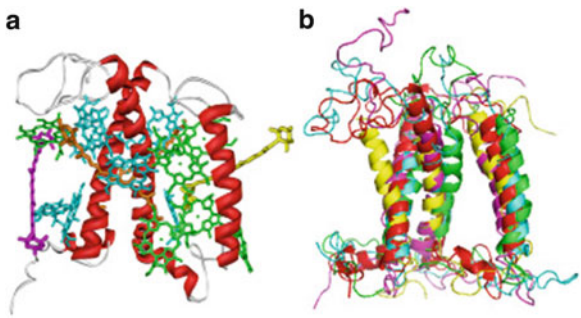

Fig. 6.4. Structure of Lhc complexes. (**a**) Structure of LHCII (**b**) Comparison of the apoprotein moiety of different members of the Lhc family for which the structure is available: LHCII (*red*), Lhca1 (*cyan*), Lhca2 (*magenta*), Lhca3 (*yellow*) and Lhca4 (*green*) (The figure was created with Pymol).

of PS II (*Lhcb1-6*) and six for that of PS I (*Lhca1-6*) (Jansson, 1999), but *Lhca5* codes for a protein present in small amounts in the thylakoid membrane (Ganeteg et al., 2004; Storf et al., 2005) and *Lhca6* is probably a pseudo-gene of *Lhca2*. Two more genes (*Lhcb7-8*) have been suggested to code for additional antenna complexes of PS II (Klimmek et al., 2006), but their products have never been characterized.

The aggregation state of these complexes differs substantially: LHCII is present in the membrane as a trimer, while CP24, CP26 and CP29 (respectively, the products of the genes *Lhcb6, Lhcb5 and Lhcb4*) are present as monomers (Dainese and Bassi, 1991). Lhca complexes form two heterodimers, Lhca1-Lhca4 and Lhca2-Lhca3 (Croce and Bassi, 1998), although the latter one has never been purified to homogeneity.

The structure of trimeric LHCII, the major antenna complex of PS II, the product of the *Lhcb1-3* genes, has been obtained at high resolution (Liu et al., 2004; Standfuss et al., 2005). The monomeric complex shows three transmembrane helices and two amphipathic helices and the position of 14 Chl molecules (8 Chl *a* and 6 Chl *b*) and 4 xanthophylls (neoxanthin, lutein and violaxanthin) (Fig. 6.4). Most of the Chls are coordinated with nucleophilic amino acids, namely 2 His, 1 Asn, 1 Gln and 3 Glu. A few other Chls are coordinated via water molecules and two by the carboxylic group of the amino acids contributing to the backbone. The last one is coordinated by a lipid molecule. The binding of all Chl *b*, with one exception, is stabilized by an H bond via a formyl group (Liu et al., 2004). Interestingly, it has been shown that several of the binding sites can accommodate both Chl *a* or Chl *b* not only upon in vitro reconstitution (Giuffra et al., 1997) but also in vivo, depending on the availability of Chl *b* (Hirashima et al., 2006). It is more difficult to obtain information about the binding of the carotenoids, which are probably bound to the protein via hydrophobic interactions. However, in the case of neoxanthin, bound to the external site N1 (Croce et al., 1999), the structure shows the presence of an H-bond with a Tyr of the lumenal loop of the protein (Liu et al., 2004; Hobe et al., 2006). Mutation of this residue indicates that this H-bond has the effect of stabilizing binding (Caffarri et al., 2007), but its absence leaves in place most of the neoxanthin thus indicating that other interactions play a major role in the stabilization of this carotenoid. Additionally, the binding sites are quite selective for the different xanthophylls, but the molecular basis for this selectivity has not yet been fully elucidated.

Sequence analysis shows high similarity between the members of the Lhc family thus suggesting a similar structural arrangement (Green and Kühlbrandt, 1995). Moreover, it also shows that the amino acids which are Chl-binding residues in LHCII are conserved in all members of the family, with only few exceptions (Bassi et al., 1997). Mutation analysis of these putative Chl-binding residues has confirmed that they are indeed all Chl ligands in the antenna complexes analyzed so far, including the four Lhca complexes (Bassi et al., 1999; Remelli et al., 1999; Rogl and Kühlbrandt, 1999; Yang et al., 1999; Morosinotto et al., 2002; Croce et al., 2004; Morosinotto et al., 2005b; Mozzo et al., 2006). Moreover, the structure of LHCII has shown that two ionic bridges involving Glu residues and Arg residues located in the two central helices stabilize the structure and in addition provide the ligands for two Chl molecules (Kühlbrandt et al., 1994). Mutations of these residues confirmed this suggestion showing that the ionic bridge between the Glu of the third helix and the Arg of the first one is strictly necessary for the folding not only of LHCII but of all Lhc complexes.

The structure of the four antenna complexes of PS I has been resolved at an intermediate resolution and although it does not show all the molecular details, it confirms the presence of the three

transmembrane helices that are characteristic of the general folding of Lhc proteins (Fig. 6.4) (Amunts et al., 2007) in agreement with the results of the mutation analysis (Mozzo et al., 2006). The structure provides the positions of the 13–14 Chls (depending on the complex), which are coordinated with each Lhca complex. However, the details of the structure should be taken with certain caution, due to the lower resolution obtained for the Lhca region as compared to the core part. For example, only a few of the residues, which have been shown to be Chl-binding residues by mutation analysis (Morosinotto et al., 2002; Croce et al., 2004; Morosinotto et al., 2005b; Mozzo et al., 2006), are located in the vicinity of the central magnesium of the Chl which they are proposed to coordinate, while most of them are far apart from their ligands and in several complexes, the Glu/Arg bridges, which stabilize the structure, are not apparent from the structure. A higher resolution structure is thus needed to perform calculations for excitation energy transfer in these complexes and to study in detail the effect of the environment on the spectroscopic properties of the chromophores.

Despite the structural similarity, the individual antenna complexes show different biochemical and spectroscopic properties. Part of this difference depends on a different pigment composition: although all complexes coordinate Chl *a*, Chl *b*, lutein and violaxanthin, their relative amounts differ for the different subunits (Bassi et al., 1997; Castelletti et al., 2003). In addition, neoxanthin was found to be bound only to the antenna complexes of PS II with the exception of Lhcb6, while β-carotene is only present in the antenna complexes of PS I (Bassi et al., 1993; Croce et al., 2002b), thus indicating a different affinity of the binding sites for the carotenoids, not a different availability of the pigments, as demonstrated by in vitro reconstitution performed in the presence of all pigments (Sandona et al., 1998; Castelletti et al., 2003). The molecular origin of this difference is unknown at the moment. The pigment binding of most of the complexes was studied by mutation analysis. Mutations of the putative Chl-binding residues followed by in vitro reconstitution (Plumley and Schmidt, 1987) leads to the production of complexes lacking individual chromophores, allowing the characterization of each chromophore in each binding site (Bassi et al., 1999; Remelli et al., 1999; Rogl and Kühlbrandt, 1999; Yang et al., 1999; Morosinotto et al., 2002; Croce et al., 2004; Morosinotto et al., 2005b; Mozzo et al., 2006). This analysis reveals that the biochemical and spectroscopic properties of Chls in several of the binding sites are conserved across the Lhc family. For example, the four Chl-binding sites located in the center of the molecule (602, 603, 610 and 612, nomenclature according to (Liu et al., 2004)) accommodate Chl *a* in all antenna complexes. On the other hand, the domain including helix C has a higher tendency to coordinate Chl *b*, although the occupancy by Chl *a* or *b* of most of the sites varies in the different complexes. This flexibility to bind Chl *a* or Chl *b* depends at least in part on the possibility to form H bonds, which can favor the binding of Chl *b*. In particular, near the C helix the structure of LHCII reveals a network of H bonds which also involves water molecules which act as ligands for several of the Chls in this region (Liu et al., 2004). LHCII has a Gln residue in position 131 which forms an H bond with Chl 607 and Chl 609, which are indeed Chl *b* in this complex, and when this Gln is changed into a Glu, the site becomes partially occupied by Chl *a* (Bassi et al., 1999; Remelli et al., 1999). In most of the other complexes, which coordinate less Chl *b* than LHCII, this Gln is naturally substituted by a Glu residue (Jansson, 1999) which partially disrupts the H-bonding network.

However, the most striking difference between Lhcb and Lhca complexes is the presence of red Chl absorption forms in the latter ones (Haworth et al., 1983). While Lhcb proteins show an emission maximum at 680 nm, all Lhca proteins have emission bands above 700 nm at low temperature, Lhca3 and Lhca4 even emit at 726 nm and 730 nm, respectively (Schmid et al., 1997; Croce et al., 2002b; Schmid et al., 2002; Castelletti et al., 2003), 50 nm more to the red as compared to the antenna systems of PS II and Lhca1 and Lhca2 have an emission band at 702 nm (Castelletti et al., 2003). The tuning of the spectroscopic properties of the Chls is due to interactions with the protein scaffold and with neighboring pigments (Croce et al., 2007). In the case of the red Chl forms it has been shown that the interaction between Chl 603 and Chl 609 is at the origin of the low-energy absorption. The difference in

energy between the red absorption of the individual complexes is mainly tuned by the ligand of Chl 603 (an Asn in Lhca3 and Lhca4 and an His in all other complexes), which helps in modulating the distance between the two interacting pigments and thus the strength of the interaction (Morosinotto et al., 2003). This demonstrates that very small structural differences can lead to major differences in the spectroscopic properties of the pigments. The Lhc family thus represents an excellent system to study the characteristics of the binding sites, to understand both the molecular basis of the affinity of the sites for different pigments and to determine the effect of the protein environment on the spectroscopic properties of the chromophores. A large set of biochemical and spectroscopic data is already available for these complexes and the future possibility to compare high-resolution structures of several members of the family might provide the necessary information for elucidating the effect of the environment on the spectroscopic properties of the chromophores.

B. Accessory Light-Harvesting Complexes of Cyanobacteria

The name CBP (Chlorophyll Binding Protein) has been introduced (Chen et al., 2008) to define a class of cyanobacterial proteins containing the prochlorophyte chlorophyll-binding protein complexes (Pcb) and the iron-stress-induced protein A (IsiA). Pcbs were first found in prochlorophytes, a special group of cyanobacteria (Lewin, 2002) which do not have phycobilisomes and they use these integral membrane proteins as major accessory antenna. The presence of antenna complexes coordinating Chl *a* and *b* (and *d*) instead of phycobilins is an adaptation to the light quality in the niche where these organisms live, which is depleted of the green light absorbed by the phycoblilins and enriched in blue light (Stomp et al., 2007). The IsiA complex (also known as CP43') is present in cyanobacteria under Fe-stress conditions (Burnap et al., 1993) and it forms a ring around PS I (Bibby et al., 2001; Boekema et al., 2001a). Pcb and IsiA are part of a large family which also includes the Chl *a* antenna complexes of the core of PS II, CP43 and CP47. They have six transmembrane helices, and can coordinate different types of Chls like *a*, *b* and *d*, depending on the organism and the strain (Partensky et al., 1999; Chen et al., 2005c). The major difference with CP43 and CP47 is the absence of the long lumenal E loop located in between helices V and VI. This difference is possibly related to the fact that the E loops of CP43 and CP47 have the role of stabilizing the oxygen-evolving complex (Eaton-Rye and Putnam-Evans, 2005; Ferreira et al., 2004), while this is not necessary in the case of the other complexes (Murray et al., 2006). Sequence alignment of Pcb and IsiA with CP43 shows that almost all the histidines which are Chl-binding residues in CP43 are conserved in both Pcb and IsiA, suggesting that the pigment organization is very similar in all cases. Due to the presence of an extra His in helix III, these complexes coordinate an additional Chl as compared to CP43 (Murray et al., 2006). Other *pcb*-like genes have been found in the genomes of several cyanobacteria, demonstrating that they are not limited to the prochlorophytes but that they are widely distributed throughout the cyanobacteria (Geiss et al., 2001).

V. The Chlorophyll of the Cytochrome b_6f Complex

The "last" chlorophyll present in the thylakoid membrane was surprisingly found to be associated with the cytochrome b_6f complex of plants, algae and cyanobacteria (Bald et al., 1992; Huang et al., 1994; Pierre et al., 1995). This Chl does not seem to play any clear role in the complex and moreover it is not present in the homologous complex of mitochondria. Pierre et al. (1997) concluded that most probably it is a relic of evolution. It is associated with subunit IV but the structure does not show a clear ligand, suggesting that it is coordinated via a water molecule (Kurisu et al., 2003; Stroebel et al., 2003). The presence of a Chl in the membrane can lead to the formation of singlet oxygen, a highly oxidative species. It was, however, shown that the lifetime of its excited state is only 200 ps versus 6 ns of a Chl in solution (Peterman et al., 1998; Dashdorj et al., 2007), thus strongly reducing the possibility of the formation of triplets. Moreover, a carotenoid molecule is also present in the structure and although located at a distance that direct triplet

quenching is prohibited, it is still a very effective quencher, possibly via the involvement of an oxygen molecule (Kim et al., 2005).

VI. Assembly of the System: Supercomplexes of Cyanobacteria and Higher Plants

In higher plants the core complexes of both the photosystems are surrounded by the outer antenna complexes forming what can be considered to be a modular system. Indeed, under different environmental and stress conditions the type and the amount of the antenna complexes can vary to allow the maintenance of an optimal photosynthetic activity.

A. Photosystem II-LHCII Supercomplex: A Flexible System

The supercomplex of PS II is composed of a dimer of the core which is surrounded by the Lhcb complexes, the amount of which can vary under different growth conditions of the organism (Melis, 1991). The basic unit is composed of a dimer of the core, 4 LHCII trimers and 2 monomeric Lhcb4, Lhcb5 and Lhcb6 complexes; these constitute the $C_2S_2M_2$ supercomplex (Boekema et al., 1999a), although smaller (C_2S_2) and larger (C_2S_2 M_2L) supercomplexes have also been observed. The two LHCII trimers present per monomeric core differ in location, subunit composition and interaction strength (S = strongly bound; M = moderately bound) within the supercomplex. The trimer (S) is located on the Lhcb5 side; it is composed of the products of the *Lhcb1* and *Lhcb2* genes and it is strongly connected with the core. The trimer (M) is attached to the supercomplex on the side of Lhcb4 and Lhcb6. In addition to Lhcb1 and Lhcb2 it also contains the product of the gene *Lhcb3*. A complex composed of Lhcb4, Lhcb6 and the trimer (M) can be purified under mild detergent conditions (Bassi and Dainese, 1992), indicating that the interactions between these subunits are rather strong. In spinach, an additional trimer (L = loosely bound) has been observed in some of the PS II supercomplexes (Dekker and Boekema, 2005). The analysis of the grana membrane reveals that up to 4 LHCII trimers can be present per core monomer (Dainese and Bassi, 1991). It is possible that there are regions of the membrane which are enriched in LHCII complexes in oligomeric form. Such oligomers (heptamers) have been observed by electron microscopy (Dekker et al., 1999).

Cross-linking experiments and analysis of mutants lacking individual subunits have shown that Lhcb4 is located near CP47, while Lhcb5 is near CP43 (Harrer et al., 1998; Yakushevska et al., 2001). In the antisense mutants of Lhcb4, no PS II-LHCII supercomplexes could be found upon mild detergent solubilization, indicating that CP29 has a crucial role in the assembly and stability of the supercomplex (Yakushevska et al., 2003). On the contrary, the absence of Lhcb5 does not seem to have any effect on the assembly, in agreement with the fact that PS II-LHCII complexes lacking Lhcb5 were also found in wild-type plants (Boekema et al., 1999b; Yakushevska et al., 2001). Lhcb6 is present only in the $C_2S_2M_2$ complexes, which are, however, formed only when Lhcb6 is present (Kovacs et al., 2006), thus confirming the interactions between this subunit and the trimer (M).

To obtain the details of the interactions and the relative orientation of the individual subunits, a higher resolution map of the supercomplex is necessary. It is, however, clear that the interactions between the subunits are not particularly strong as demonstrated by the fact that the supercomplexes disassemble upon very mild detergent treatment, under conditions in which PS I-LHCI remains intact (Ballottari et al., 2004), indicating that the strength and/or the nature of the interactions between the core and the Lhc complexes differ for PS I and PS II. It is well known that the amount of antenna complexes varies when plants are grown under different conditions. For example, in low light the amount of LHCII strongly increases, while it decreases in high light (Anderson and Andersson, 1988; Pfannschmidt, 2003). It is not clear at present where the additional LHCII subunits are located and electron microscopy studies of the supercomplex of plants grown under different conditions are necessary.

B. Photosystem I-LHCI Supercomplex: A Less Flexible System

Electron microscopy analyses of higher plant PS I have shown that the Lhca complexes are located on one side of the core, near subunits PsaF and

PsaG (Boekema et al., 2001b). Subsequently, the X ray structure has confirmed this finding, showing the location of the four complexes (Ben-Shem et al., 2003). The sequence of the complexes, Lhca1-Lhca4-Lhca2-Lhca3 going from the G-pole (PsaG side) to the K-pole (PsaK side) was based on biochemical evidence and was later confirmed by the new higher resolution structure, where the bulky aromatic amino acids could be used for fitting the electronic density (Amunts et al., 2007). Biochemical data showed that Lhca complexes are organized as functional dimers: Lhca1-4 and Lhca2-3 (Jansson et al., 1996; Ihalainen et al., 2000). The dimerization is mediated by small contact surfaces at the lumenal and stromal sides of the membrane, while there are no interactions between the transmembrane helices (Ben-Shem et al., 2003), although mutation analysis suggests that helix II of Lhca4 also plays an important role in dimerization (Corbet et al., 2007). Additionally, the interactions between the Lhca proteins and the subunits of the core complex appear to be weak according to the structure. The strongest one is between the Lhca1 and the PsaG proteins, while Lhca4 interacts with PsaF, Lhca2 with PsaJ and Lhca3 with PsaA (Amunts et al., 2007). Analysis of mutants lacking individual subunits indicates that the binding of Lhca4 plays a key role for the connection of the outer antenna complexes to the core: in the absence of Lhca4 a large part of the antenna does not assemble (Klimmek et al., 2005; Morosinotto et al., 2005a). On the other hand, the presence of Lhca2 is necessary to stabilize the binding of Lhca3, which is partially lost in the PS I preparations from Lhca2 antisense plants (Ganeteg et al., 2001). This is reasonable when we take into account that Lhca2 and Lhca4 are stabilized by interactions with the core subunits and with two other Lhcas, while the binding of Lhca1 and Lhca3, which are located at the periphery of the half-ring, is only stabilized by interactions with the core subunits and with one Lhca complex, thus making their association with the supercomplex less tight. Interestingly, the structure shows that Lhca1 interacts with both PsaG and Lhca4 (Ben-Shem et al., 2003), but analysis of the knockout mutants of these two subunits indicates that Lhca1 is only lost in the mutant lacking Lhca4 (Jensen et al., 2002; Klimmek et al., 2005), suggesting that the interactions between the complexes in the Lhca1-Lhca4 heterodimer are stronger than the interactions between Lhca1 and the core subunit. Despite the fact that the structure points to weak interactions between the core and the antenna, the PS I-LHCI supercomplex is very stable and it does not fall apart upon detergent treatment, as is the case for PS II (Ballottari et al., 2004) (see below). This high stability seems to correlate with the fact that the antenna composition of PS I does not vary under different environmental conditions, at variance with the composition of PS II (Ballottari et al., 2004). Moreover, Lhca complexes are not interchangeable: in mutants in which one of the subunits is not expressed, its position is not taken over by any of the other Lhcas (Ganeteg et al., 2001; Klimmek et al., 2005). This again is different from PS II for which it has been shown that in the absence of Lhcb1 and Lhcb2, the principal component of the LHCII trimers, CP26 is overexpressed and forms trimers which are organized exactly as the LHCII trimers in the wild type (Ruban et al., 2003).

One of the biggest surprises of the plant PS I structure was the presence of "gap" and "linker" Chls located between the Lhca half-ring and the core and between the individual Lhcas (Ben-Shem et al., 2003). The binding of these chromophores is apparently stabilized by interactions between the outer antenna and the core, since they are lost when the antenna is detached from the core (Ballottari et al., 2004). Klimmek et al. (2005) have proposed that these Chls facilitate the energy transfer from the antenna to the reaction center.

Under low-light conditions a mechanism known as the state transition balances the absorption cross section of the two photosystems (Allen, 1992; Wollman, 2001; Kanervo et al., 2005). The state transition involves the phosphorylation of Lhcb1 and Lhcb2 by a membrane-bound protein kinase (Bellafiore et al., 2005). The phosphorylated complexes then dissociate from PS II in the grana stacks, and diffuse to the stroma lamellae where they associate with the PS I complexes on the side of PsaH, forming the PS I-LHCI-LHCII supercomplex (Lunde et al., 2000; Kouril et al., 2005).

C. Supercomplexes in Cyanobacteria: PS I-Pcb, PS II-Pcb and PS I-IsiA

The proteins, PS I-Pcb, PS II-Pcb and PS I-IsaA, can act as a light-harvesting system for both PS I and PS II. The IsiA and Pcb proteins form a ring

composed of 18 different subunits around trimeric PS I (Bibby et al., 2001; Boekema et al., 2001a; Bumba et al., 2005; Chen et al., 2005a). Yeremenko et al. (2004) have shown that that after prolonged growth of *Synechocystis* sp. PCC 6803 in an iron-deficient medium, the number of bound IsiA proteins increases: the largest complexes bind 12–14 units in an inner ring and 19–21 units in an outer ring around a PSI monomer. In mutants depleted in PsaF and PsaJ, a ring of 17 IsiA subunits was observed (Kouril et al., 2003). The supercomplexes show tight interaction between the subunits in the antenna rings, but only few connections with the PS I trimer (Melkozernov et al., 2006). However, spectroscopic data show that the energy transfer from the ring to the reaction center occurs in around 2 ps, indicating that IsiA is an efficient antenna system (Melkozernov et al., 2003; Andrizhiyevskaya et al., 2004). Pcb proteins act as antenna complexes of PS II: a giant supercomplex composed of a core dimer with a flanking region accommodating four or five Pcb proteins along each edge was observed (Bibby et al., 2003a; Bibby et al., 2003b; Chen et al., 2005b). The addition of Pcb increases the absorption cross section of the PSII reaction center core by almost 200%.

VII. Chlorophyll-Binding Proteins: The Right Pigment in the Right Site — Selectivity of the Pigment-Binding Site or Control of Pigment Availability

The core complexes of PS I and PS II coordinate Chl *a* molecules in both cyanobacteria and higher plants. In plants, Chl *b* is never found in the core complexes of wild-type plants. However, Chl *b* has been found associated with the core complexes in a cyanobacterial mutant transformed with the chlorophyll *a* oxygenase (CAO) gene (Satoh et al., 2001; Xu et al., 2001b and in a *Arabidopsis thaliana* mutant transformed with the CAO from *Prochlorothrix hollandica* (Hirashima et al., 2006) which lacks the regulatory domain and thus produces more Chl *b* than in higher plants. The presence of Chl *b* in the core complexes of these mutants suggests that the availability of Chl *b* is the principal requisite for its binding. Moreover, it was shown by Ikegami et al. (2007) that in the core of PS I the Chl-binding sites have the same affinity for Chl *a* and Chl *b*, suggesting that there is no Chl *b* available during the folding of the core complexes in vivo. Regarding the outer antenna complex, it is clear that Chl *b* is necessary for the correct assembly of LHCII and of several Lhc complexes (Plumley and Schmidt, 1987; Paulsen et al., 1993; Giuffra et al., 1996; Pagano et al., 1998; Croce et al., 2002a). Five of the Chl-binding sites of LHCII are selective for Chl *b*, while at least 7 others, although showing some preference for Chl *a*, can also bind Chl *b* (Hobe et al., 2003). This led Harald Paulsen's group to propose that during the folding of the complexes the availability of Chl *b* is limited: enough Chl *b* to fill the Chl *b*-specific binding site, but not enough to allow Chl *b* to compete with Chl *a* for the other sites (Horn et al., 2007). This requires a regulation of the CAO enzyme, and Yamasato et al. (2005) have indeed shown that the N-terminal domain of CAO is a regulatory domain which makes the enzyme unstable in response to Chl *b* accumulation. Information about the environment in which the folding of the different complexes occurs is needed to fully clarify this point. In addition, the analysis of sites, which are Chl *b*-specific in one of the Lhc complexes and Chl *a*-specific in another, might provide further information about the "affinity rules".

VIII. Conclusions

During the last several years, the structures of most of the photosynthetic complexes have been resolved, allowing the organization, in a "visual framework", of the large body of information obtained by genetics, biochemical and spectroscopic methods. The only structure that is still to be solved is that of PS II-LHCII from higher plants. This is, however, going to be an even bigger challenge not only because of the size of the complex, but also because of its instability, which at present does not allow us to obtain homogeneous preparations that can be used for crystallization. In this respect electron microscopy seems to be the most practicable method to obtain, in the near future, the structure of the complex at an intermediate resolution. The next challenge is to discover the higher level of organization represented by the thylakoid membrane in order to

determine the spatial organization of the complexes and to understand how a very packed membrane can still be highly dynamic allowing for the rapid diffusion of several complexes between grana and stroma lamellae.

While this review was being processed for publication, the structures of several photosynthetic complexes were obtained at higher resolution. An updated structure of the PS I-LHCI complex of plants (Amunts et al., 2010) allows now a more accurate positioning of PsaK and reveals the location of 15 more pigments. The PS II core structure was improved from 2.9 to 1.9 Å resolution, revealing the detailed structure of the catalytic center of water splitting (Umena et al., 2011). The structure of CP29, the first structure of a minor antenna complex, has also become recently available at 2.8 Å resolution (Pan et al., 2011), allowing for a direct comparison with the structure of LHCII and opening the way to our understanding of the structural bases of the different spectroscopic properties among the Lhc complexes. Finally, a projection map at 12 Å of the PS II-LHCII supercomplex was obtained by electron microscopy, allowing determination of the orientation of the individual antenna complexes and the energy transfer pathways between the outer antenna and the core (Caffarri et al., 2009).

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References

Allen JF (1992) Protein phosphorylation in regulation of photosynthesis. Biochim Biophys Acta 1098: 275–335

Allen JF (1995) Thylakoid protein-phosphorylation, state-1-state-2 transitions, and photosystem stoichiometry adjustment – redox control at multiple levels of gene-expression. Physiol Plant 93: 196–205

Amunts A, Drory O and Nelson N (2007) The structure of a plant photosystem I supercomplex at 3.4 angstrom resolution. Nature 447: 58–63

Amunts A, Toporik H, Borovikova A and Nelson N (2010) Structure determination and improved model of plant photosystem I. J Biol Chem 285: 3478–3486

Anderson JM and Andersson B (1988) The dynamic photosynthetic membrane and regulation of solar-energy conversion. Trends Biochem Sci 13: 351–355

Andrizhiyevskaya EG, Frolov D, Van Grondelle R and Dekker JP (2004) Energy transfer and trapping in the photosystem I complex of *Synechococcus* PCC 7942 and in its supercomplex with IsiA. Biochim Biophys Acta 1656: 104–113

Aro EM, Virgin I and Andersson B (1993) Photoinhibition of photosystem-2 – inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Bald D, Kruip J, Boekema E and Rögner M (1992) Structural investigations on Cytb_6f-complex and PS-I complex from the cyanobacterium *Synechocystis* PCC-6803. Photosynth Res 34: 133–133

Ballottari M, Govoni C, Caffarri S and Morosinotto T (2004) Stoichiometry of LHCI antenna polypeptides and characterization of gap and linker pigments in higher plants photosystem I. Eur J Biochem 271: 4659–4665

Barber J (2002) Photosystem II: a multisubunit membrane protein that oxidises water. Curr Opin Struc Biol 12: 523–530

Barber J (2006) Structure of photosystem II and its implications for understanding the water splitting reaction it catalyses. Biochim Biophys Acta 34: 619–631

Barter LMC, Durrant JR and Klug DR (2003) A quantitative structure-function relationship for the photosystern II reaction center: Supermolecular behavior in natural photosynthesis. Proc Natl Acad Sci USA 100: 946–951

Bassi R and Dainese P (1992) A supramolecular light-harvesting complex from chloroplast photosystem-II membranes. Eur J Biochem. 204: 317–326

Bassi R, Pineau B, Dainese P and Marquardt J (1993) Carotenoid-binding proteins of photosystem-II. Eur J Biochem. 212: 297–303

Bassi R, Sandona D and Croce R (1997) Novel aspects of chlorophyll *a*/*b*-binding proteins. Physiol Plant 100: 769–779

Bassi R, Croce R, Cugini D and Sandona D (1999) Mutational analysis of a higher plant antenna protein provides identification of chromophores bound into multiple sites. Proc Natl Acad Sci USA 96: 10056–10061

Bellafiore S, Barneche F, Peltier G and Rochaix JD (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895

Ben-Shem A, Frolow F and Nelson N (2004) Light-harvesting features revealed by the structure of plant photosystem I. Photosynth Res 81: 239–250

Ben-Shem A, Frolow F and Nelson N (2003) Crystal structure of plant photosystem I. Nature 426: 630–635

Bibby TS, Nield J and Barber J (2001) Iron deficiency induces the formation of an antenna ring around trimeric photosystem I in cyanobacteria. Nature 412: 743–745

Bibby TS, Mary I, Nield J, Partensky F and Barber J (2003a) Low-light-adapted *Prochlorococcus* species possess specific antennae for each photosystem. Nature 424: 1051–1054

Bibby TS, Nield J, Chen M, Larkum AWD and Barber J (2003b) Structure of a photosystem II supercomplex isolated from *Prochlorion didemni* retaining its chlorophyll

a/*b* light-harvesting system. Proc Natl Acad Sci USA 100: 9050–9054

Blankenship RE (1992) Origin and early evolution of photosynthesis. Photosynth Res 33: 91–111

Boekema EJ, Van Roon H, Calkoen F, Bassi R and Dekker JP (1999a) Multiple types of association of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. Biochemistry 38: 2233–2239

Boekema EJ, Van Roon H, Van Breemen JF and Dekker JP (1999b) Supramolecular organization of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. Eur J Biochem 266: 444–452

Boekema EJ, Hifney A, Yakushevska AE, Piotrowski M, Keegstra W, Berry S, Michel KP, Pistorius EK and Kruip J (2001a) A giant chlorophyll-protein complex induced by iron deficiency in cyanobacteria. Nature 412: 745–748

Boekema EJ, Jensen PE, Schlodder E, Van Breemen JF, Van Roon H, Scheller HV and Dekker JP (2001b) Green plant photosystem I binds light-harvesting complex I on one side of the complex. Biochemistry 40: 1029–1036

Brettel K (1997) Electron transfer and arrangement of the redox cofactors in photosystem I. Biochim Biophys Acta 1318: 322–373

Bruggemann B, Sznee K, Novoderezhkin V, Van Grondelle R and May V (2004) From structure to dynamics: Modeling exciton dynamics in the photosynthetic antenna PS1. J Phys Chem B 108: 13536–13546

Buchel C and Kühlbrandt W (2005) Structural differences in the inner part of photosystem II between higher plants and cyanobacteria. Photosynth Res 85: 3–13

Bumba L, Prasil O and Vacha F (2005) Antenna ring around trimeric photosystem I in chlorophyll *b* containing cyanobacterium *Prochlorothrix hollandica*. Biochim Biophys Acta 1708: 1–5

Burnap RL, Troyan T and Sherman LA (1993) The highly abundant chlorophyll-protein complex of iron-deficient *Synechococcus* sp. PCC7942 (CP43) is encoded by the *isiA* gene. Plant Physiol 103: 893–902

Butler WL (1978) Energy distribution in the photochemical apparatus of photosynthesis. Ann Rev Plant Physiol 29: 345–378

Byrdin M, Jordan P, Krauss N, Fromme P, Stehlik D and Schlodder E (2002) Light harvesting in photosystem I: Modeling based on the 2.5-angstrom structure of photosystem I from *Synechococcus elongatus*. Biophys J 83: 433–457

Caffarri S, Passarini F, Bassi R and Croce R (2007) A specific binding site for neoxanthin in the monomeric antenna proteins CP26 and CP29 of photosystem II. FEBS Lett 581: 4704–4710

Caffarri S, Kouril R, Kereiche S, Boekema EJ and Croce R (2009) Functional architecture of photosystem II supercomplexes. EMBO J 28: 3052–3063

Castelletti S, Morosinotto T, Robert B, Caffarri S, Bassi R and Croce R (2003) Recombinant Lhca2 and Lhca3 subunits of the photosystem I antenna system. Biochemistry 42: 4226–4234

Chen M, Bibby TS, Nield J, Larkum A and Barber J (2005a) Iron deficiency induces a chlorophyll *d*-binding Pcb antenna system around photosystem I in *Acaryochloris marina*. Biochim Biophys Acta 1708: 367–374

Chen M, Bibby TS, Nield J, Larkum AWD and Barber J (2005b) Structure of a large photosystem II supercomplex from *Acaryochloris marina*. FEBS Lett 579: 1306–1310

Chen M, Telfer A, Lin S, Pascal A, Larkum AWD, Barber J and Blankenship RE (2005c) The nature of the photosystem II reaction centre in the chlorophyll *d*-containing prokaryote, *Acaryochloris marina*. Photoch Photobio Sci 4: 1060–1064

Chen M, Zhang Y and Blankenship RE (2008) Nomenclature for membrane-bound light-harvesting complexes of cyanobacteria. Photosynth Res 95: 147–154

Chitnis PR, Purvis D and Nelson N (1991) Molecular cloning and targeted mutagenesis of the gene *psaF* encoding subunit III of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. J Biol Chem 266: 20146–20151

Chitnis PR (2001) Photosystem I: Function and physiology. Ann Rev Plant Physiol 52: 593–626

Corbet D, Schweikardt T, Paulsen H and Schmid VHR (2007) Amino acids in the second transmembrane helix of the Lhca4 subunit are important for formation of stable heterodimeric light-harvesting complex LHCI-730. J Mol Biol 370: 170–182

Croce R and Bassi R (1998). The light-harvesting complex of photosystem I: pigment composition and stoichiometry. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 421–424. Kluwer Academic Publishers, Dordrecht

Croce R, Zucchelli G, Garlaschi FM and Jennings RC (1998) A thermal broadening study of the antenna chlorophylls in PSI-200, LHCI, and PSI core. Biochemistry 37: 17355–17360

Croce R, Remelli R, Varotto C, Breton J and Bassi R (1999) The neoxanthin binding site of the major light harvesting complex (LHC II) from higher plants. FEBS Lett 456: 1–6

Croce R, Canino G, Ros F and Bassi R (2002a) Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry 41: 7334–7343

Croce R, Morosinotto T, Castelletti S, Breton J and Bassi R (2002b) The Lhca antenna complexes of higher plants photosystem I. Biochim Biophys Acta 1556: 29–40

Croce R, Morosinotto T, Ihalainen JA, Chojnicka A, Breton J, Dekker JP, Van Grondelle R and Bassi R (2004) Origin of the 701-nm fluorescence emission of the Lhca2 subunit of higher plant photosystem I. J Biol Chem 279: 48543–48549

Croce R, Chojnicka A, Morosinotto T, Ihalainen JA, Van Mourik F, Dekker JP, Bassi R and Van Grondelle R (2007) The low-energy forms of photosystem I light-harvesting

complexes: Spectroscopic properties and pigment-pigment interaction characteristics. Biophys J 93: 2418–2428

Dainese P and Bassi R (1991) Subunit stoichiometry of the chloroplast photosystem-II antenna system and aggregation state of the component chlorophyll *a*/*b* binding proteins. J Biol Chem 266: 8136–8142

Damjanovic A, Vaswani HM, Fromme P and Fleming GR (2002) Chlorophyll excitations in photosystem I of *Synechococcus elongatus*. J Phys Chem B 106: 10251–10262

Damm I, Steinmetz D and Grimme LH (1990). Multiple functions of β-carotene in Photosystem I. In: Baltscheffsky M (ed) Current Research in Photosynthesis vol II, pp 607–610. Kluwer Academic Publishers, Dordrecht

Dashdorj N, Yamashita E, Schaibley J, Cramer WA and Savikhin S (2007) Ultrafast optical pump-probe studies of the cytochrome b_6f complex in solution and crystalline states. J Phys Chem B 111: 14405–14410

Dekker JP, Van Roon H and Boekem EJ (1999) Heptameric association of light-harvesting complex II trimers in partially solubilized photosystem II membranes. FEBS Lett 449: 211–214

Dekker JP and Boekema EJ (2005) Supramolecular organization of thylakoid membrane proteins in green plants. Biochim Biophys Acta 1706: 12–39

Diner BA, Schlodder E, Nixon PJ, Coleman WJ, Rappaport F, Lavergne J, Vermaas WFJ and Chisholm DA (2001) Site-directed mutations at D1-His198 and D2-His 97 of photosystem II in *Synechocystis* PCC 6803: Sites of primary charge separation and cation and triplet stabilization. Biochemistry 40: 9265–9281

During U, Ossenbuhl F and Wilde A (2007) Late assembly steps and dynamics of the cyanobacterial photosystem I. J Biol Chem 282: 10915–10921

Durrant JR, Klug DR, Kwa SL, Van Grondelle R, Porter G and Dekker JP (1995) A multimer model for P680, the primary electron donor of photosystem II. Proc Natl Acad Sci USA 92: 4798–4802

Eaton-Rye JJ and Putnam-Evans C (2005) The CP47 and CP43 core antenna components. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 45–70. Springer, Dordrecht

Farah J, Rappaport F, Choquet Y, Joliot P and Rochaix JD (1995) Isolation of a PsaF-deficient mutant of *Chlamydomonas reinhardtii* – efficient interaction of plastocyanin with the photosystem-I reaction center is mediated by the PsaF subunit. EMBO J 14: 4976–4984

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Ganeteg U, Strand A, Gustafsson P and Jansson S (2001) The properties of the chlorophyll *a*/*b*-binding proteins Lhca2 and Lhca3 studied in vivo using antisense inhibition. Plant Physiol 127: 150–158

Ganeteg U, Klimmek F and Jansson S (2004) Lhca5 – an LHC-type protein associated with photosystem I. Plant Mol Biol 54: 641–651

Geiss U, Vinnemeier J, Kunert A, Lindner I, Gemmer B, Lorenz M, Hagemann M and Schoor A (2001) Detection of the *isiA* gene across cyanobacterial strains: Potential for probing iron deficiency. Appl Environ Microb 67: 5247–5253

Giuffra E, Cugini D, Croce R and Bassi R (1996) Reconstitution and pigment binding protein of recombinant CP29. Eur J Biochem 238: 112–120

Giuffra E, Zucchelli G, Sandona D, Croce R, Cugini D, Garlaschi FM, Bassi R and Jennings RC (1997) Analysis of some optical properties of a native and reconstituted photosystem II antenna complex, CP29: Pigment binding sites can be occupied by chlorophyll *a* or chlorophyll *b* and determine spectral forms. Biochemistry 36: 12984–12993

Gobets B and Van Grondelle R (2001) Energy transfer and trapping in photosystem I. Biochim Biophys Acta 1057: 80–99

Gobets B, Valkunas L and Van Grondelle R (2003a) Bridging the gap between structural and lattice models: A parameterization of energy transfer and trapping in photosystem I. Biophys J 85: 3872–3882

Gobets B, Van Stokkum IHM, Van Mourik F, Dekker JP and Van Grondelle R (2003b) Excitation wavelength dependence of the fluorescence kinetics in photosystem I particles from *Synechocystis* PCC 6803 and *Synechococcus elongatus*. Biophys J 85: 3883–3898

Golbeck JH (2006) (ed) Photosystem I The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24. Springer, Dordrecht

Green BR and Kühlbrandt W (1995) Sequence conservation of light-harvesting and stress- response proteins in relation to the three-dimensional molecular structure of LHCII. Photosynth Res 44: 139–148

Green BR, and Parson WW (2003) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13. Kluwer Academic Publishers, Dordrecht

Grimm BP, Porra RJ, Rüdiger W and Scheer H (2006) Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications, Advances in Photosynthesis and Respiration, Vol. 25. Springer, Dordrecht

Groot ML, Pawlowicz NP, Van Wilderen LJGW, Breton J, Van Stokkum IHM and Van Grondelle R (2005) Initial electron donor and acceptor in isolated photosystem II reaction centers identified with femtosecond mid-IR spectroscopy. Proc Natl Acad Sci USA 102: 13087–13092

Grotjohann I, Jolley C and Fromme P (2004) Evolution of photosynthesis and oxygen evolution: Implications from the structural comparison of photosystems I and II. Phys Chem Chem Phys 6: 4743–4753

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A and Saenger W (2009) Cyanobacterial photosystem II at 2.9-angstrom resolution and the role of quinones, lipids, channels and chloride. Nat Struct Mol Biol 16: 334–342

Haldrup A, Naver H and Scheller HV (1999) The interaction between plastocyanin and photosystem I is inefficient in transgenic Arabidopsis plants lacking the PSI-N subunit of photosystem I. Plant J 17: 689–698

Harrer R, Bassi R, Testi MG and Schäfer C (1998) Nearest-neighbor analysis of a photosystem II complex from *Marchantia polymorpha* L. (liverwort), which contains reaction center and antenna proteins. Eur J Biochem 255: 196–205

Haworth P, Watson JL and Arntzen CJ (1983) The detection, isolation and characterization of a light-harvesting complex which is specifically associated with photosystem I. Biochim Biophys Acta 724: 151–158

He WZ and Malkin R (1992) Specific release of a 9-kDa extrinsic polypeptide of photosystem-I from spinach chloroplasts by salt washing. FEBS Lett 308: 298–300

Hippler M, Reichert J, Sutter M, Zak E, Altschmied L, Schröer U, Herrmann RG and Haehnel W (1996) The plastocyanin binding domain of photosystem I. EMBO J 15: 6374–6384

Hippler M, Drepper F, Farah J and Rochaix JD (1997) Fast electron transfer from cytochrome c_6 and plastocyanin to photosystem I of *Chlamydomonas reinhardtii* requires PsaF. Biochemistry 36: 6343–6349

Hippler M, Drepper F, Haehnel W and Rochaix JD (1998) The N-terminal domain of PsaF: Precise recognition site for binding and fast electron transfer from cytochrome c_6 and plastocyanin to photosystem I of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 95: 7339–7344

Hirashima M, Satoh S, Tanaka R and Tanaka A (2006) Pigment shuffling in antenna systems achieved by expressing prokaryotic chlorophyllide *a* oxygenase in *Arabidopsis*. J Biol Chem 281: 15385–15393

Hobe S, Fey H, Rogl H and Paulsen H (2003) Determination of relative chlorophyll binding affinities in the major light-harvesting chlorophyll *a*/*b* complex. J Biol Chem 278: 5912–5919

Hobe S, Trostmann I, Raunser S and Paulsen H (2006) Assembly of the major light-harvesting chlorophyll *a*/*b* complex – thermodynamics and kinetics of neoxanthin binding. J Biol Chem 281: 25156–25166

Holzwarth AR, Muller MG, Reus M, Nowaczyk M, Sander J and Rögner M (2006) Kinetics and mechanism of electron transfer in intact photosystem II and in the isolated reaction center: Pheophytin is the primary electron acceptor. Proc Natl Acad Sci USA 103: 6895–6900

Horn R, Grundmann G and Paulsen H (2007) Consecutive binding of chlorophylls *a* and *b* during the assembly in vitro of light-harvesting chlorophyll *a*/*b* protein (LHCIIb). J Mol Biol 366: 1045–1054

Horton P and Ruban AV (2005) Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. J Exp Bot 56: 365–373

Huang D, Everly RM, Cheng RH, Heymann JB, Schagger H, Sled V, Ohnishi T, Baker TS and Cramer WA (1994) Characterization of the chloroplast cytochrome b_6f complex as a structural and functional dimer. Biochemistry 33: 4401–4409

Ihalainen JA, Gobets B, Sznee K, Brazzoli M, Croce R, Bassi R, Van Grondelle R, Korppi-Tommola JEI and Dekker JP (2000) Evidence for two spectroscopically different dimers of light-harvesting complex I from green plants. Biochemistry 39: 8625–8631

Ihalainen JA, Jensen PE, Haldrup A, Van Stokkum IHM, Van Grondelle R, Scheller HV and Dekker JP (2002) Pigment organization and energy transfer dynamics in isolated, photosystem I (PSI) complexes from *Arabidopsis thaliana* depleted of the PSI-G, PSI-K, PSI-L, or PSI-N subunit. Biophys J 83: 2190–2201

Ihalainen JA, Van Stokkum IHM, Gibasiewicz K, Germano M, Van Grondelle R and Dekker JP (2005) Kinetics of excitation trapping in intact photosystem I of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*. Biochim Biophys Acta 1706: 267–275

Ikegami I, Satoh S and Aoki M (2007) Binding affinity of Chl *b* for the Chl *a*-binding sites in PSI core complexes. Plant Cell Physiol 48: 1092–1097

Jansson S, Andersen B and Scheller HV (1996) Nearest-neighbor analysis of higher-plant photosystem I holocomplex. Plant Physiol 112: 409–420

Jansson S (1999) A guide to the Lhc genes and their relatives in Arabidopsis. Trends Plant Sci 4: 236–240

Jensen PE, Gilpin M, Knoetzel J and Scheller HV (2000) The PSI-K subunit of photosystem I is involved in the interaction between light-harvesting complex I and the photosystem I reaction center core. J Biol Chem 275: 24701–24708

Jensen PE, Rosgaard L, Knoetzel J and Scheller HV (2002) Photosystem I activity is increased in the absence of the PSI-G subunit. J Biol Chem 277: 2798–2803

Jensen PE, Haldrup A, Zhang SP and Scheller HV (2004) The PSI-O subunit of plant photosystem I is involved in balancing the excitation pressure between the two photosystems. J Biol Chem 279: 24212–24217

Jensen PE, Bassi R, Boekema EJ, Dekker JP, Jansson S, Leister D, Robinson C and Scheller HV (2007) Structure, function and regulation of plant photosystem I. Biochim Biophys Acta 1767: 335–352

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W and Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. Nature 411: 909–917

Kühlbrandt W, Wang DN and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Kamiya N and Shen JR (2003) Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7-angstrom resolution. Proc Natl Acad Sci USA 100: 98–103

Kanervo E, Suorsa M and Aro EM (2005) Functional flexibility and acclimation of the thylakoid membrane. Photochem Photobiol Sci 4: 1072–80

Khrouchtchova A, Hansson M, Paakkarinen V, Vainonen JP, Zhang SP, Jensen PE, Scheller HV, Vener AV, Aro EM and Haldrup A (2005) A previously found thylakoid membrane protein of 14 kDa (TMP14) is a novel subunit of plant photosystem I and is designated PSI-P. FEBS Lett 579: 4808–4812

Kim H, Dashdorj N, Zhang HM, Yan JS, Cramer WA and Savikhin S (2005) An anomalous distance dependence of intraprotein chlorophyll-carotenoid triplet energy transfer. Biophys J 89: L28–L30

Klimmek F, Ganeteg U, Ihalainen JA, Van Roon H, Jensen PE, Scheller HV, Dekker JP and Jansson S (2005) Structure of the higher plant light harvesting complex I: In vivo characterization and structural interdependence of the Lhca proteins. Biochemistry 44: 3065–3073

Klimmek F, Sjodin A, Noutsos C, Leister D and Jansson S (2006) Abundantly and rarely expressed Lhc protein genes exhibit distinct regulation patterns in plants. Plant Physiol 140: 793–804

Knoetzel H, Mant A, Haldrup A, Jensen PE and Scheller HV (2002) PSI-O, a new 10-kDa subunit of eukaryotic photosystem I. FEBS Lett 510: 145–148

Kos PB, Deak Z, Cheregi O and Vass I (2008) Differential regulation of *psbA* and *psbD* gene expression, and the role of the different D1 protein copies in the cyanobacterium *Thermosynechococcus elongatus* BP-1. Biochim Biophys Acta 1777: 74–83

Kouril R, Yeremenko N, D'Haene S, Yakushevska AE, Keegstra W, Matthijs HCP, Dekker JP and Boekema EJ (2003) Photosystem I trimers from *Synechocystis* PCC 6803 lacking the PsaF and PsaJ subunits bind an IsiA ring of 17 units. Biochim Biophys Acta 1607: 1–4

Kouril R, Zygadlo A, Arteni AA, de Wit CD, Dekker JP, Jensen PE, Scheller HV and Boekema EJ (2005) Structural characterization of a complex of photosystem I and light-harvesting complex II of *Arabidopsis thaliana*. Biochemistry 44: 10935–10940

Kovacs L, Damkjaer J, Kereiche S, Ilioaia C, Ruban AV, Boekema EJ, Jansson S and Horton P (2006) Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts. Plant Cell 18: 3106–3120

Kurisu G, Zhang HM, Smith JL and Cramer WA (2003) Structure of the cytochrome b_6f complex of oxygenic photosynthesis: Tuning the cavity. Science 302: 1009–1014

Lelong C, Setif P, Lagoutte B and Bottin H (1994) Identification of the amino acids involved in the functional interaction between photosystem I and ferredoxin from *Synechocystis* sp. PCC 6803 by chemical cross-linking. J Biol Chem 269: 10034–10039

Lewin RA (2002) Prochlorophyta – a matter of class distinctions. Photosynth Res 73: 59–61

Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X and Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. Nature 428: 287–292

Loll B, Kern J, Saenger W, Zouni A and Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 angstrom resolution structure of photosystem II. Nature 438: 1040–1044

Lunde C, Jensen PE, Haldrup A, Knoetzel J and Scheller HV (2000) The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis. Nature 408: 613–615

Melis A (1991) Dynamics of photosynthetic membrane composition and function. Biochim Biophys Acta 1058: 87–106

Melkozernov AN, Lin S and Blankenship RE (2000) Excitation dynamics and heterogeneity of energy equilibration in the core antenna of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry 39: 1489–1498

Melkozernov AN, Bibby TS, Lin S, Barber J and Blankenship RE (2003) Time-resolved absorption and emission show that the CP43' antenna ring of iron-stressed *Synechocystis* sp. PCC6803 is efficiently coupled to the photosystem I reaction center core. Biochemistry 42: 3893–3903

Melkozernov AN, Barber J and Blankenship RE (2006) Light harvesting in photosystem I supercomplexes. Biochemistry 45: 331–345

Merry SAP, Nixon PJ, Barter LMC, Schilstra M, Porter G, Barber J, Durrant JR and Klug DR (1998) Modulation of quantum yield of primary radical pair formation in photosystem II by site-directed mutagenesis affecting radical cations and anions. Biochemistry 37: 17439–17447

Mimuro M and Kichchi, H. (2003). Antenna systems and energy transfer in Cyanophyta and Rhodophyta. In: Green BHP and Parson WW (eds) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 281–306. Kluwer Academic Publishers, Dordrecht

Minagawa J and Takahashi Y (2004) Structure, function and assembly of photosystem II and its light-harvesting proteins. Photosynth Res 82: 241–263

Morosinotto T, Castelletti S, Breton J, Bassi R and Croce R (2002) Mutation analysis of Lhca1 antenna complex – low energy absorption forms originate from pigment-pigment interactions. J Biol Chem 277: 36253–36261

Morosinotto T, Breton J, Bassi R and Croce R (2003) The nature of a chlorophyll ligand in Lhca proteins determines the far red fluorescence emission typical of photosystem I. J Biol Chem 278: 49223–49229

Morosinotto T, Ballottari M, Klimmek F, Jansson S and Bassi R (2005a) The association of the antenna system to photosystem I in higher plants. J Biol Chem 280: 31050–31058

Morosinotto T, Mozzo M, Bassi R and Croce R (2005b) Pigment-pigment interactions in Lhca4 antenna complex of higher plants photosystem I. J Biol Chem 280: 20612–20619

Mozzo M, Morosinotto T, Bassi R and Croce R (2006) Probing the structure of Lhca3 by mutation analysis. Biochim Biophys Acta 1757: 1607–1613

Muh F, Renger T and Zouni A (2008) Crystal structure of cyanobacterial photosystem II at 3.0 Å resolution: a closer look at the antenna system and the small membrane-intrinsic subunits. Plant Physiol Biochem 46: 238–264

Muller MG, Niklas J, Lubitz W and Holzwarth AR (2003) Ultrafast transient absorption studies on photosystem I reaction centers from *Chlamydomonas reinhardtii*. 1. A new interpretation of the energy trapping and early electron transfer steps in photosystem I. Biophys J 85: 3899–3922

Mullineaux CW (2005) Function and evolution of grana. Trend Plant Sci 10: 521–525

Murray JW, Duncan J and Barber J (2006) CP43-like chlorophyll binding proteins: structural and evolutionary implications. Trend Plant Sci 11: 152–158

Nakamoto H and Hasegawa M (1999) Targeted inactivation of the gene *psaK* encoding a subunit of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol. 40: 9–16

Nelson N and Yocum CF (2006) Structure and function of photosystems I and II. Ann Rev Plant Biol 57: 521–565

Nield J, Kruse O, Ruprecht J, da Fonseca P, Buchel C and Barber J (2000) Three-dimensional structure of *Chlamydomonas reinhardtii* and *Synechococcus elongatus* photosystem II complexes allows for comparison of their oxygen-evolving complex organization. J Biol Chem. 275: 27940–27946

Pagano A, Cinque G and Bassi R (1998) In vitro reconstitution of the recombinant photosystem II light-harvesting complex CP24 and its spectroscopic characterization. J Biol Chem. 273: 17154–17165

Palsson LO, Dekker JP, Schlodder E, Monshouwer R and Van Grondelle R (1996) Polarized site-selective fluorescence spectroscopy of the long-wavelength emitting chlorophylls in isolated photosystem I particles of *Synechococcus elongatus*. Photosynth Res 48: 239–246

Pan X, Li M, Wan T, Wang L, Jia C, Hou Z, Zhao X, Zhang J and Chang W (2011) Structural insights into energy regulation of light-harvesting complex CP29 from spinach. Nat Struct Mol Biol 18: 309–315

Partensky F, Hess WR and Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol Mol Biol Rev 63: 106–127

Paulsen H, Finkenzeller B and Kuhlein N (1993) Pigments induce folding of light-harvesting chlorophyll *a*/*b* binding protein. Eur J Biochem 215: 809–816

Peterman EJ, Wenk SO, Pullerits T, Palsson LO, Van Grondelle R, Dekker JP, Rögner M and Van Amerongen H (1998) Fluorescence and absorption spectroscopy of the weakly fluorescent chlorophyll *a* in cytochrome b_6f of *Synechocystis* PCC6803. Biophys J 75: 389–398

Pfannschmidt T (2003) Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33–41

Pierre Y, Breyton C, Kramer D and Popot JL (1995) Purification and characterization of the cytochrome b_6f complex from *Chlamydomonas reinhardtii.* J Biol Chem 270: 29342–29349

Pierre Y, Breyton C, Lemoine Y, Robert B, Vernotte C and Popot JL (1997) On the presence and role of a molecule of chlorophyll *a* in cytochrome b_6f complex. J Biol Chem. 272: 21901–21908

Plumley FG and Schmidt GW (1987) Reconstitution of chloroform *a*/*b* light-harvesting complexes: Xanthophyll-dependent assembly and energy transfer. Proc Natl Acad Sci USA 84: 146–150

Prokhorenko VI and Holzwarth AR (2000) Primary process and structure of the photosystem II reaction center: A photon echo study. J Phys Chem B 104: 11563–11578

Remelli R, Varotto C, Sandona D, Croce R and Bassi R (1999) Chlorophyll binding to monomeric light-harvesting complex. A mutation analysis of chromophore-binding residues. J Biol Chem 274: 33510–33521

Rogl H and Kühlbrandt W (1999) Mutant trimers of light-harvesting complex II exhibit altered pigment content and spectroscopic features. Biochemistry 38: 16214–16222

Roose JL, Wegener KM and Pakrasi HB (2007) The extrinsic proteins of photosystem II. Photosynth Res 92: 369–387

Ruban AV, Wentworth M, Yakushevska AE, Andersson J, Lee PJ, Keegstra W, Dekker JP, Boekema EJ, Jansson S and Horton P (2003) Plants lacking the main light-harvesting complex retain photosystem II macro-organization. Nature 421: 648–652

Ruffle SV, Wang J, Johnston HG, Gustafson TL, Hutchison RS, Minagawa J, Crofts A and Sayre RT (2001) Photosystem II peripheral accessory chlorophyll mutants in *Chlamydomonas reinhardtii*. Biochemical characterization and sensitivity to photo-inhibition. Plant Physiol 127: 633–644

Sandona D, Croce R, Pagano A, Crimi M and Bassi R (1998) Higher plants light harvesting proteins. Structure and function as revealed by mutation analysis of either protein or chromophore moieties. Biochim Biophys Acta 1365: 207–214

Satoh S, Ikeuchi M, Mimuro M and Tanaka A (2001) Chlorophyll *b* expressed in cyanobacteria functions as a light-harvesting antenna in photosystem I through flexibility of the proteins. J Biol Chem 276: 4293–4297

Schmid VHR, Cammarata KV, Bruns BU and Schmidt GW (1997) In vitro reconstitution of the photosystem I light-harvesting complex LHCI-730: Heterodimerization is required for antenna pigment organization. Proc Natl Acad Sci USA 94: 7667–7672

Schmid VHR, Potthast S, Wiener M, Bergauer V, Paulsen H and Storf S (2002) Pigment binding of photosystem I light-harvesting proteins. J Biol Chem 277: 37307–37314

Schubert WD, Klukas O, Saenger W, Witt HT, Fromme P and Krauss N (1998) A common ancestor for oxygenic and anoxygenic photosynthetic systems: a comparison based on the structural model of photosystem I. J Mol Biol 280: 297–314

Sener MK, Lu DY, Park SH, Schulten K and Fromme P (2002) Spectral disorder and excitation transfer dynamics in cyanobacterial photosystem I. Biophys J 82: 292A–292A

Siefermann-Harms D (1985) Carotenoids in photosynthesis. I. Location in photosynthetic membranes and light-harvesting function. Biochim Biophys Acta 811: 325–355

Standfuss R, Van Scheltinga ACT, Lamborghini M and Kühlbrandt W (2005) Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. Embo J 24: 919–928

Stomp M, Huisman J, Stal LJ and Matthijs HCP (2007) Colorful niches of phototrophic microorganisms shaped by vibrations of the water molecule. ISME J 1: 271–282

Storf S, Jansson S and Schmid VHR (2005) Pigment binding, fluorescence properties, and oligomerization behavior of Lhca5, a novel light-harvesting protein. J Biol Chem 280: 5163–5168

Stroebel D, Choquet Y, Popot JL and Picot D (2003) An atypical haem in the cytochrome b_6f complex. Nature 426: 413–418

Tang HD and Chitnis PR (2000) Addition of C-terminal histidyl tags to PsaL and PsaK1 proteins of cyanobacterial photosystem I. Indian J Biochem Biophys 37: 433–440

Umena Y, Kawakami K, Shen J-R and Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473: 55–60

Vaitekonis S, Trinkunas G and Valkunas L (2005) Red chlorophylls in the exciton model of photosystem I. Photosynth Res 86: 185–201

Varotto C, Pesaresi P, Jahns P, Lessnick A, Tizzano M, Schiavon F, Salamini F and Leister D (2002) Single and double knockouts of the genes for photosystem I subunits G, K, and H of Arabidopsis. Effects on photosystem I composition, photosynthetic electron flow, and state transitions. Plant Physiol 129: 616–624

Wang J, Gosztola D, Ruffle SV, Hemann C, Seibert M, Wasielewski MR, Hille R, Gustafson TL and Sayre RT (2002) Functional asymmetry of photosystem II D1 and D2 peripheral chlorophyll mutants of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 99: 4091–4096

Wollman FA (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J 20: 3623–30

Wydrzynski TJ and Satoh K (eds) (2005) Photosystem II The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22. Springer, Dordrecht

Xu H, Vavilin D and Vermaas W (2001) Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria. Proc Natl Acad Sci USA 98: 14168–14173

Xu Q, Jung YS, Chitnis VP, Guikema JA, Golbeck JH and Chitnis PR (1994a) Mutational analysis of photosystem-I polypeptides in *Synechocystis* sp. PCC 6803 – subunit requirements for reduction of $NADP^+$ mediated by ferredoxin and flavodoxin. J Biol Chem 269: 21512–21518

Xu Q, Odom WR, Guikema JA, Chitnis VP and Chitnis PR (1994b) Targeted deletion of PsaJ from the cyanobacterium *Synechocystis* sp. PCC 6803 indicates structural interactions between the PsajJ and PsaF subunits of photosystem I. Plant Mol Biol 26: 291–302

Xu W, Tang HD, Wang YC and Chitnis PR (2001) Proteins of the cyanobacterial photosystem I. Biochim Biophys Acta 1507: 32–40

Yakushevska AE, Jensen PE, Keegstra W, Van Roon H, Scheller HV, Boekema EJ and Dekker JP (2001) Supermolecular organization of photosystem II and its associated light-harvesting antenna in *Arabidopsis thaliana*. Eur J Biochem 268: 6020–6028

Yakushevska AE, Keegstra W, Boekema EJ, Dekker JP, Andersson J, Jansson S, Ruban AV and Horton P (2003) The structure of photosystem II in *Arabidopsis*: Localization of the CP26 and CP29 antenna complexes. Biochemistry 42: 608–613

Yamasato A, Nagata N, Tanaka R and Tanaka A (2005) The N-terminal domain of chlorophyllide *a* oxygenase confers protein instability in response to chlorophyll *b* accumulation in Arabidopsis. Plant Cell 17: 1585–1597

Yang CH, Kosemund K, Cornet C and Paulsen H (1999) Exchange of pigment-binding amino acids in light-harvesting chlorophyll *a*/*b* protein. Biochemistry 38: 16205–16213

Yeremenko N, Kouril R, Ihalainen JA, D'Haene S, Van Oosterwijk N, Andrizhiyevskaya EG, Keegstra W, Dekker HL, Hagemann M, Boekema EJ, Matthijs HCP and Dekker JP (2004) Supramolecular organization and dual function of the IsiA chlorophyll-binding protein in cyanobacteria. Biochemistry 43: 10308–10313

Zouni A, Witt HT, Kern J, Fromme P, Krauss N, Saenger W and Orth P (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. Nature 409: 739–743

Zygadlo A, Jensen PE, Leister D and Scheller HV (2005) Photosystem I lacking the PSI-G subunit has a higher affinity for plastocyanin and is sensitive to photodamage. Biochim Biophys Acta 1708: 154–163

Chapter 7

Plant Proteomics and Photosynthesis

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Summary

Peptide mass finger printing and tandem mass spectrometry-based identification of proteins emerged in the mid 1990s, with its first application on the study of photosynthesis and chloroplasts about a decade ago. From there on, the impact of proteomics and protein mass spectrometry has been very significant, with initially most efforts placed on the identification of chloroplast proteins, followed by phosphorylation, comparative proteomics and work on protein-protein interactions. Most of this work has been carried out with *Arabidopsis thaliana*, the first plant for which the genome was sequenced. This chapter will focus on new results in proteomics of photosynthesis published since 2004, as well as on emerging concepts, challenges and opportunities to make discoveries relevant to photosynthesis. The chapter is restricted to photosynthesis and its regulatory proteins in higher plants and the green alga *Chlamydomonas reinhardtii*. With the exception of some small, hydrophobic thylakoid membrane proteins, most of the photosynthetic machinery has been observed by mass spectrometry, but many of the post-translational modifications remain to be discovered. One of the biggest challenges of proteomics in the area of photosynthesis will be to identify and characterize proteins with regulatory functions, and proteins involved in chloroplast development, biogenesis and adaptation.

I. Introduction

A. A Brief History of Proteomics and its Importance for Photosynthesis

Characterization of the chloroplast proteins is important to understand photosynthesis and its dynamic response to changing developmental states and abiotic conditions. Such characterization not only includes protein identification and their intra-chloroplast localization, but also determination of protein accumulation levels, possible protein-protein interactions and post-translational modifications. This information will help us in the understanding of the role of proteins in photosynthesis and would also allow us to test their functions through e.g., reverse genetics. Indeed reverse and forward genetics of *Arabidopsis thaliana*, as well as the green alga *Chlamydomonas reinharditii* has advanced our understanding of the biogenesis of the photosynthesis apparatus and the regulation of photosynthesis.

Advances in proteomics have been driven by mass spectrometry (MS) using the soft ionization techniques of Matrix Assisted Laser Desorption Ionization (MALDI) and Electro Spray Ionization (ESI), as well as high throughput data-dependent acquisition (DDA) techniques and the exponentially increasing amount of genome and expressed-sequence tag (EST) data from different species. The latest MS approaches using linear trap quadrupole (LTQ)-Orbitrap (Hu et al., 2005; Olsen et al., 2005; Scigelova and Makarov, 2006; Yates et al., 2006) and LTQ-Fourier transform ion cyclotron resonance (FTICR) instruments (Haas et al., 2006; Macek et al., 2006) have now such a high speed, sensitivity, accuracy and mass resolution, that literally several thousands of proteins can be identified in a sample (Adachi et al., 2006). General aspects of proteomics and mass spectrometry have been reviewed and I refer to excellent reviews from some of the leading labs (Steen and Mann, 2004; Domon and Aebersold, 2006;

Abbreviations: 2D – Two dimensional; BN-PAGE – Blue native gel electrophoresis; cICAT – Cleavable isotope coded affinity tag; CN-PAGE – Colorless native gel electrophoresis; cTP – Chloroplast transit peptide; DDA – Data dependent acquisition; ESI – Electro-spray ionization; EST – Expressed sequence tag; FTICR – Fourier transform ion cyclotron resonance; IEF – Isoelectric focusing; IPG – Immobilized pH gradient; iTRAQ – Isobaric tag reagent for quantification; LC – Liquid chromatography; Lhca/b – Light-harvesting proteins from the LHCI/II complexes; LHCI/II – Light-harvesting complex 1 (or II); LTQ – Linear trap quadrupole; MALDI – Matrix assisted laser desorption ionization; MetSo – Methionine sulfoxide; MS – Mass spectrometry; MS/MS – Tandem mass spectrometry; MSR – Methionine sulfoxide reductase; NDH – NADPH dehydrogenase complex; PGs – Plastoglobules; PMF – Peptide mass finger printing; PS – Photosystem; PTM – Post-translational modification; Q-TOF – Quadrapole time of flight; TOF – Time of flight; TRX – Thioredoxin; VIPP1 – Vesicle-inducing protein in plastids 1

Jensen, 2006b). These developments in MS now allow for an in-depth characterization of the photosynthetic machinery identifying most, if not all, of the more abundant structural proteins, as well as regulatory proteins typically of lower abundance. Studies concerning identification of the photosynthetic machinery and its regulators will be discussed in Section II. More in-depth discussions on the functions of many of these proteins are found in various chapters throughout this book.

Proteomics has also been able to capture information on protein-protein interactions and protein complexes. This relies on various biochemical techniques, such as affinity purification of tagged proteins, native gel-based protein separation and affinity columns with clever 'baits'. Whereas large scale protein-protein interaction studies have been carried out in yeast (Gavin et al., 2002; Ho et al., 2002; Gavin et al., 2006; Krogan et al., 2006) and *Escherichia coli* (Butland et al., 2005), no large scale protein interaction studies have (yet) been pursued successfully for photosynthetic organisms. However, several examples of newly identified protein-protein interactions and protein complexes involved in photosynthesis will be discussed in Section III.

There are now a wide variety of techniques that allow for comparative and quantitative proteome analysis, for instance to study the response of the photosynthetic apparatus to abiotic stress and to address cell-type specific differences, such as chloroplasts in bundle sheath and mesophyll cells of C4 plants. Developments in this area have been particularly rapid in the last several years. Many new and existing studies related to chloroplasts will be discussed in Section IV.

After their synthesis, proteins are often modified in reversible or irreversible ways. Examples are phosphorylation, lipid modifications, protein processing and glycosylation. Thus post-translational modifications (PTMs) play an important role in protein subcellular localization, protein stability, as well as protein function and protein-protein interactions. High resolution mass spectrometry, in combination with enrichment techniques for individual PTMs is now used widely and successfully (Jensen, 2004, 2006a). Although still in its infancy, significant progress has been made in the study of PTMs of the photosynthetic apparatus, particularly regarding phosphorylation of the thylakoid proteome and PTMs induced by oxidative stress. The last few years have seen a real break-through regarding regulation of state transitions by phosphorylation and these will be discussed in Section V.

Finally, bioinformatics and web-based resources have become essential in biology. The vast amount of information generated from genome sequencing, large-scale transcript analysis, proteomics and protein structure analyses, as well as metabolomics have provided amazing possibilities to study chloroplasts and photosynthesis. Section VI briefly discusses some of the web-based resources for proteomics of plastids and outlines challenges and opportunities in this area.

B. Recent Reviews in Proteomics of Plants, Plastids and Photosynthesis

In addition to the numerous reviews on plant and *C. reinhardtii* proteomics (Stauber and Hippler, 2004; Peck, 2005; Glinski and Weckwerth, 2006; Rossignol et al., 2006), a number of reviews have been published over the last several years that have specifically focused on the proteomics of non-green plastids, chloroplast and the photosynthetic apparatus (Van Wijk, 2000, 2001; Kieselbach and Schroder, 2003; Schroder and Kieselbach, 2003; Baginsky and Gruissem, 2004; Van Wijk, 2004a, b; Weber et al., 2005; Van Wijk, 2006). In addition, several reviews have focused on technical aspects of plant proteomics, with particular attention towards thylakoid and envelope membrane proteomics (Whitelegge, 2003, 2004; Rolland et al., 2006) and an isolation method of chloroplasts from *A. thaliana* for proteomics (Van Wijk et al., 2007). Redox regulation is central in regulation of photosynthesis, with thioredoxins and peroxiredoxins playing a central role in this process; affinity purification in combination with mass spectrometry has accelerated this areas and was reviewed earlier (Buchanan and Luan, 2005; Meyer et al., 2005; Dietz et al., 2006).

In light of these various reviews in books and journals, this chapter will focus on new results in proteomics of photosynthesis published from late 2004 and onwards, as well as on emerging concepts, challenges and opportunities to make discoveries relevant to photosynthesis. The chapter will be restricted to photosynthesis and its regulatory proteins in higher plants and the green alga *C. reinhardtii*.

II. Identification of Proteins in the Photosynthetic Apparatus

A. Introduction

The chloroplast proteins involved in photosynthesis can be divided in two groups based on their localization in either the stroma or the thylakoid membrane. Most enzymes involved in the "dark reactions" are soluble in the stroma, although several of them have shown interactions with the thylakoid membrane. The proteins involved in the "light reactions" are thylakoid-bound and are either located at the lumenal side of the thylakoid membrane, are integral to the thylakoid membrane bilayer or are associated with the stromal side of the thylakoid membrane. There are numerous proteins that are not primarily involved in photosynthesis, but that have regulatory or auxiliary roles in: (1) activation or deactivation of enzymes through PTMs; (2) governing specific protein-protein interactions, and (3) facilitating assembly and disassembly of the photosynthetic machinery. The first category of such regulatory proteins includes protein kinases, phosphatases, and redox regulators such as the thioredoxins. The second category includes CP12 involved in the formation of a heterotetradecamer with eight copies of glyceraldehyde 3-phosphate dehydrogenase, four copies of phoshoribulose kinase and two copies of CP12. The third category is the largest and most diverse and consists of chaperones, isomerases (e.g., TPL40), proteases (e.g., DegP, FtsH, Clp), metal transporters and chaperones (e.g., PAA2), and specific assembly factors (e.g., HFC136 involved in Photosystem (PS) II assembly; HCF101 involved in 2Fe-2S clusters). Identification of all photosynthetic enzymes and structural proteins, as well as regulatory proteins involved in photosynthesis is an important objective in proteomics studies.

Identification of proteins through MS is now well established and there are several approaches, either based on so-called peptide mass finger printing (PMF) or based on fragmentation of selected precursor ions in tandem mass spectrometry (MS/MS). These approaches have been extensively described in many original papers and reviews. Using PMF or MS/MS data, proteins are typically identified by matching this information against expressed sequence tags (ESTs) or annotated genome data with clearly defined gene/protein models. However, even in well annotated genomes such as that of *A. thaliana*, not all proteins are correctly predicted. This can be due to incorrect intron/exon splice site predictions, incorrect N-terminus predictions or simply because genes are so small that they have escaped gene annotation programs. This results in a mismatch of mass spectral data and predicted protein information; examples for thylakoid and stromal proteins have been presented and discussed in several papers (Van Wijk, 2001; Peltier et al., 2002). As of now, several software routines are available from various labs to automatically match (small or large scale) mass spectral data to sequenced genomes without relying on genome annotation (Kuster et al., 1999, 2001; Wisz et al., 2004; Allmer et al., 2006; Shadforth et al., 2006; Tanner et al., 2007). Relevant to photosynthesis, Michael Hippler and colleagues developed a search routine to match spectral data obtained from thylakoid membrane proteins from *C. reinhardtii*. They identified 98 intron-split peptides, resulting in the identification of novel proteins, improved annotation of gene models, and evidence of alternative splicing (Allmer et al., 2004, 2006). Given the multitude of different programs developed in this area, it should soon be a 'mainstream' procedure in any proteomics study (using MS/MS data).

The first chloroplast proteomics paper was published in 2000 and was focused on the thylakoid proteome (Peltier et al., 2000). Subsequent chloroplast proteomics papers initially focused on the identification of more chloroplast proteins, either in or associated with the thylakoid (Gómez et al., 2002; Peltier et al., 2002, 2004; Schubert et al., 2002; Gomez et al., 2003; Friso et al., 2004; Storf et al., 2004; Ciambella et al., 2005; Ytterberg et al., 2006), in the envelope membranes (Seigneurin-Berny et al., 1999; Ferro et al., 2000, 2002, 2003; Froehlich et al., 2003), in the stroma of maize and *A. thaliana* (Majeran et al., 2005; Peltier et al., 2006) or including the whole chloroplast or etioplast (Kleffmann et al., 2004; von Zychlinski et al., 2005; Baginsky et al., 2007; Kleffmann et al., 2007).

The shift from PMF and off-line, manual ESI/MS/MS to online LC-ESI-MS/MS (with data-dependent acquisition i.e., DDA) that occurred over the last decade has allowed analysis of

increasingly complex samples, thereby reducing the need for extensive protein separation prior to MS analysis. Collectively, these studies identified some 750 chloroplast proteins in *A. thaliana* and lower numbers in other plant species (mostly pea, maize, rice, barley) and in *C. reinhardtii*. The accession numbers for these proteins can be found in the respective papers, and are also collected by chloroplast proteomics databases in the case of *A. thaliana*, maize and rice (see Section VI). This section will discuss some of the conclusions that can be extracted from these studies. In addition, several particular interesting new observations will be highlighted.

B. Identification of the Thylakoid Proteome

The thylakoid proteome has been analyzed in significant depth in *A. thaliana* over the last several years. This showed that nearly every known subunit of the photosynthetic electron transport chain (PS I, PS II, cytochrome b_6f, ATP synthase) can and has been identified by MS. The exceptions are mostly the very small, one transmembrane-helix proteins, as well as some of the transiently expressed light stress proteins with chlorophyll-binding domains (e.g., Elips and Lils). Several members of small groups of homologues (e.g., psaH1,2, several LHCII proteins) cannot easily be identified individually, but only as a group. Nevertheless, this shows that most subunits of the photosynthetic electron transport chain can be accessed by MS. This should allow a better study of quantitative changes in protein composition in response to environmental conditions and during biogenesis and leaf development (see Section IV).

The NDH complex is a large thylakoid complex involved in cyclic electron flow and chlororespiration. It is of lower abundance than the four complexes discussed above, as is reflected in a lower success rate for identification by MS and much less knowledge about its composition and structure. To identify missing subunits, tobacco NDH-H was (His) 6 tagged at its N terminus using plastid transformation (Rumeau et al., 2005). A functional NDH subcomplex was purified by Ni^{2+} affinity chromatography and its subunit composition analyzed by MS. Five plastid encoded subunits (NDH-A, -H, -I, -J, and -K) were identified, as well as three new subunits (NDH-M, -N, and -O). Six of these subunits have been identified in two proteomics studies of the thylakoid proteome (Friso et al., 2004; Peltier et al., 2004).

The lumenal thylakoid proteome has been analyzed in several studies and the study has collectively identified some 60 proteins. In addition to several proteins of the oxygen-evolving complex and their (distant) homologues, as well as some lumenal proteins of PS I, the majority of proteins appears to be involved in various aspects of protein homeostasis (folding, degradation), as well as unidentified activities. Predictions based on lumenal transit peptides suggest that there exist between 80 (Schubert et al., 2002) and 200 lumenal proteins (Peltier et al., 2002; Sun et al., 2004). Analysis of lumenal preparations with higher sensitivity MS instruments should provide clarity on the lumenal proteome and possibly shed more light on the lumenal proteome function.

Several thylakoid proteome studies identified the so-called fibrillin proteins (Friso et al., 2004; Peltier et al., 2004). A protein in this family had been shown to be located on thylakoid associated plastid lipid particles, named plastoglobules (PGs) (Kessler et al., 1999). In 2006, two papers analyzed the proteome of these PGs in great detail (Vidi et al., 2006; Ytterberg et al., 2006) and, surprisingly, identified more than 30 proteins, some with established metabolic functions. Additional research analyzed the metabolic content of these PGs (Lohmann et al., 2006; Gaude et al., 2007) and determined the precise location and topology of PGs using tomography (Austin et al., 2006). This showed that PGs are contiguous with the outer lipid leaflet of the thylakoid membrane and have a transient storage function of quionone, phylloquinone, tocopherols and other lipid molecules. It is likely that PGs can channel these products to and from the thylakoid bilayer and possibly the inner envelope membrane. Moreover, PGs are likely an important storage place for toxic breakdown products of chlorophylls and membrane lipids. For a review, see Brehelin et al. (2007). The dynamics of the PG proteome in response to light conditions will be discussed in Section IV.

In addition to the PGs and the proteome of the photosynthetic electron transport chain, more than 200 additional proteins have been identified in thylakoid samples of *A. thaliana*. A number of those are mostly stromal proteins interacting with the thylakoid membrane surface, serving a

particular function or simply as a consequence of ionic or hydrophobic interactions - for discussion see Van Wijk (2006).

Finally, K. Lilley and colleagues devised an original procedure (abbreviated as LOPIT) to assign proteins to various cellular membrane systems (Dunkley et al., 2006). They used density gradient centrifugation of mixed cellular membrane and iTRAQ labeling of individual fractions and mass spectrometry for quantification and identification. A principle component analysis was then used to assign proteins to various cellular locations. Thylakoid and mitochondrial proteins clustered together, but were separated from endoplasmatic reticulum and plasma membranes (Dunkley et al., 2006).

C. Identification of the Stromal Proteome

Although best known for their role in photosynthesis, chloroplasts (and plastids in general) synthesize many essential compounds, such as plant hormones, fatty acids and lipids, amino acids, vitamins, purine and pyrimidine nucleotides, tetrapyrroles and isoprenoids (Buchanan et al., 2000). Chloroplasts are required for nitrogen and sulfur assimilation, and contain numerous protein chaperones and assembly factors, peptidases and proteases (Buchanan et al., 2000). To facilitate chloroplast gene expression, chloroplasts contain the plastid transcriptional and translation machinery, including many mRNA binding proteins involved in mRNA processing, stability and translation (Barkan and Goldschmidt-Clermont, 2000; Monde et al., 2000).

Two proteomics studies have specifically analyzed the stromal proteome of *A. thaliana* (Peltier et al., 2006) and of maize (Majeran et al., 2005). Other studies analyzed the total chloroplast proteome of *A. thaliana*, including the stroma (Kleffmann et al., 2004), while two other studies focused on the rice (non-photosynthetic) etioplasts (von Zychlinski et al., 2005; Kleffmann et al., 2007).

Chloroplasts of maize leaves differentiate into specific bundle sheath and mesophyll types to accommodate C4 photosynthesis (Edwards and Walker, 1983; Nelson and Langdale, 1992). Bundle sheath cells have thick cell walls and contain centrifugally arranged chloroplasts with large starch granules and unstacked thylakoid membranes, whereas the mesophyll cells contain randomly arranged chloroplasts with stacked thylakoids and little or no starch (Edwards and Walker, 1983). The fully differentiated bundle sheath and mesophyll chloroplasts each accumulate a distinct set of photosynthetic enzymes and proteins that enables them to cooperate in carbon fixation (Hatch and Osmond, 1976; Edwards and Walker, 1983) (reviewed in Langdale (1998) and Sheen (1999)). Differentiation of many other chloroplast functions (reviewed in Neuhaus and Emes (2000)), such as synthesis of fatty acids, nucleotides, hormones, amino acids are largely unknown in C4 plants. To compare bundle sheath and mesophyll chloroplast functions, stromal proteomes of bundle sheath and mesophyll chloroplasts were purified and analyzed using three independent techniques, as will be discussed in more detail in Section III (Majeran et al., 2005). Collectively, 400 ZmGI (this is the Tigr database with maize assembled ESTs) accessions were identified from searches against ZmGI. 327 proteins were identified by searching against the maize genome sequences (AZM), and 277 by homology-based searches against the predicted rice proteome (OsGI – this is the Tigr database for rice sequences). To determine the functions of the identified stromal proteomes, ZmGI accessions were *blasted* to the predicted rice and *A. thaliana* proteome. Proteins were then functionally classified using the non-redundant MapMan functional classification system (see http://gabi.rzpd.de/projects/MapMan/) developed for *A. thaliana* (Thimm et al., 2004) . Functions of identified maize protein included primary carbon metabolism and photosynthesis (Calvin-Benson cycle, Oxidative PhosphoPentose Pathway, glycolysis, starch metabolism) totaling 16%; amino-acid metabolism (6%); lipid metabolism (3%) and nitrogen and sulfur assimilation (2%). Proteins involved in protein synthesis, folding and degradation represented 24% of all identified proteins. Bundle sheath and mesophyll localized enzymes of the C4 shuttle and the photosynthetic electron transport chain were also found. Several enzymes in tetrapyrrole synthesis and vitamin biosynthesis were identified. Twenty-one percent of the identified proteins were classified in a miscellaneous category with a majority of unknown proteins. Thus, the analysis covered a wide range of plastid functions.

The objectives of the stromal analysis of the *A. thaliana* chloroplast study (Peltier et al., 2006) were to: (i) identify the stromal proteome and determine the expression of chloroplast paralogues within protein families; (ii) obtain a semi-quantitative overview of the chloroplast proteome, and (iii) begin building resources for unraveling the protein interaction network in chloroplasts of *A. thaliana*. Given the complexity of the chloroplast proteome, the dynamic nature of many protein interactions and the wide protein expression range (likely more than nine orders of magnitude), this is a challenging task. This aspect of protein-protein interactions will be discussed in Section III. The stromal proteome was separated by 2-dimensional gels, with non-denaturing colorless PAGE (CN-PAGE) (Schagger et al., 1994) as the first dimension and denaturing SDS-PAGE as the second dimension. Two hundred and forty-one proteins were identified, representing about 99% of the stromal protein mass; 88% of the proteome was predicted by TargetP to be plastid localized, which is indicative of the purity of the stromal sample and/or the low false positive rate of protein identification. This is also consistent with the notion that most chloroplast proteins have typical and predictable (by TargetP) N-terminal transit peptides (see Section VI for discussion). This stromal dataset complements the existing chloroplast membrane proteome datasets (Ferro et al., 2000, 2002, 2003; Peltier et al., 2002, 2004; Schubert et al., 2002; Froehlich et al., 2003; Gomez et al., 2003; Friso et al., 2004) as about 40% of the identified stromal proteins were not present in these published studies. The analysis covered most known chloroplast functions, ranging from protein biogenesis and protein fate, to primary and secondary metabolism, as well as proteins without any obvious function. Importantly, since false-positive paralogue identification was largely avoided, this dataset will help to assign individual gene family members to subcellular locations and tissues. The Calvin-Benson cycle, the oxidative pentose phosphate pathway and glycolysis together represent some 75% of the total mass, with protein synthesis, folding and biogenesis about 6.5% and nitrogen and sulfur assimilation an additional 7.5%. This leaves less than 10% of the total stromal mass for all other stromal functions. The large investments in carbon metabolism and the Calvin-Benson cycle in particular, reflect the inefficiency of Rubisco, as well as the high metabolic flux of reduced carbohydrates. The large protein mass dedicated to protein synthesis (ribosomes, RNA binding proteins and elongation factors), protein folding and proteolysis is likely a reflection of the abundance (and need for synthesis) of the chloroplast-encoded large subunit of Rubisco, and chloroplast-encoded proteins of the thylakoid photosynthetic apparatus. The abundance of the chaperones is likely a reflection of the need for folding and assembly of the thousands of nuclear-encoded proteins imported into the stroma. The identified proteins span at least an expression range of five orders of magnitude.

Quantification of molar ratios between different proteins in complex proteomes is generally difficult and has not been attempted at any large scale, in contrast to relative quantification of the same protein in different samples (see Section III). Quantitative techniques to determine changes in protein accumulation levels of the same protein in a complex mixture between samples are now quite powerful, as will be discussed in Section III. However, these techniques cannot be used to compare accumulation levels of different proteins within a sample and are thus not applicable to answer questions raised in the current studies. A new approach has been introduced recently in which isotope labeled peptides matching the individual proteins are added as internal standards into complex mixtures. This technique (assigned the name AQUA) is most appropriate for comparing expression levels of a small number of known proteins and it is not suitable for quantification of unidentified proteins (Kirkpatrick et al., 2005). Denaturing IsoElectric Focusing (IEF) using commercial immobilized pH gradient (IPG) strips is not quantitative when comparing stoichiometries of different protein species, due to the underestimation of high molecular mass proteins, proteins with significant hydrophobicity or extreme pI. The study of by Peltier et al. (2006) showed that although certainly not perfect, two-dimensional (2D) separation with CN-PAGE in the first dimension, currently provides a convenient semi-quantitative comparison of larger sets of protein species.

It is interesting to contrast the proteome of chloroplasts with those of non-green plastids.

Non-green plastids exist in roots, non-green flowers, fruits and in seeds. Several papers on proteomics were published concerning (non-green) wheat amyloplasts and tobacco (colorless) BY-2 cell culture plastids (Andon et al., 2002; Baginsky et al., 2004). It is hard to compare these identified proteomes to the current chloroplast proteome analysis, since these studies did not quantify protein accumulation, but also because wheat, tobacco and *A. thaliana* are quite distant in evolution, with relatively low levels of sequence homology. Nevertheless, the most striking difference between the BY-2 plastid proteome and the chloroplast proteome is that a significantly higher percentage of identified proteins are involved in amino acid metabolism in the BY-2 cells (25% versus 7%). Using 2D electrophoresis gels with IPG strips in the first dimension, the rice etioplast proteome was analyzed (Kleffmann et al., 2007). This showed that the etioplast allocates the main proportion of total protein mass to carbohydrate and amino acid metabolism and a surprisingly high number of proteins to the regulation and expression of plastid genes. Chaperones, proteins for photosynthetic energy metabolism, and enzymes of the tetrapyrrole pathway were identified among the most abundant etioplast proteins.

D. Cross-Correlating Proteomics Information to Functional Studies

Chloroplast proteome analyses typically do not provide much functional information about specific proteins. In addition, when new proteins are discovered it is often very hard to make a good prediction of their molecular function, even if a protein has predicted functional domains. Therefore, it is important to cross-correlate protein accessions identified in proteomics studies to published literature. This can be a very time consuming process, although some of this is now carried out in a centralized way by the various organismal databases, such as TAIR (http://www.arabidopsis.org/) for *A. thaliana* and Gramene (http://www.gramene.org/) for maize, rice and other grasses. My lab is making a specific effort in this respect on chloroplast proteins identified in *A. thaliana* (http://ppdb.tc.cornell.edu/) (see further Section VI).

III. Protein Complexes and Dynamic Protein-Protein Interactions in Photosynthesis

A. Tools and Techniques

The function of many proteins requires stable or transient protein-protein interactions. Identifying these interactions by MS relies on various biochemical techniques, such as affinity purification of transgenic proteins fused to specific epitopes (e.g., hemagglutinin, Strep, $(His)_6$, tandem affinity tag or TAP) and native gel-based protein separation. Large scale protein-protein interaction studies, using expression of affinity-tagged proteins and MS, identified hundreds of protein complexes in yeast (Gavin et al., 2002, 2006; Ho et al., 2002; Krogan et al., 2006) and *E. coli* (Butland et al., 2005). However, no such large scale experimental protein interaction studies (other than yeast 2 hybrid) have (yet) been pursued successfully for photosynthetic organisms. However, several smaller scale analyses of protein-protein interactions and protein complexes involved in (regulation of) photosynthesis have been carried out and will be discussed in this section. Studies on individual complexes (with the exception of the His-tagged NDH complex and the assembly of fascinating dynamic vesicle-inducing protein in plastids 1 (VIPP1) structures) will not be discussed here.

B. Protein Complexes and Protein-Protein Interactions Identified by Native Gels and Affinity Chromatography

As mentioned in Section II, the NDH complex is of much lower abundance than the other complexes in the photosynthetic electron transport chain and its composition has not been clearly established. To identify the NDH composition, tobacco chloroplast-encoded NDH-H was His tagged at its N-terminus using plastid transformation, purified by Ni^{2+} affinity chromatography and its subunit composition analyzed by MS (Rumeau et al., 2005). Five plastid-encoded subunits (NDH-A, -H, -I, -J, and -K) were identified as well as three new subunits (NDH-M, -N, and -O). *A. thaliana* mutants missing one of these new subunits lack a functional NDH complex, and

NDH-M and NDH-N are not detected in a tobacco transformant lacking the NDH complex (Rumeau et al., 2005). Other mutant studies have suggested two additional NDH subunits, assigned Crr3 and Crr7 (Kamruzzaman Munshi et al., 2005).

The procedure for 'Blue Native' gel electrophoresis (BN-PAGE) was originally developed to separate membrane complexes (Schagger and von Jagow, 1991; Schagger et al., 1994). With the advent of proteomics and MS, these BN-PAGE gels have become increasingly popular and have been widely used to study the dynamics (see Section V) and steady state of the thylakoid proteome. A refreshed protocol was published in 2006 (Wittig et al., 2006). As described in the papers from the research group(s) of Hermann Schagger and Gebhard von Jagov, BN-PAGE is a "charge shift" method developed for the isolation of native membrane protein complexes solubilized by non-ionic detergent from biological membranes. To facilitate migration of both basic and acidic proteins and complexes in proportion to their native mass, proteins are 'coated', in this method, with negatively charged Coomassie Blue (similar to SDS as in SDS-PAGE). When followed by (denaturing) SDS-PAGE, it provides an analytical method for the determination of molecular mass and oligomeric state of complexes as well as its subunit composition (Schagger et al., 1994).

BN-PAGE was used to study the oligomeric state and composition of (abundant) protein complexes of thylakoids of *A. thaliana* (Heinemeyer et al., 2004), spinach (Danielsson et al., 2006), maize (Darie et al., 2005) and barley (Ciambella et al., 2005; Granvogl et al., 2006). As will be discussed in Section IV, BN-PAGE was also used for comparative studies. These published 2D BN-PAGE studies in combination with MS have mainly served to identify most of the ~50–70 proteins of PS II, PS I, ATP synthase, and the cytochrome b_6f complex, and to determine the various oligomerization states. A few of the subunits of the NDH complex were also identified. Here, we highlight one of the earlier studies using BN-PAGE gels and MS to investigate the supramolecular structure of photosystems in *A. thaliana* using the detergent digitonin rather than β-dodecylmaltoside. Nine photosystem supercomplexes were described with apparent molecular masses between 600 and 3,200 kDa on BN gels. Identities of the supercomplexes were determined on the basis of their subunit compositions, as documented by 2D BN/SDS-PAGE and BN/BN-PAGE. Two supercomplexes of 1,060 and ~1,600 kDa represent dimeric and trimeric forms of PS I, which include tightly bound LHCI proteins. Compared to monomeric PS I, these protein complexes are of low abundance. In contrast, PS II mainly forms part of dominant supercomplexes of 850, 1,000, 1,050 and 1,300 kDa. It was suggested that these supercomplexes contain dimeric PS II, 1–4 LHCII trimers and additionally monomeric LHCII proteins. The 1,300-kDa PS II supercomplex (containing four LHCII trimers) is partially converted into the 1,000-kDa PS II supercomplex (containing two LHCII trimers) in the presence of dodecylmaltoside on 2D BN/BN gels. An excellent review on the various assembly states of these photosynthetic complexes has been presented by Aro et al. (2005). It is important to point out that BN-PAGE has not been used for the discovery of low abundant proteins and their association into complexes. However, analysis of thylakoid and envelope membranes of bundle sheath and mesophyll cells from maize using just one dimensional separation with BN-PAGE gels, in combination with highly sensitive MS/MS analysis (LTQ-Orbitrap), has identified about 500 proteins in or associated with the chloroplast membranes (Majeran et al., 2008).

H. Schagger and G. von Jagow also developed so called colorless native (CN) PAGE, which is a milder procedure than BN-PAGE, retaining better complex stability and is particularly useful for soluble complexes (Schagger et al., 1994). As mentioned in Section II, CN-PAGE was used to produce a 'snap-shot' of the oligomeric chloroplast proteome of *A. thaliana*, isolated during the first half of the 10 h light period from complete rosettes of 6-week-old plants (Peltier et al., 2006). The effective native mass range on the CN-PAGE gels was about 10–950 kDa. Hardly any destabilization of complexes took place during separation in the first dimension, as evidenced by the near absence of 'streaking' in that dimension. About 10% of the 241 proteins identified in this study were found in a monomeric state. More than 85% of the proteins were essentially (within a ~10 kDa mass range) found at a single native mass. The remaining 15%

were found at multiple native masses, either because they were abundant – and trace amounts of streaking were enough to identify the protein by the sensitive MS measurements, or because they likely interacted with different partners or formed different homo-oligomeric states. Searching the literature for native mass information for each of the identified stromal proteins or their homologues, it was surprising to find that ~60% of the complexes reported in the literature are homomeric rather than heteromeric. These complexes are involved in a wide variety of metabolic activity without any particular bias for metabolic function. In a few cases, it appeared that these complexes are homomeric in chloroplasts, but heteromeric in non-plant species. It is not clear if there is a more general trend for a higher degree of homo-oligomerization in plastids than elsewhere in the plant or in other non-plant species. The prevalence of homomeric complexes over heteromeric complexes in the published literature is striking and warrants examination. Does this reflect a bias due to the methodology used (mostly non-denaturing chromatography involving salt) and proteins studied, or does this truly reflect the protein-interaction network in plastids? If indeed, homo-oligomers prevail over heteromers, what could be its benefit for the function of the plastids? It might be easier to regulate protein complex concentrations if they consist of only one gene product. Maybe many interactions in vivo are in fact between different homo-oligomers forming larger hetero-oligomers, but these interactions are more dynamic and less stable than those between homomers themselves. Currently, it is difficult to evaluate any of these explanations. Different types of protein-protein interaction studies are required, as well as in vivo monitoring of protein-protein interactions using techniques such as fluorescent resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) (Kluge et al., 2004; Subramanian et al., 2004; Bhat et al., 2005).

Finally, worth mentioning is a very interesting oligomerizing protein named VIPP1 (vesicle-inducing protein in plastids 1) with homology to phage shock protein A (PspA) in *E. coli*. VIPP1 was initially identified from a highly successful forward genetics screen of photosynthetic T-DNA mutants in *A. thaliana*; Loss of VIPP1 expression is deleterious to thylakoid membrane formation (Kroll et al., 2001). A subsequent study suggested that VIPP1 is needed for the formation of the thylakoid bilayer, rather than the functional assembly of thylakoid protein complexes (Aseeva et al., 2007). VIPP1 is mentioned in this section on protein-protein interactions, because multiple lines of evidence show that VIPP1 in *C. reinhardtii* and *A. thaliana* forms protein complexes of various sizes and shapes (homomeric dimers, rings and rods), likely driven by the HSP70 chaperone machinery (DnaK/J) in the chloroplast. (Aseeva et al., 2004, 2007; Liu et al., 2005; Liu et al., 2007; Willmund et al., 2007). Oligomerization appears important for VIPP1 function and a minimal concentration is needed in the chloroplast to establish these oligomers and carry out VIPP1 function. The VIPP1 complex is found in several chloroplast fractions (thylakoid, stroma and inner envelope) which could be a reflection of functional interactions.

Thioredoxins (TRX) and glutaredoxins (GRX) are small multi-functional redox active proteins present in many organisms and their interaction with other proteins is typically of transient nature. In chloroplasts, four types of thioredoxins (f, m, x, y) and at least one glutaredoxin (GRXcp) are known, as well as a dozen or more thioredoxin-like protein (e.g., NTRC, CDSP32, HCF164) (Collin et al., 2003; Buchanan and Balmer, 2005; Michelet et al., 2006; Lemaire et al., 2007). These redox active proteins play central roles in regulating enzyme activity in the Calvin-Benson cycle as well as in other chloroplast metabolic pathways and even in components of the chloroplast biogenesis machinery; more than 100 putative targets have now been identified in chloroplasts of higher plants and *C. reinhardtii* (Lemaire et al., 2007). Proteomics has played a significant role in the identification of targets for these redox proteins. Two types of proteomics experiments have been used to identify targets: (i) affinity purification using immobilized monocysteinic thioredoxin or glutaredoxin, using dithiothreitol for elution and MS for identification, and (ii) in vitro labeling of target proteins by labeling with exposed thiols after reduction by thioredoxin. For details, refer to reviews by Buchanan and Balmer (2005) and Lemaire et al. (2007). The challenge for the near future is to determine target specificities and unravel further interplay between the different redox active components in chloroplasts. For further discussion, see others (Motohashi et al., 2001; Buchanan and Balmer, 2005; Michelet et al., 2006).

IV. Differential Protein Accumulation and Dynamic Changes of the Chloroplast Proteome

A. Background

The possibilities to compare quantitative differences in protein accumulation between different protein samples have greatly improved over the last decade. Initially, the most viable technique was based on the image analysis of stained proteins separated on various types of 2D gels. The 2D gel approach has been widely used for quantitative studies of the photosynthetic apparatus as will be discussed below. Over the last several years various techniques based on relative quantification in the mass spectrometer have provided excellent opportunities to improve throughput, obtain higher accuracies and also quantification of membrane proteins. To accommodate such MS-based quantification, proteins from the samples that are to be compared are differentially labeled with stable isotopes. This can be done through metabolic labeling using C-12/C-13, N-14/N-15 during plant or cell growth and in the case of cell cultures also using stable isotope labeled amino acids (Gruhler et al., 2005; Naumann et al., 2005; Huttlin et al., 2007; Lanquar et al., 2007; Nelson et al., 2007). Alternatively, isotope labels can be introduced after purification of the plant proteins, using cross-linking of small, isotope-labeled peptides, such as cICAT (Majeran et al., 2005), iTRAQ (Rudella et al., 2006) and D,H-formaldehyde (Ytterberg et al., 2006) or via trypsin catalyzed ^{18}O labeling (Nelson et al., 2006). Several of these techniques have been used to study differential accumulation of chloroplast proteins as will be reviewed below.

In the last few years, the so-called label free comparative proteomics has emerged as a possible alternative, in particular when using fast and highly accurate mass spectrometers (Wiener et al., 2004; Listgarten and Emili, 2005; Majeran et al., 2005; Old et al., 2005; Wang et al., 2006; Meng et al., 2007). We can expect that this technique will be used to study changes in the photosynthetic apparatus in the near future. For general information on quantitative proteomics, see reviews by Goshe and Smith (2003), Tao and Aebersold (2003) Julka and Regnier (2004) and Domon and Aebersold (2006).

B. Biogenesis and Differentiation of the Photosynthetic Apparatus Studied by Proteomics

Since the discovery that the D1 reaction center protein of PS II has a much shorter life-time than the rest of the PS II complex, hundreds of published studies have investigated the repair cycle of PS II – reviewed by Aro et al. (2005). BN-PAGE in combination with pulse-chase labeling, Western blots and MS, has become a crucial tool to study the process of PS II disassembly and reassembly (Aro et al., 2005; Rokka et al., 2005). These studies identified the most abundant proteins in the thylakoid membrane although accumulation levels were not quantified, but rather assessed 'semi-quantitatively'. As cited above, these studies were reviewed and will not be further discussed here. It is relevant to point out that these studies did not identify auxiliary proteins, possibly involved in this process, since these auxiliary proteins are not detectable by protein staining due to their much lower accumulation levels (several orders of magnitude lower than the photosynthetic apparatus).

Dark grown plants accumulate non-photosynthetic etioplasts with a distinctive prolamellar body. Upon transfer to light, etioplasts develop into chloroplasts over the period of 1–2 days. Two papers on proteomics addressed this transition in maize (Lonosky et al., 2004) and rice (Kleffmann et al., 2007), both using 2D gels, with denaturing IEF in the first dimension and SDS PAGE in the second dimension. The maize study was mostly of a technical nature and focused on the evaluation of statistical methods to analyze the patterns of expression of 526 "high-quality," unique spots on the 2D gels (Lonosky et al., 2004). The rice study (Kleffmann et al., 2007) showed that the etioplast allocates the main proportion of total protein mass to carbohydrate and amino acid metabolism and a surprisingly high number of proteins to the regulation and expression of plastid genes. Chaperones, proteins for photosynthetic energy metabolism, and enzymes of the tetrapyrrole pathway were identified among the most abundant etioplast proteins. The transition from heterotrophic metabolism to photosynthesis-supported autotrophic metabolism was already detectable 2 h after illumination and affected most essential metabolic modules. Enzymes in carbohydrate metabolism, photosynthesis, and gene

expression were up-regulated, whereas enzymes in amino acid and fatty acid metabolism were significantly decreased in relative abundance. Enzymes involved in nucleotide metabolism, tetrapyrrole biosynthesis, and redox regulation remained unchanged. Phosphoprotein-staining with Diamond Q revealed light-induced phosphorylation of a nuclear-encoded plastid RNA-binding protein. Quantitative information about all the identified proteins and their regulation by light is now available in plprot, a plastid proteome database (http://www.plprot.ethz.ch) (see Section VI).

To better understand bundle sheath and mesophyll chloroplast functions in maize leaves, stromal proteomes of bundle sheath and mesophyll chloroplasts were purified and analyzed using three independent techniques, namely 2D electrophoresis PAGE, isotope coded affinity tags (ICAT) and label-free analysis by Q-TOF MS (Majeran et al., 2005). It was found that enzymes involved in lipid biosynthesis, nitrogen import, and tetrapyrrole and isoprenoid biosynthesis are preferentially located in the mesophyll chloroplasts. By contrast, enzymes involved in starch synthesis and sulfur import preferentially accumulate in bundle sheath chloroplasts. The different soluble antioxidative systems, in particular peroxiredoxins, accumulate at higher levels in mesophyll chloroplasts. We also observed differential accumulation of proteins involved in expression of plastid-encoded proteins (e.g., EF-Tu, EF-G, and mRNA binding proteins) and thylakoid formation (VIPP1), whereas others were equally distributed. Enzymes related to the C4 shuttle, the carboxylation and regeneration phase of the Calvin-Benson cycle, and several regulators (e.g., CP12) distributed as expected. However, enzymes involved in triose phosphate reduction and triose phosphate isomerase are primarily located in the mesophyll chloroplasts, indicating that the mesophyll-localized triose phosphate shuttle should be viewed as part of the bundle sheath-localized Calvin-Benson cycle, rather than a parallel pathway. Majeran et al. (2005) also point out the strengths and the weaknesses of the three methodologies and comment on the success rates with the various genome/EST databases for MS-based protein identification. Proteomics data from these projects were integrated and made accessible in the Plastid Proteome Data Base (http://ppdb.tc.cornell.edu/) (Majeran et al., 2005).

Wolfgang Haehnel and colleagues analyzed the composition of the thylakoid NDH complex from differentiated bundle sheath and mesophyll cells using native gels (Darie et al., 2005). The NDH complex forms dimers at 1,000–1,100 kDa in both bundle sheath and mesophyll chloroplasts. Native/PAGE of the bundle sheath and mesophyll chloroplasts allowed us to determine that the NDH complex contains at least 14 different subunits. The molecular mass of the NDH complex (550 kDa) can be dissociated into a 300 kDa membrane subcomplex (containing NDHE) and a 250 kDa subcomplex (containing NDHH, -J and -K) (Darie et al., 2005).

C. Changes in the Chloroplast Proteome in Response to Changes in the Environment and Abiotic Stress

In this section, studies analyzing the proteome response to light stress, cold stress and iron deficiency of chloroplast subcompartments in a variety of higher plants and in *C.reinhardtii* are briefly reviewed. Most of these studies used 2D electrophoresis with IEF in the first dimension, quantifying the differential protein accumulation by spot image analysis (Andaluz et al., 2006; Giacomelli et al., 2006; Goulas et al., 2006). The proteome responses in two studies were determined from MS-based quantification using metabolic labeling (Naumann et al., 2005) or H,D formaldehyde labeling (Ytterberg et al., 2006).

1. Cold Stress

Changes in the soluble thylakoid lumen and stromal proteomes in *A. thaliana* were determined after 1 day of cold shock, and short (10 days) and long-term (40 days) acclimation to 5°C. (Goulas et al., 2006). Differential 2D PAGE stained with fluorescence dyes (see Marouga et al. (2005)) – was used for protein separation and quantification. Cold shock resulted in minimal changes, while short-term (10 days) acclimation resulted in several changes in the stromal, but few changes in the lumen proteome. Further, long-term acclimation (40 days) resulted in differential accumulation of 8 lumenal proteins (oxygen-evolving complex proteins, isomerases and HCF136) and 35 stromal or peripheral thylakoid proteins (several Calvin-Benson cycle enzymes), other plastid

metabolic functions, hormone changes and several stress responses (Goulas et al., 2006).

2. Light Stress

The thylakoid proteome of chloroplasts contains multiple proteins involved in anti-oxidative defense, protein folding, and repair. To understand this functional protein network, the quantitative response of the thylakoid-associated proteome of *A. thaliana* wild type and the ascorbate-deficient mutant *vtc2-2* was studied after transition to high light (HL; 1,000 µmol photons m^{-2} s^{-1}). The soluble thylakoid proteomes of wild type and *vtc2-2* were compared after 0, 1, 3, and 5 day of HL using 2D-PAGE in three independent experiments, followed by a multi-variant statistical analysis and MS/MS. After 5 day of HL, both wild-type and *vtc2-2* plants accumulated anthocyanins, increased their total ascorbate content, and lost 10% of PS II efficiency, but showed no bleaching. Forty-five protein spots significantly changed as a consequence of genotype, light treatment, or both. Independent confirmation was obtained from western blots. The most significant response was the up-regulation of thylakoid YCF37, likely involved in PS I assembly, and specific fibrillins, a flavin reductase-like protein, and an aldolase, each located in thylakoid-associated PGs. Their assignment to PGs was based on studies of the PG proteome (Vidi et al., 2006; Ytterberg et al., 2006). Fe-superoxide dismutase was down-regulated in *vtc2-2* plants, while Cu,Zn-superoxide dismutase was up-regulated. The *vtc2-2* mutant also showed a systematic up-regulation of a steroid dehydrogenase-like protein. A number of other stress-related proteins, several thylakoid proteases, and lumenal isomerases did not change, while PsbS increased in wild type upon light stress. These findings were discussed in terms of plastid metabolism and oxidative stress defense; they emphasize that an understanding of the chloroplast stress-response network must include the enzymatic role of PGs (Giacomelli et al., 2006). In a separate study, the quantitative differences in PG composition in response to high light stress and degreening were determined by differential stable-isotope labeling using H,D formaldehyde (Ytterberg et al., 2006). Significant differential accumulation was observed for several fibrillins, carotenoid cleavage dioxygenase CCD4, an ABC1 kinase, and several PG specific proteins with unknown function(s). This study is in agreement with the idea that PGs play an active role in stress defense.

3. Iron Deficiency

Changes induced in the thylakoid proteome by iron deficiency from *Beta vulgaris* (sugar beet) grown in hydroponics were analyzed by IEF-SDS PAGE and BN-SDS PAGE (Andaluz et al., 2006). There were significant iron deficiency-induced changes in the thylakoid proteome; in particular the relative amounts of electron transfer protein complexes were reduced, whereas those of proteins participating in leaf carbon fixation were increased. The response of the thylakoid proteome to iron deficiency in *C. reinhardtii* was analyzed in several studies (Moseley et al., 2002; Naumann et al., 2005). The main conclusion from these studies was that the iron deficiency induces a remodeling of the photosynthetic apparatus. A key event in the remodeling process of PS I and its associated light-harvesting proteins (LHCI) is the N-terminal processing of Lhca3; this was also shown independently by 2D electrophoresis and MS/MS, as well as by quantitative comparative MS/MS peptide profiling using metabolic labeling with 12C,13C-arginine. In addition, accumulation of Lhca5 was down-regulated, whereas Lhca4 and Lhca9 were up-regulated. These events correlated with a loss of excitation energy transfer efficiency between LHCI and PS I. The responses of *C. reinhardtii* to various abiotic stresses have also been reviewed (Nield et al., 2004; Merchant et al., 2006).

V. Post-Translational Protein Modifications of the Photosynthetic Proteome

A. Introduction

After their synthesis, proteins are often modified in reversible or irreversible ways. Examples of such modifications include: phosphorylation, lipidation, protein processing and glycosylation (Huber and Hardin, 2004). These PTMs play an important role in protein subcellular localization, protein stability, as well as protein function and

protein-protein interactions. High resolution MS, in combination with enrichment techniques for individual PTMs (e.g., metal affinity columns for phosphopeptides) is now used widely. Several excellent reviews summarize the various approaches (Jensen, 2004, 2006a). Although still in its infancy, significant progress has been made in the study of PTMs of the photosynthetic apparatus, particularly regarding phosphorylation of the thylakoid proteome and PTMs induced by oxidative stress. The last few years have seen a real break-through in the understanding of the role of phosphorylation in regulation of state transition and thylakoid adaptation to light conditions.

B. Regulation of Thylakoid State Transitions, Photosystem II Repair and Acclimation by Reversible Phosphorylation

Phosphorylation plays a major role in cellular regulation at nearly every level imaginable. Proteomics and MS now provide enormous opportunities to determine phosphorylation of specific amino acid residues on a large scale, identifying thousands of phosphorylated proteins in a protein sample – for methodology see various reviews (Glinski and Weckwerth, 2006; Jensen, 2006a; Larsen et al., 2006; Turkina and Vener, 2007).

Developments in MS played an important role in determining the phosphorylation status of the thylakoid proteins and in capturing quantitative changes in the phosphorylation status, particularly in response to changes in abiotic conditions (e.g., light quality and intensity), as well as in the context of thylakoid kinase mutants in *C. reinhardtii* and *A. thaliana*. Within the context of photosynthesis, progress in the area of thylakoid phosphoproteomics and state transitions has been impressive in the last several years. Excellent reviews on thylakoid phosphorylation, thylakoid kinases, state transitions, acclimation and other related aspects were recently published (Tikkanen et al., 2006; Rochaix, 2007). I will only briefly summarize this topic.

After a long search, a forward genetic approach on a *C. reinhardtii* mutant, using chlorophyll fluorescence as the primary screen, identified a chloroplast thylakoid-associated serine-threonine protein kinase, Stt7. The protein Stt7 is required for the phosphorylation of the major light-harvesting protein (LHCII) and for state transitions (Depege et al., 2003). The orthologue of Stt7 in *A. thaliana* is STN7. Analysis of *stn7* mutant plants showed that loss of STN7 blocks state transitions (locking the thylakoid in state 1) and LHCII phosphorylation and growth is impaired under changing light conditions. This indicates that STN7, and probably state transitions, have an important role in response to environmental changes (Bellafiore et al., 2005). In an independent study, it was shown that the STN7 kinase also phosphorylates Lhcb4.2 (CP29). Upon growth of *A. thaliana* wild type and *stn7* plants under low and moderate light conditions, the wild type favored state 2 whereas *stn7* plants were locked in state 1. The lack of the STN7 kinase and state transitions in *stn7* plants also modified the thylakoid protein content upon long-term low light acclimation resulting, for example, in the reduction of Lhcb1 and increases in Lhca1 and Lhca2. Adjustment in thylakoid protein content probably occurred at the post-transcriptional level since DNA microarray experiments from each growth condition did not reveal any significant differences between the transcriptomes of *stn7* and wild-type plants. The resulting high Lhcb2/Lhcb1 ratio in the *stn7* mutant upon growth at low light was accompanied by a lowered capacity for non-photochemical quenching compared with wild type. In contrast, higher amounts of thylakoid protein PsbS in *stn7* plants under moderate and high light growth conditions resulted in higher non-photochemical quenching compared to wild type and consequently also in the protection of PS II against photoinhibition. STN7 kinase and the state transitions are suggested to have a physiological significance for dynamic acclimation to low but fluctuating growth light conditions. They have been shown to function as a buffering system upon short high light illumination peaks by shifting the thylakoids from state 2 to state 1 and thereby down regulating the induction of stress-responsive genes, a likely result from transient over-reduction of PS I acceptors (Tikkanen et al., 2006).

A. thaliana has a second thylakoid protein kinase, STN8 (homologous to Stl8 in *C. reinhardtii*) which specifically phosphorylates the N-terminal threonine residues in D1, D2, and CP43 proteins, and Thr-4 in the PsbH protein of PS II (Vainonen et al., 2005). Phosphorylation of Thr-4 in the wild type required both light and

prior phosphorylation at Thr-2, indicating that STN8 is a light-activated kinase that phosphorylates Thr-4 only after another kinase phosphorylates Thr-2. PS I activity under high-intensity light is affected only slightly in *stn8* mutants, and D1 turnover is indistinguishable from the wild type (Bonardi et al., 2005).

In a related study, thylakoid proteome phosphorylation in *C. reinhardtii* was studied under four different conditions: (i) dark aerobic, corresponding to photosynthetic state 1; (ii) dark under nitrogen atmosphere, corresponding to photosynthetic state 2; (iii) moderate light; and (iv) high light (Turkina et al., 2006a): Nineteen phosphorylation sites were identified in a total of 15 proteins. The state 1-to-state 2 transition induced phosphorylation of the PS II core components D2 and PsbR and quadruple phosphorylation of a minor LHCII antennae subunit, CP29, as well as phosphorylation of constituents of a major LHCII complex, Lhcbm1 and Lhcbm10. Exposure to either moderate or high light caused additional phosphorylation of the D1 and CP43 proteins of the PS II core. The high light treatment led to specific hyperphosphorylation of CP29 at seven residues, phosphorylation of another minor LHCII constituent, CP26, at a single threonine, and double phosphorylation of additional subunits of a major LHCII complex including Lhcbm4, Lhcbm6, Lhcbm9, and Lhcbm11. It was concluded that protein phosphorylation at the interface of the PS II core and the associated antenna proteins, particularly multiple differential phosphorylations of the CP29 linker protein, play a central role in LHCII uncoupling from PS II (Turkina et al., 2006b). Thylakoid phosphorylation studies on the acclimation of *C. reinhardtii* to limiting environmental CO_2 identified several phosphopeptides on the stroma-exposed side of LHCI-5 protein, which is encoded by a low-CO_2-inducible gene. Phosphorylation of LHCI-5 occurred strictly at limiting CO_2, but was not induced by light and did not require Stn7 (Turkina et al., 2006a).

C. N-Terminal Modifications are Required for Functional Chloroplasts

As discussed by Van Wijk (2004a, b), N-terminal modifications of chloroplast proteins are important for chloroplast functions. Since not much progress has been made in this area, I briefly summarize some of the main observations. Nuclear-encoded chloroplast proteins undergo N-terminal processing in the stroma during and immediately after translocation through the envelope translocon. It has been shown that N-terminal acetylation in the cytosol of nuclear-encoded chloroplast proteins is required for chloroplast functions (Pesaresi et al., 2003). Chloroplast-encoded proteins are synthesized with formylated methione. Chloroplast deformylase is present to remove the formyl group (Giglione et al., 2000; Dirk et al., 2001, 2002; Giglione and Meinnel, 2001) likely followed by removal of the methione by an methione endopeptidase (Giglione et al., 2003; Ross et al., 2005). With the availability of highly accurate (experimental error less than 3–6 ppm), highly resolving (100,000) and sensitive (femtomole range) mass spectrometers (LTQ-Orbitrap and various FTICR instruments), it will be possible to study these N-terminal modifications in far greater detail.

D. Reversible and Irreversible Protein Modifications of the Photosynthetic Machinery as a Consequence of Oxidative Stress

Considerable progress has been made in the detection of irreversible protein carbonylation and the reversible methionine sulfoxide reaction, both resulting from oxidative stress in the chloroplast. Protein carbonylation is an irreversible oxidative process leading to a loss of the function of the modified proteins; in a variety of model systems, including worms, flies, and mammals, carbonyl levels gradually increase with age (Nystrom, 2005). However, in *A. thaliana* an initial increase in protein oxidation during the first 20 days of the life cycle of the plant is followed by a drastic reduction in protein carbonyls prior to bolting and flower development (Johansson et al., 2004). Protein carbonylation prior to the transition to flowering, targets specific proteins such as Hsp70, ATP synthases, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and proteins involved in light harvesting/energy transfer and the C2 oxidative photosynthetic carbon cycle. It should be noted that this study only identified carbonylation of the most abundant (and photosynthetic) proteins in *A. thaliana*. An *A. thaliana* mutant with loss of expression of

a chloroplast glutaredoxin displayed increased protein carbonylation within chloroplasts (Cheng et al., 2006). It seems logical to extrapolate this observation and postulate that the extensive oxidative stress defense network in chloroplasts likely plays a significant role in the protection against protein carbonylation.

Generation of methionine sulfoxide (MetSo) results in the modification of activity and conformation of proteins, including those in the chloroplasts. The MetSo modification can be reversed by methionine sulfoxide reductase (MSR). Chloroplasts contain three MSR proteins (MSRA4, MSRB1, MSRB2) (Sadanandom et al., 2000; Vieira Dos Santos et al., 2005) that are highly expressed in photosynthetic organs. Thioredoxins (TRXs) constitute very likely physiological electron donors to plant MSR proteins for the catalysis of MetSO reduction, but the specificity between the numerous TRXs and methionine sulfoxide reductases (MSRs) present in plants remains to be investigated. The essential role of plant MSRs in protecting plants against oxidative damage was demonstrated in 2006 in transgenic *A. thaliana* plants modified in the content of cytosolic or plastidic MSRA (Rouhier et al., 2006). Transgenic *A. thaliana* lines were generated in which MSRA4 expression ranged from 95% to 40% (antisense) and more than 600% overexpressing lines of wild-type plants (Romero et al., 2004). Under optimal growth conditions, there was no effect of the transgene on the phenotype of the plants. When exposed to different oxidative stress conditions – methyl viologen, ozone, and high light – differences were observed in the rate of photosynthesis, the maximum quantum yield of PS II (as measured by the ratio of variable to maximal chlorophyll fluorescence, i.e., the Fv/Fm ratio), and the Met sulfoxide content of the isolated chloroplasts. Plants that overexpressed PMSR4 were more resistant to oxidative damage localized in the chloroplast, and plants that underexpressed PMSR4 were more susceptible. The Met sulfoxide levels in proteins of the soluble fraction of chloroplasts were increased by methyl viologen and ozone, but not by high light treatment. Under stress conditions, the overexpression of PMSR4 lowered the sulfoxide content and underexpression resulted in an overall increase in content (Romero et al., 2004).

VI. Proteome Resources on the Web and *in silico* Analysis

A. Prediction of Plastid Proteins in Different Plant Species and Chlamydomonas reinhardtii

The vast majority of chloroplast proteins is encoded by the nuclear genome and is targeted into the chloroplast based on information in the N-terminal transit peptide (cTP). Shared features in these cTPs have allowed training of various chloroplast localization predictors each with different levels of false positive- and false negative rates. Various subcellular predictors and reflections on strengths, weaknesses and pitfalls were discussed by Emanuelsson et al. (2007). The increasing amount of established chloroplast proteins (for positive test/training sets), as well as established 'non-chloroplast' proteins (for negative test/training sets) through proteome analysis will provide new opportunities to retrain or redesign these chloroplast predictors for different plant and algal species.

B. Plastid Databases

The increasing amounts of chloroplast (and other) proteomics data are only fully meaningful when they can be easily accessed and judged on their quality. Accessibility of data, quality control, redundancy removal and connectivity to other types of biological information are therefore essential. Two databases have been created for plastid proteome information: Plprot (http://www.plprot.ethz.ch/) (Kleffmann et al., 2006) integrates protein accessions identified by proteomics in different plastid types, including etioplasts, chloroplasts, chromoplasts and non-green plastids from tobacco BY2 cells. PPDB (http://ppdb.tc.cornell.edu/) (Friso et al., 2004) is focused on the (cell-type specific) chloroplast proteomes from maize and *A. thaliana* and provides also the in-house experimental information on the MS based identifications (e.g., Mowse scores and peptide sequences). In addition, it provides information on the predicted localizations, protein function, post-translational modifications and sub-chloroplast locations, as well as dynamic cross-referencing to the experimental literature.

VII. Conclusions and Challenges

Peptide mass finger printing and MS/MS based identification of proteins emerged about a decade ago, with its first application on the study of photosynthesis and chloroplasts in 2000. From there on, the impact of proteomics and protein MS has been very significant, with initially most efforts placed on the identification of chloroplast proteins, followed by phosphorylation, comparative proteomics and work on protein-protein interactions. Most of this work has been carried out with *A. thaliana*, the first plant to have its genome fully sequenced. With the exception of some small, very hydrophobic thylakoid membrane proteins, most of the photosynthetic machinery has been observed by mass spectrometry, but many post-translational modifications remain to be discovered. One of the biggest challenges of proteomics in the area of photosynthesis will be to identify and characterize proteins with regulatory functions, as well as proteins involved in chloroplast development and biogenesis and adaptation. The increasing amount of established chloroplast proteins (for positive test/training sets), as well as established 'non-chloroplast' proteins (for negative test/training sets) through proteome analysis will provide new opportunities to retrain or redesign these chloroplast predictors. Of course, although *A. thaliana* serves as an excellent experimental system, the use of other sequenced C-3 plant species such as rice, as well as C-4 species such as maize and sorghum should help us accelerate the exploration of the biological variations, and the study of cell-specific differentiation, that are relevant to photosynthesis.

Note: Since the completion of this chapter, the field has progressed and we refer the readers to see a focus issue of the journal *Plant Physiology* on "Plastid Biology: Focus on the Defining organelle of Plastids (Volume 154, # 4, April, 2011, pp. 1475–1588). For update and further current information on the topic of this chapter, see Van Wijk and Baginsky (2011).

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References

Adachi J, Kumar C, Zhang Y, Olsen JV and Mann M (2006) The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 7: R80

Allmer J, Markert C, Stauber EJ and Hippler M (2004) A new approach that allows identification of intron-split peptides from mass spectrometric data in genomic databases. FEBS Lett 562: 202–206

Allmer J, Naumann B, Markert C, Zhang M and Hippler M (2006) Mass spectrometric genomic data mining: Novel insights into bioenergetic pathways in *Chlamydomonas reinhardtii*. Proteomics 6: 6207–6220

Andaluz S, Lopez-Millan AF, De las Rivas J, Aro EM, Abadia J and Abadia A (2006) Proteomic profiles of thylakoid membranes and changes in response to iron deficiency. Photosynth Res 89: 141–155

Andon NL, Hollingworth S, Koller A, Greenland AJ, Yates JR, 3rd and Haynes PA (2002) Proteomic characterization of wheat amyloplasts using identification of proteins by tandem mass spectrometry. Proteomics 2: 1156–1168

Aro EM, Suorsa M, Rokka A, Allahverdiyeva Y, Paakkarinen V, Saleem A, Battchikova N and Rintamaki E (2005) Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. J Exp Bot 56: 347–356

Aseeva E, Ossenbühl F, Eichacker LA, Wanner G, Soll J and Vothknecht UC (2004) Complex formation of Vipp1 depends on its alpha-helical PspA-like domain. J Biol Chem 279: 35535–35541

Aseeva E, Ossenbuhl F, Sippel C, Cho WK, Stein B, Eichacker LA, Meurer J, Wanner G, Westhoff P, Soll J and Vothknecht UC (2007) Vipp1 is required for basic thylakoid membrane formation but not for the assembly of thylakoid protein complexes. Plant Physiol Biochem 45: 119–128

Austin JR, 2nd, Frost E, Vidi PA, Kessler F and Staehelin LA (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell 18: 1693–1703

Baginsky S and Gruissem W (2004) Chloroplast proteomics: potentials and challenges. J Exp Bot 55: 1213–1220

Baginsky S, Siddique A and Gruissem W (2004) Proteome analysis of tobacco bright yellow-2 (BY-2) cell culture

plastids as a model for undifferentiated heterotrophic plastids. J Proteome Res 3: 1128–1137

Baginsky S, Grossmann J and Gruissem W (2007) Proteome analysis of chloroplast mRNA processing and degradation. J Proteome Res 6: 809–820

Barkan A and Goldschmidt-Clermont M (2000) Participation of nuclear genes in chloroplast gene expression. Biochimie 82: 559–572

Bellafiore S, Barneche F, Peltier G and Rochaix JD (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895

Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P and Panstruga R (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc Natl Acad Sci USA 102: 3135–3140

Bonardi V, Pesaresi P, Becker T, Schleiff E, Wagner R, Pfannschmidt T, Jahns P and Leister D (2005) Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. Nature 437: 1179–1182

Brehelin C, Kessler F and Van Wijk KJ (2007) Plastoglobules: versatile lipoprotein particles in plastids. Trends Plant Sci 12: 260–266

Buchanan BB and Balmer Y (2005) Redox regulation: A broadening horizon. Annu Rev Plant Biol 56: 187–220

Buchanan BB and Luan S (2005) Redox regulation in the chloroplast thylakoid lumen: a new frontier in photosynthesis research. J Exp Bot 56: 1439–1447

Buchanan B, Gruissem W and Jones RL (eds) (2000) Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville

Butland G, Peregrin-Alvarez JM, Li J, Yang W, Yang X, Canadien V, Starostine A, Richards D, Beattie B, Krogan N, Davey M, Parkinson J, Greenblatt J and Emili A (2005) Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature 433: 531–537

Cheng NH, Liu JZ, Brock A, Nelson RS and Hirschi KD (2006) AtGRXcp, an *Arabidopsis* chloroplastic glutaredoxin, is critical for protection against protein oxidative damage. J Biol Chem 281: 26280–26288

Ciambella C, Roepstorff P, Aro EM and Zolla L (2005) A proteomic approach for investigation of photosynthetic apparatus in plants. Proteomics 5: 746–757

Collin V, Issakidis-Bourguet E, Marchand C, Hirasawa M, Lancelin JM, Knaff DB and Miginiac-Maslow M (2003) The *Arabidopsis* plastidial thioredoxins: new functions and new insights into specificity. J Biol Chem 21: 21

Danielsson R, Suorsa M, Paakkarinen V, Albertsson PA, Styring S, Aro EM and Mamedov F (2006) Dimeric and monomeric organization of photosystem II. Distribution of five distinct complexes in the different domains of the thylakoid membrane. J Biol Chem 281: 14241-14249

Darie CC, Biniossek ML, Winter V, Mutschler B and Haehnel W (2005) Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays.* FEBS J 272: 2705–2716

Depege N, Bellafiore S and Rochaix JD (2003) Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in *Chlamydomonas*. Science 299: 1572–1575

Dietz KJ, Jacob S, Oelze ML, Laxa M, Tognetti V, de Miranda SM, Baier M and Finkemeier I (2006) The function of peroxiredoxins in plant organelle redox metabolism. J Exp Bot 57: 1697–1709

Dirk LM, Williams MA and Houtz RL (2001) Eukaryotic peptide deformylases. Nuclear-encoded and chloroplast-targeted enzymes in Arabidopsis. Plant Physiol 127: 97–107

Dirk LM, Williams MA and Houtz RL (2002) Specificity of chloroplast-localized peptide deformylases as determined with peptide analogs of chloroplast-translated proteins. Arch Biochem Biophys 406: 135–141

Domon B and Aebersold R (2006) Mass spectrometry and protein analysis. Science 312: 212–217

Dunkley TP, Hester S, Shadforth IP, Runions J, Weimar T, Hanton SL, Griffin JL, Bessant C, Brandizzi F, Hawes C, Watson RB, Dupree P and Lilley KS (2006) Mapping the *Arabidopsis* organelle proteome. Proc Natl Acad Sci USA 103: 6518–6523

Edwards G and Walker DA (1983) C3,C4: mechanisms, and cellular and environmental regulation, of photosynthesis. University of California Press, Berkeley

Emanuelsson O, Brunak S, von Heijne G and Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2: 953–971

Ferro M, Seigneurin-Berny D, Rolland N, Chapel A, Salvi D, Garin J and Joyard J (2000) Organic solvent extraction as a versatile procedure to identify hydrophobic chloroplast membrane proteins. Electrophoresis 21: 3517–3526

Ferro M, Salvi D, Riviere-Rolland H, Vermat T, Seigneurin-Berny D, Grunwald D, Garin J, Joyard J and Rolland N (2002) Integral membrane proteins of the chloroplast envelope: Identification and subcellular localization of new transporters. Proc Natl Acad Sci USA 99: 11487–11492

Ferro M, Salvi D, Brugiere S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J and Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. Mol Cell Proteomics 28: 28

Friso G, Giacomelli L, Ytterberg AJ, Peltier JB, Rudella A, Sun Q and Wijk KJ (2004) In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. Plant Cell 16: 478–499

Froehlich JE, Wilkerson CG, Ray WK, McAndrew RS, Osteryoung KW, Gage DA and Phinney BS (2003) Proteomic study of the *Arabidopsis thaliana* chloroplastic envelope membrane utilizing alternatives to traditional two-dimensional electrophoresis. J Proteome Res 2: 413–425

Gaude N, Brehelin C, Tischendorf G, Kessler F and Dormann P (2007) Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. Plant J 49: 727–739

Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G and Superti-Furga G (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415: 141–147

Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, Marzioch M, Rau C, Jensen LJ, Bastuck S, Dumpelfeld B, Edelmann A, Heurtier MA, Hoffman V, Hoefert C, Klein K, Hudak M, Michon AM, Schelder M, Schirle M, Remor M, Rudi T, Hooper S, Bauer A, Bouwmeester T, Casari G, Drewes G, Neubauer G, Rick JM, Kuster B, Bork P, Russell RB and Superti-Furga G (2006) Proteome survey reveals modularity of the yeast cell machinery. Nature 440: 631–636

Giacomelli L, Rudella A and Van Wijk KJ (2006) High light response of the thylakoid proteome in Arabidopsis wild type and the ascorbate-deficient mutant *vtc2-2*. A comparative proteomics study. Plant Physiol 141: 685–701

Giglione C and Meinnel T (2001) Organellar peptide deformylases: universality of the N-terminal methionine cleavage mechanism. Trends Plant Sci 6: 566–572

Giglione C, Serero A, Pierre M, Boisson B and Meinnel T (2000) Identification of eukaryotic peptide deformylases reveals universality of N-terminal protein processing mechanisms. EMBO J 19: 5916-5929

Giglione C, Vallon O and Meinnel T (2003) Control of protein life-span by N-terminal methionine excision. EMBO J 22: 13–23

Glinski M and Weckwerth W (2006) The role of mass spectrometry in plant systems biology. Mass Spectrom Rev 25: 173–214

Gómez SM, Nishio JN, Faull KF and Whitelegge JP (2002) The chloroplast grana proteome defined by intact mass measurements from liquid chromatography mass spectrometry. Mol Cell Proteomics 1: 46–59

Gomez SM, Bil KY, Aguilera R, Nishio JN, Faull KF and Whitelegge JP (2003) Transit peptide cleavage sites of integral thylakoid membrane proteins. Mol Cell Proteomics 2: 1068–1085

Goshe MB and Smith RD (2003) Stable isotope-coded proteomic mass spectrometry. Curr Opin Biotechnol 14: 101–109

Goulas E, Schubert M, Kieselbach T, Kleczkowski LA, Gardestrom P, Schroder W and Hurry V (2006) The chloroplast lumen and stromal proteomes of *Arabidopsis thaliana* show differential sensitivity to short- and long-term exposure to low temperature. Plant J 47: 720–734

Granvogl B, Reisinger V and Eichacker LA (2006) Mapping the proteome of thylakoid membranes by de novo sequencing of intermembrane peptide domains. Proteomics 6: 3681–3695

Gruhler A, Schulze WX, Matthiesen R, Mann M and Jensen ON (2005) Stable isotope labeling of *Arabidopsis thaliana* cells and quantitative proteomics by mass spectrometry. Mol Cell Proteomics

Haas W, Faherty BK, Gerber SA, Elias JE, Beausoleil SA, Bakalarski CE, Li X, Villen J and Gygi SP (2006) Optimization and use of peptide mass measurement accuracy in shotgun proteomics. Mol Cell Proteomics 5: 1326–1337

Hatch MD and Osmond CB (1976) Compartmentation and transport in C4 photosynthesis. In: Heber U and Stocking C (eds) Encyclopedia of Plant Physiology, Ed New Series, Vol 3, pp 144–184. Springer-Verlag, Berlin

Heinemeyer J, Eubel H, Wehmhoner D, Jansch L and Braun HP (2004) Proteomic approach to characterize the supramolecular organization of photosystems in higher plants. Phytochemistry 65: 1683–1692

Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreault M, Muskat B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CW, Figeys D and Tyers M (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature 415: 180–183

Hu Q, Noll RJ, Li H, Makarov A, Hardman M and Graham Cooks R (2005) The Orbitrap: a new mass spectrometer. J Mass Spectrom 40: 430–443

Huber SC and Hardin SC (2004) Numerous posttranslational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels. Curr Opin Plant Biol 7: 318–322

Huttlin EL, Hegeman AD, Harms AC and Sussman MR (2007) Comparison of full versus partial metabolic labeling for quantitative proteomics analysis in *Arabidopsis thaliana*. Mol Cell Proteomics 6: 860–881

Jensen ON (2004) Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. Curr Opin Chem Biol 8: 33–41

Jensen ON (2006a) Automated phosphorylation site mapping. Nat Biotechnol 24: 1226–1227

Jensen ON (2006b) Interpreting the protein language using proteomics. Nat Rev Mol Cell Biol 7: 391–403

Johansson E, Olsson O and Nystrom T (2004) Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. J Biol Chem 279: 22204–22208

Julka S and Regnier F (2004) Quantification in proteomics through stable isotope coding: a review. J Proteome Res 3: 350–363

Kamruzzaman Munshi M, Kobayashi Y and Shikanai T (2005) Identification of a novel protein, CRR7, required for the stabilization of the chloroplast NAD(P)H dehydrogenase complex in Arabidopsis. Plant J 44: 1036–1044

Kessler F, Schnell D and Blobel G (1999) Identification of proteins associated with plastoglobules isolated from pea (*Pisum sativum* L.) chloroplasts. Planta 208: 107–113

Kieselbach T and Schroder WP (2003) The proteome of the chloroplast lumen of higher plants. Photosynth Res 78: 249–264

Kirkpatrick DS, Gerber SA and Gygi SP (2005) The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods 35: 265–273

Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjolander K, Gruissem W and Baginsky S (2004) The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol 14: 354–362

Kleffmann T, Hirsch-Hoffmann M, Gruissem W and Baginsky S (2006) plprot: a comprehensive proteome database for different plastid types. Plant Cell Physiol 47: 432–436

Kleffmann T, von Zychlinski A, Russenberger D, Hirsch-Hoffmann M, Gehrig P, Gruissem W and Baginsky S (2007) Proteome dynamics during plastid differentiation in rice. Plant Physiol 143: 912–923

Kluge C, Seidel T, Bolte S, Sharma SS, Hanitzsch M, Satiat-Jeunemaitre B, Ross J, Sauer M, Golldack D and Dietz KJ (2004) Subcellular distribution of the V-ATPase complex in plant cells, and in vivo localisation of the 100 kDa subunit VHA-a within the complex. BMC Cell Biol 5: 29

Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, Punna T, Peregrin-Alvarez JM, Shales M, Zhang X, Davey M, Robinson MD, Paccanaro A, Bray JE, Sheung A, Beattie B, Richards DP, Canadien V, Lalev A, Mena F, Wong P, Starostine A, Canete MM, Vlasblom J, Wu S, Orsi C, Collins SR, Chandran S, Haw R, Rilstone JJ, Gandi K, Thompson NJ, Musso G, St Onge P, Ghanny S, Lam MH, Butland G, Altaf-Ul AM, Kanaya S, Shilatifard A, O'Shea E, Weissman JS, Ingles CJ, Hughes TR, Parkinson J, Gerstein M, Wodak SJ, Emili A and Greenblatt JF (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature 440: 637–643

Kroll D, Meierhoff K, Bechtold N, Kinoshita M, Westphal S, Vothknecht UC, Soll J and Westhoff P (2001) VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. Proc Natl Acad Sci USA 98: 4238–4242

Kuster B, Mortensen P and Mann M (1999) Identifying proteins in genome databases using mass spectrometry. In: Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, pp 1897–1989, Dallas

Kuster B, Mortensen P, Andersen JS and Mann M (2001) Mass spectrometry allows direct identification of proteins in large genomes. Proteomics 1: 641-650

Langdale JA (1998) Cellular differentiation in the leaf. Curr Opin Cell Biol 10: 734–738

Lanquar V, Kuhn L, Lelievre F, Khafif M, Espagne C, Bruley C, Barbier-Brygoo H, Garin J and Thomine S (2007) ^{15}N-metabolic labeling for comparative plasma membrane proteomics in Arabidopsis cells. Proteomics 7: 750–754

Larsen MR, Trelle MB, Thingholm TE and Jensen ON (2006) Analysis of posttranslational modifications of proteins by tandem mass spectrometry. Biotechniques 40: 790–798

Lemaire SD, Michelet L, Zaffagnini M, Massot V and Issakidis-Bourguet E (2007) Thioredoxins in chloroplasts. Curr Genet 51: 343–365

Listgarten J and Emili A (2005) Statistical and computational methods for comparative proteomic profiling using liquid chromatography-tandem mass spectrometry. Mol Cell Proteomics 4: 419–434

Liu C, Willmund F, Whitelegge JP, Hawat S, Knapp B, Lodha M and Schroda M (2005) J-domain protein CDJ2 and HSP70B are a plastidic chaperone pair that interacts with vesicle inducing protein in plastids 1 (VIPP1). Mol Biol Cell 16: 1165–1177

Liu C, Willmund F, Golecki JR, Cacace S, Hess B, Markert C and Schroda M (2007) The chloroplast HSP70B-CDJ2-CGE1 chaperones catalyse assembly and disassembly of VIPP1 oligomers in *Chlamydomonas*. Plant J 50: 265–277

Lohmann A, Schottler MA, Brehelin C, Kessler F, Bock R, Cahoon EB and Dormann P (2006) Deficiency in phylloquinone (vitamin K(1)) methylation affects prenyl quinone distribution, photosystem I abundance, and anthocyanin accumulation in the *Arabidopsis AtmenG* mutant. J Biol Chem 281: 40461–40472

Lonosky PM, Zhang X, Honavar VG, Dobbs DL, Fu A and Rodermel SR (2004) A proteomic analysis of maize chloroplast biogenesis. Plant Physiol 134: 560–574

Macek B, Waanders LF, Olsen JV and Mann M (2006) Top-down protein sequencing and MS3 on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer. Mol Cell Proteomics 5: 949–958

Majeran W, Cai Y, Sun Q and Van Wijk KJ (2005) Functional differentiation of bundle sheath and mesophyll maize chloroplasts determined by comparative proteomics. Plant Cell 17: 3111–3140

Majeran W, Zybailov B, Ytterberg AJ, Dunsmore J, Sun Q, and Van Wijk KJ (2008) Consequences of C4 differentiation for chloroplast membrane proteomes in maize mesophyll and bundle sheath cells. Mol Cell Proteomics 7: 1609–1638

Marouga R, David S and Hawkins E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 382: 669–678

Meng F, Wiener MC, Sachs JR, Burns C, Verma P, Paweletz CP, Mazur MT, Deyanova EG, Yates NA and Hendrickson RC (2007) Quantitative analysis of complex peptide mixtures using FTMS and differential mass spectrometry. J Am Soc Mass Spectrom 18: 226–233

Merchant SS, Allen MD, Kropat J, Moseley JL, Long JC, Tottey S and Terauchi AM (2006) Between a rock and a hard place: trace element nutrition in *Chlamydomonas*. Biochim Biophys Acta 1763: 578–594

Meyer Y, Reichheld JP and Vignols F (2005) Thioredoxins in *Arabidopsis* and other plants. Photosynth Res 86: 419–433

Michelet L, Zaffagnini M, Massot V, Keryer E, Vanacker H, Miginiac-Maslow M, Issakidis-Bourguet E and Lemaire SD (2006) Thioredoxins, glutaredoxins, and glutathionylation: new crosstalks to explore. Photosynth Res 89: 225–245

Monde RA, Schuster G and Stern DB (2000) Processing and degradation of chloroplast mRNA. Biochimie 82: 573–582

Moseley JL, Allinger T, Herzog S, Hoerth P, Wehinger E, Merchant S and Hippler M (2002) Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus. Embo J 21: 6709–6720

Motohashi K, Kondoh A, Stumpp MT and Hisabori T (2001) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci USA 98: 11224–11229

Naumann B, Stauber EJ, Busch A, Sommer F and Hippler M (2005) N-terminal processing of Lhca3 Is a key step in remodeling of the photosystem I-light-harvesting complex under iron deficiency in *Chlamydomonas reinhardtii*. J Biol Chem 280: 20431–20441

Nelson CJ, Hegeman AD, Harms AC and Sussman MR (2006) A quantitative analysis of *Arabidopsis* plasma membrane using trypsin-catalyzed ^{18}O labeling. Mol Cell Proteomics 5: 1382–1395

Nelson CJ, Huttlin EL, Hegeman AD, Harms AC and Sussman MR (2007) Implications of ^{15}N-metabolic labeling for automated peptide identification in *Arabidopsis thaliana*. Proteomics 7: 1279–1292

Nelson T and Langdale J (1992) Developmental genetics of C4 photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 43: 25–47

Neuhaus HE and Emes MJ (2000) Nonphotosynthetic metabolism in plastids. Annu Rev Plant Physiol Plant Mol Biol 51: 111–140

Nield J, Redding K and Hippler M (2004) Remodeling of light-harvesting protein complexes in *Chlamydomonas* in response to environmental changes. Eukaryot Cell 3: 1370–1380

Nystrom T (2005) Role of oxidative carbonylation in protein quality control and senescence. Embo J 24: 1311–1317

Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA and Ahn NG (2005) Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics 4: 1487–1502

Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S and Mann M (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 4: 2010–2021

Peck SC (2005) Update on proteomics in Arabidopsis. Where do we go from here? Plant Physiol 138: 591–599

Peltier JB, Friso G, Kalume DE, Roepstorff P, Nilsson F, Adamska I and Van Wijk KJ (2000) Proteomics of the chloroplast. Systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. Plant Cell 12: 319–342

Peltier JB, Emanuelsson O, Kalume DE, Ytterberg J, Friso G, Rudella A, Liberles DA, Soderberg L, Roepstorff P, von Heijne G and Van Wijk KJ (2002) Central functions of the lumenal and peripheral thylakoid proteome of Arabidopsis determined by experimentation and genome-wide prediction. Plant Cell 14: 211–236

Peltier JB, Ytterberg AJ, Sun Q and Van Wijk KJ (2004) New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. J Biol Chem 279: 49367–49383

Peltier JB, Cai Y, Sun Q, Zabrouskov V, Giacomelli L, Rudella A, Ytterberg AJ, Rutschow H and Van Wijk KJ (2006) The oligomeric stromal proteome of *Arabidopsis thaliana* chloroplasts. Mol Cell Proteomics 5: 114–133

Pesaresi P, Gardner NA, Masiero S, Dietzmann A, Eichacker L, Wickner R, Salamini F and Leister D (2003) Cytoplasmic N-terminal protein acetylation is required for efficient photosynthesis in Arabidopsis. Plant Cell 15: 1817–1832

Rochaix JD (2007) Role of thylakoid protein kinases in photosynthetic acclimation. FEBS Lett 581: 2768–2775

Rokka A, Suorsa M, Saleem A, Battchikova N and Aro EM (2005) Synthesis and assembly of thylakoid protein complexes: multiple assembly steps of photosystem II. Biochem J 388: 159–168

Rolland N, Ferro M, Ephritikhine G, Marmagne A, Ramus C, Brugiere S, Salvi D, Seigneurin-Berny D, Bourguignon J, Barbier-Brygoo H, Joyard J and Garin J (2006) A versatile method for deciphering plant membrane proteomes. J Exp Bot 57: 1579–1589

Romero HM, Berlett BS, Jensen PJ, Pell EJ, Tien M (2004) Investigations into the role of the plastidial peptide methionine sulfoxide reductase in response to oxidative stress in Arabidopsis. Plant Physiol 136: 3784–3794

Ross S, Giglione C, Pierre M, Espagne C and Meinnel T (2005) Functional and developmental impact of cytosolic protein N-terminal methionine excision in Arabidopsis. Plant Physiol 137: 623–637

Rossignol M, Peltier JB, Mock HP, Matros A, Maldonado AM and Jorrin JV (2006) Plant proteome analysis: a 2004–2006 update. Proteomics 6: 5529–5548

Rouhier N, Vieira Dos Santos C, Tarrago L and Rey P (2006) Plant methionine sulfoxide reductase A and B multigenic families. Photosynth Res 89: 247–262

Rudella A, Friso G, Alonso JM, Ecker JR and Van Wijk KJ (2006) Downregulation of ClpR2 leads to reduced accumulation of the ClpPRS protease complex and defects in chloroplast biogenesis in *Arabidopsis*. Plant Cell 18: 1704–1721

Rumeau D, Becuwe-Linka N, Beyly A, Louwagie M, Garin J and Peltier G (2005) New subunits NDH-M, -N, and -O, encoded by nuclear genes, are essential for plastid Ndh complex functioning in higher plants. Plant Cell 17: 219–232

Sadanandom A, Poghosyan Z, Fairbairn DJ and Murphy DJ (2000) Differential regulation of plastidial and cytosolic isoforms of peptide methionine sulfoxide reductase in Arabidopsis.Plant Physiol 123: 255–264

Schagger H, Cramer WA and von Jagow G (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal Biochem 217: 220–230

Schagger H and von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 199: 223–231

Schroder WP and Kieselbach T (2003) Update on chloroplast proteomics. Photosynth Res 78: 181–193

Schubert M, Petersson UA, Haas BJ, Funk C, Schröder WP and Kieselbach T (2002) Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. J Biol Chem 277: 8354–8365

Scigelova M and Makarov A (2006) Orbitrap mass analyzer – overview and applications in proteomics. Proteomics 6 Suppl 2: 16–21

Seigneurin-Berny D, Rolland N, Garin J and Joyard J (1999) Technical advance: Differential extraction of hydrophobic proteins from chloroplast envelope membranes: a subcellular-specific proteomic approach to identify rare intrinsic membrane proteins. Plant J 19: 217–228

Shadforth I, Xu W, Crowther D and Bessant C (2006) GAPP: a fully automated software for the confident identification of human peptides from tandem mass spectra. J Proteome Res 5: 2849–2852

Sheen J (1999) C-4 gene expression. Annu Rev Plant Physiol Plant Mol Biol 50: 187–217

Stauber EJ and Hippler M (2004) *Chlamydomonas reinhardtii* proteomics. Plant Physiol Biochem 42: 989–1001

Steen H and Mann M (2004) The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol 5: 699–711

Storf S, Stauber EJ, Hippler M and Schmid VH (2004) Proteomic analysis of the photosystem I light-harvesting antenna in tomato (*Lycopersicon esculentum*). Biochemistry 43: 9214–9224

Subramanian C, Xu Y, Johnson CH and von Arnim AG (2004) In vivo detection of protein-protein interaction in plant cells using BRET. Methods Mol Biol 284: 271–286

Sun Q, Emanuelsson O and Van Wijk KJ (2004) Analysis of curated and predicted plastid subproteomes of Arabidopsis. Subcellular compartmentalization leads to distinctive proteome properties. Plant Physiol 135: 723–734

Tanner S, Shen Z, Ng J, Florea L, Guigo R, Briggs SP and Bafna V (2007) Improving gene annotation using peptide mass spectrometry. Genome Res 17: 231–239

Tao WA and Aebersold R (2003) Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr Opin Biotechnol 14: 110–118

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY and Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37: 914–939

Tikkanen M, Piippo M, Suorsa M, Sirpio S, Mulo P, Vainonen J, Vener AV, Allahverdiyeva Y and Aro EM (2006) State transitions revisited-a buffering system for dynamic low light acclimation of *Arabidopsis*. Plant Mol Biol 62: 779–793

Turkina MV and Vener AV (2007) Identification of phosphorylated proteins. Methods Mol Biol 355: 305–316

Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV and Villarejo A (2006a) CO_2 limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. Proteomics 6: 2693–2704

Turkina MV, Kargul J, Blanco-Rivero A, Villarejo A, Barber J and Vener AV (2006b) Environmentally modulated phosphoproteome of photosynthetic membranes in the green alga *Chlamydomonas reinhardtii*. Mol Cell Proteomics 5: 1412–1425

Vainonen JP, Hansson M and Vener AV (2005) STN8 protein kinase in *Arabidopsis thaliana* is specific in phosphorylation of photosystem II core proteins. J Biol Chem 280: 33679–33686

Van Wijk KJ (2000) Proteomics of the chloroplast: experimentation and prediction. Trends Plant Sci 5: 420–425

Van Wijk KJ (2001) Challenges and prospects of plant proteomics. Plant Physiol 126: 501–508

Van Wijk KJ (2004a) Chloroplast proteomics. In: Leister D (ed) Plant Functional Genomics, pp 329–358. The Haworth Press, Inc., Book Division, Binghamton, NY

Van Wijk KJ (2004b) Plastid proteomics. Plant Physiol Biochem 42: 963–977

Van Wijk KJ (2006) Expression, prediction and function of the thylakoid proteome in higher plants and green algae. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 125–143. Springer, Dordrecht

Van Wijk KJ and Baginsky S (2011) Plastid proteomics in higher plants: Current state and future goals. Plant Physiol 155: 1578–1588

Van Wijk KJ, Peltier JB and Giacomelli L (2007) Isolation of chloroplast proteins from *Arabidopsis thaliana* for proteome analysis. Methods Mol Biol 355: 43–48

Vidi PA, Kanwischer M, Baginsky S, Austin JR, Csucs G, Dörmann P, Kessler F and Bréhélin C (2006) Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J Biol Chem 281: 11225–11234

Vieira Dos Santos C, Cuine S, Rouhier N and Rey P (2005) The Arabidopsis plastidic methionine sulfoxide reductase B proteins. Sequence and activity characteristics, comparison of the expression with plastidic methionine sulfoxide reductase A, and induction by photooxidative stress. Plant Physiol 138: 909–922

von Zychlinski A, Kleffmann T, Krishnamurthy N, Sjolander K, Baginsky S and Gruissem W (2005) Proteome analysis of the rice etioplast: metabolic and regulatory networks and novel protein functions. Mol Cell Proteomics 4: 1072–1084

Wang G, Wu WW, Zeng W, Chou CL and Shen RF (2006) Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and application with complex proteomes. J Proteome Res 5: 1214–1223

Weber AP, Schwacke R and Flugge UI (2005) Solute transporters of the plastid envelope membrane. Annu Rev Plant Biol 56: 133–164

Whitelegge JP (2003) Thylakoid membrane proteomics. Photosynth Res 78: 265–277

Whitelegge JP (2004) Mass spectrometry for high throughput quantitative proteomics in plant research: lessons from thylakoid membranes. Plant Physiol Biochem 42: 919–927

Wiener MC, Sachs JR, Deyanova EG and Yates NA (2004) Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. Anal Chem 76: 6085–6096

Willmund F, Muhlhaus T, Wojciechowska M and Schroda M (2007) The NH2-terminal domain of the chloroplast GrpE homolog CGE1 is required for dimerization and cochaperone function in vivo. J Biol Chem 282: 11317–11328

Wisz MS, Suarez MK, Holmes MR and Giddings MC (2004) GFSWeb: a web tool for genome-based identification of proteins from mass spectrometric samples. J Proteome Res 3: 1292–1295

Wittig I, Braun HP and Schagger H (2006) Blue native PAGE. Nat Protoc 1: 418–428

Yates JR, Cociorva D, Liao L and Zabrouskov V (2006) Performance of a linear ion trap-Orbitrap hybrid for peptide analysis. Anal Chem 78: 493–500

Ytterberg AJ, Peltier JB and Van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts; a surprising site for differential accumulation of metabolic enzymes. Plant Physiol 140: 984–997

Chapter 8

Intracellular Signaling from Plastids to the Nucleus

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Summary

Signaling from chloroplasts or other plastids to the nucleus is part of the intracellular communication network of plants. Several signals originate in the plastids from processes like plastid gene expression, photosynthetic electron transport, or chlorophyll biosynthesis. In some cases, signaling compounds like reactive oxygen species or Mg-protoporphyrin are suggested to be involved in this process. Signaling leads to up- and down-regulation in the expression of certain nuclear genes. While the origins of signaling pathways in the plastids and the effect in the nucleus have been accessible to various experimental approaches, none of the signaling pathways has been completely elucidated. According to the present view, several signaling pathways are connected to a communication network in which light signals are also involved.

I. Introduction

Intracellular communication between the different cellular compartments is an essential prerequisite for a functioning cell. The communication between nucleus and plastids of plant cells, dealt with in this article, functions in both directions: from nucleus to plastids (anterograde signaling) and from plastids to nucleus (retrograde signaling) (Bräutigam et al., 2008). The ancestors of plastids, presumably prokaryotes related to contemporary cyanobacteria, were taken up as endosymbionts by the eukaryotic host cell, and the subsequent transfer of most symbiotic genes to the nucleus established far-reaching genetic control of the host cell over functions of the endosymbionts. In present-day plants, more than 95% of chloroplast proteins are encoded by nuclear genes, their

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mRNAs are translated in the cytoplasm as precursor proteins that in turn are imported into the plastids where they are processed to mature proteins. Many of these proteins are eventually combined with plastid-encoded proteins and pigments to functioning complexes in the envelope, stroma and thylakoid membrane. Many other nuclear-encoded proteins have a regulatory function, for example on plastid genome expression, on import, sorting and assembly, and on plastid enzyme activities; the number of regulatory proteins is possibly tenfold higher than the number of proteins necessary for the biochemical reactions within plastids (Bräutigam et al., 2008). Thus anterograde signaling uses a variety of nuclear-encoded proteins that are imported into the plastids.

The existence of retrograde signaling was first deduced from observations made under various conditions whereby this signaling pathway was apparently disrupted (Fig. 8.1). Conditions that lead to early proposals of retrograde signaling include the use of plants that lacked plastid translation, either by mutation or after application of specific inhibitors (pathway 1 in Fig. 8.1); further, retrograde signaling was postulated for plants in which the redox state of the plastoquinone pool was manipulated (pathway 2). Pathway 3 in Fig. 8.1 signifies carotenoid-lacking plants that show typical photodamage of plastids in strong light, this correlates with the accumulation of Mg-protoporphyrin (MgProto); some details of this experimental system will be discussed below. Production of reactive oxygen species (ROS) in the plastids or in the mitochondria is likewise a source of signaling to the nucleus, different for singlet oxygen (pathway 4a) and for hydrogen peroxide (pathway 4b). In all of these cases, transcription of certain nuclear genes was affected, under conditions 1–3 mainly transcription of genes encoding plastid proteins. Interestingly, expression of most affected genes can be induced by light. Although it is clear that several signaling pathways, from plastids to the nucleus, exist, we are far from a complete understanding of the signaling pathways and their possible interaction. In the following pages, experimental results leading to the concept of retrograde signaling will be discussed with emphasis on those cases that are already known in some detail. Several excellent recent reviews in the field are available with varying emphasis on different aspects (Strand, 2004; Vasileuskaya et al., 2004; Baier and Dietz, 2005; Beck, 2005; Fey et al., 2005; Gray, 2005; Leister, 2005; Nott et al., 2006; Pesaresi et al., 2007; Tanaka and Tanaka, 2007; Bräutigam et al., 2008; Fernandez and Strand, 2008; Oelze et al., 2008).

An early proposal for a plastid factor that is involved in regulation of nuclear genes came from experiments with the *albostrians* mutant of *Hordeum vulgare* (Bradbeer et al., 1979). The offspring of this recessive nuclear mutant consists of entirely green, entirely white, and green-white striped leaves. The albino tissue lacks plastid ribosomes and the content of chlorophylls and carotenoids is less than 1% of that of the wild type; likewise, the level of a number of plastid proteins encoded by nuclear genes, e.g., subunits of the ATP synthase, ferredoxin-NADPH oxidoreductase, the small subunit of ribulose bisphosphate carboxylase (RBCS), enzyme activities of NADP-GAPDH and phosphoribulokinase, and the level of the cytoplasmic enzyme, nitrate reductase, are very low in the mutant (Hess et al., 1994). A plastid-derived factor was postulated, related to the function of plastid ribosomes that was believed to somehow control the expression of certain nuclear genes (Bradbeer et al., 1979). Although the transcripts of *LHCB*, the gene family encoding the light-harvesting chlorophyll *a*/*b*-binding proteins, and *RBCS* were detected only at a very low level, they respond to up-regulation by light; moreover, the photoreceptor phytochrome is present at a level of 50% compared to that in the wild type, is active in the mutant and is down-regulated by light (Hess et al., 1991).

Much early work has been performed with photodamaged plastids, reviewed by Oelmüller (1989)

Abbreviations: ABA – Abscisic acid; APX – Ascorbate peroxidase; CAO – Chlorophyllide *a* oxygenase; 2CPA – 2-cystein peroxiredoxin A; CRY – Cryptochrome; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; *ELIP2* – Early light-induced gene; GUN – Genomes uncoupled; *HEMA* – Gene encoding glutamyl-tRNA reductase; HSP70 – 70 kDa heat shock protein; LHC – Light harvesting complex; LHCB – Light-harvesting chlorophyll *a*/*b*-binding protein; MgProto – Mg-protoporphyrin; *PETE* – Gene encoding plastocyanin; PQ – Plastoquinone; PS I, PS II – Photosystem I, photosystem II; PSAD, PSAF – Subunits of PS I; RBCS – Ribulose bisphosphate carboxylase small subunit; ROS – Reactive oxygen species

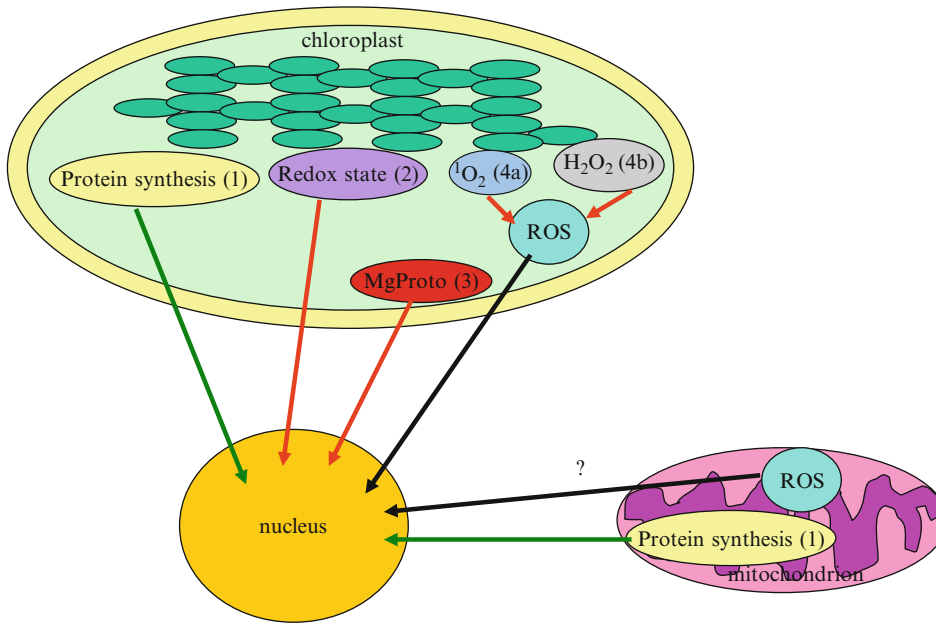

Fig. 8.1. Signaling from chloroplasts to the nucleus. Signals originate from plastid protein synthesis, which can in part be substituted by mitochondrial protein synthesis (1), the redox state of parts of the photosynthetic apparatus (2), overproduction of chlorophyll precursors (3) and reactive oxygen species (*ROS*), singlet oxygen (*4a*) and hydrogen peroxide (*4b*).

and Taylor (1989). Either mutants with carotenoid deficiency or plants treated with the inhibitor norflurazon were investigated. The experimental conditions can briefly be characterized as follows. Norflurazon inhibits phytoene desaturase; the accumulated colorless phytoene does not protect chlorophyll (and membrane lipids) against photodestruction, which is recognized as bleaching of plants and disappearance of plastid ribosomes and of the thylakoid membranes of chloroplasts in strong light. The same is true for carotenoid-free mutants: the plastid envelope remains intact, and no visible damage is detected outside the plastids. By contrast to exposure to strong light, norflurazon-treated plants develop like control plants in dim white or red light. Photodamaged plants placed under such dim, non-destructive light conditions recover from the photodamage within a couple of days, indicating that some residual functionality remains even under severe destruction of thylakoid membranes. In normal daylight, development of carotenoid-free plastids is arrested at early stages; it is believed that accumulated protochlorophyllide or chlorophyllide suffices to impair plastid development by photooxidation (Oelmüller, 1989). The key observation for the question of signaling was the lack of transcription of several nuclear genes when the plastids suffered from photodamage. The affected genes, as far as they were studied, encoded either plastid proteins or proteins related to plastid functions; most sensitive to plastid photooxidation was the transcription of *LHCB* genes. The existence of a "plastid factor" or "plastid signal" was deduced from these observations. Oelmüller (1989) and Taylor (1989) and others suggested that this plastid factor should be released from intact plastids and its presence should be necessary to maintain expression of the set of genes related to plastid function; further, that the plastid factor should be short-lived and immediately be destroyed by photooxidation together with the thylakoid membranes and the plastid pigments. The nature of the presumed plastid factor remained entirely unknown; since a chlorophyll-deficient maize mutant, grown with norflurazon under strong light, allowed *LHCB* mRNA accumulation, Taylor et al. (1986) and Oelmüller (1989) concluded that reduced *LHCB* gene transcription does not operate by a feedback mechanism connected to chlorophyll or carotenoid biosynthesis. In the view of more recent results, discussed below, these hypotheses must be corrected.

II. Mg-Protoporphyrin (MgProto)

Early indication that chlorophyll precursors can act as signals from plastids to the nucleus came from inhibitor experiments with the green alga *Chlamydomonas reinhardtii* (Johanningmeier and Howell, 1984; Johanningmeier, 1988). The authors found that light-dependent accumulation of *LHCB* mRNA and *RBCS1* mRNA was abolished by dipyridyl, that was presumed to inhibit late steps of chlorophyll biosynthesis leading to accumulation of chlorophyll precursors, whereas no disruption was observed by heme, dioxoheptanoic acid and levulinic acid, that inhibit very early steps of chlorophyll biosynthesis so that no tetrapyrrole precursors of chlorophyll accumulate (Fig. 8.2). Dipyridyl certainly has unspecific side effects: the authors mentioned that dipyridyl reduces accumulation of total RNA, largely composed of ribosomal RNA; nevertheless, the specific effect on *LHCB* and *RBCS1* mRNA was partly reversed by the addition of Fe or Zn to the treated cells indicating that dipyridyl had acted through its metal-chelating property. Based on earlier work in other laboratories, the authors assumed that oxidative cyclase, an iron-requiring enzyme, was inhibited and its substrate, MgProto monomethylester, had accumulated; however, no porphyrin analysis was performed to confirm this.

Similar experiments were also performed with higher plants. Inhibition of protochlorophyllide synthesis in etiolated cress seedlings by thujaplicin resulted in accumulation of Mg protoporphyrin IX (i.e., MgProto) and its monomethylester, and in reduction of mRNA levels of various *LHCB* genes after induction with a red light pulse (Oster et al., 1996). Kittsteiner et al. (1991a) found reduced transcription of *LHCB* mRNA after treatment of cress seedlings with aminolevulinic acid or metal chelators (dipyridyl, hydroxyquinoline), conditions under which several chlorophyll precursors (protophorphyrin, MgProto and its monomethylester, protochlorophyllide) accumulated. A problem inherent in this type of experiment is photooxidative damage mediated by the chlorophyll precursors, especially at the transition from darkness to light conditions (Kittsteiner et al., 1991b). To avoid such damage as far as possible, Kittsteiner et al. (1991a) used far-red light for induction of *LHCB* mRNA expression. The same chlorophyll precursors also accumulate when carotenoid biosynthesis is inhibited in barley

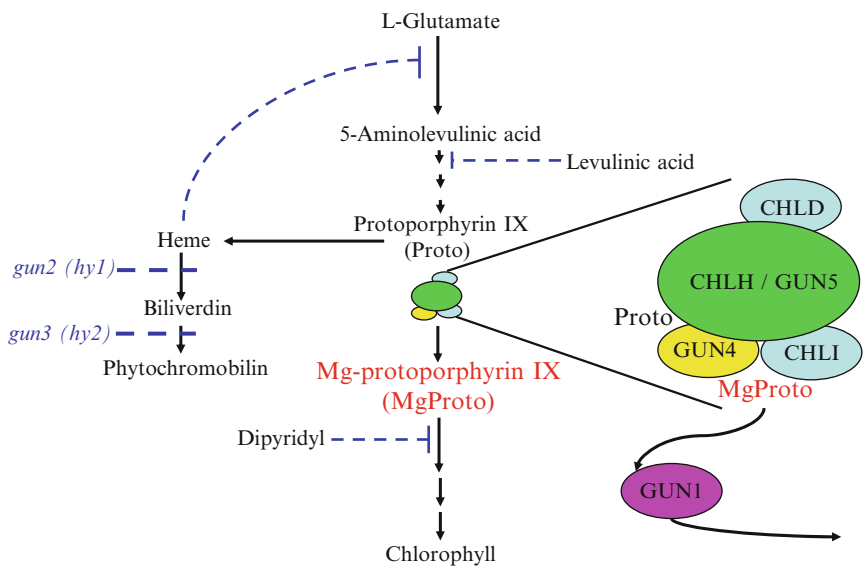

Fig. 8.2. Pathway of chlorophyll biosynthesis. Only steps discussed in the text are presented. Steps inhibited by heme, levulinic acid and dipyridyl are indicated. Heme accumulates by interruption of the pathway in the mutants *gun2* (synonym *hy1*) and *gun3* (synonym *hy2*). Mg chelatase is enlarged at the right side with the subunits CHLD, CHLI and CHLH (synonym GUN5). The GUN4 protein is attached to Mg chelatase and is believed to be responsible for the transport of protoporphyrin to the enzyme and MgProto away from the enzyme. The retrograde signal originating from MgProto is mediated by GUN1.

seedlings at the lycopene cyclase step (La Rocca et al., 2001, 2007). After treatment with either aminotriazole or 2-(4-chlorophenylthio)triethylamine, the authors found decreased levels of *LHCB* mRNA and *RBCS* mRNA together with perturbation of the plastid inner membrane structure, accumulation of lycopene and of chlorophyll precursors. Because these effects were not only observed in the light but also in absolute darkness, photooxidation was excluded as effector.

The first direct evidence for a regulation of nuclear genes by defined chlorophyll precursors was achieved in experiments with light induction of nuclear heat-shock genes of *C. reinhardtii* (Kropat et al., 1997, 1999). It had been known that a mutant, blocked in the step from protophorphyrin to MgProto, did not show any light induction of the heat-shock genes *HSP70A* and *HSP70B* while a mutant blocked in the step from protochlorophyllide to chlorophyllide showed normal light induction; thus induction seemed to be correlated with the presence of a compound of the tetrapyrrole pathway between MgProto and chlorophyllide. Kropat et al. (1997, 1999) demonstrated that feeding the *C. reinhardtii* cells with MgProto in darkness resulted in induction of the *HSP70* genes; thus, MgProto could substitute for light for this induction. Feeding of earlier (protophorphyrin) or later (protochlorophyllide, chlorophyllide) chlorophyll precursors did not induce *HSP70* genes. Likewise, induction of the *HEMA* gene, encoding glutamyl-tRNA reductase, was achieved by MgProto as substitute for light (Vasileuskaya et al., 2005). Instead of MgProto, hemin can be used as inducer while ZnProto, CoProto, or MnProto have no effect (von Gromoff et al., 2008). The effect of MgProto or hemin is not a general stress response: promoter deletion leading to the loss of light induction resulted in the loss of response to MgProto and hemin while the heat-shock response remained intact. Detailed promoter analysis and alignment with other genes inducible by MgProto or hemin revealed short enhancer sequences responsive to light, hemin and MgProto; the essential sequence motif was elucidated as GCGACNAN$_{15}$TA and named "plastid response element" (von Gromoff et al., 2006, 2008). The close relationship between induction by light, heme and MgProto was demonstrated in another biochemical approach (Kropat et al., 2000; von Gromoff et al., 2008). A shift of the algal cultures from darkness to light resulted in a rapid and transient increase of MgProto that preceded induction of the *HSP70* gene. Lack of light induction of the *HSP70* gene, for example in cells pretreated with nitrogen-free medium (pregametes) and in cells pretreated with cycloheximide, paralleled the lack of increase in the MgProto level; however, addition of MgProto to such pretreated cells resulted in normal induction of the *HSP70* gene. Preincubation with hemin abolished further induction of *HSP70A* by light or MgProto. Taken together, the results led to the proposal that light induction may be mediated by MgProto and/or heme (von Gromoff et al., 2006, 2008). A puzzling result was obtained by feeding the *C. reinhardtii* cells with protophorphyrin that did not cause *HSP70* gene induction but neither did it interfere with induction by exogenously applied MgProto: the applied protophorphyrin was taken up and metabolized to MgProto; however, this MgProto did not induce *HSP70* genes (Kropat et al., 2000). The authors argued that because Mg chelatase is exclusively located in the plastids, the newly-formed MgProto must initially be present in the plastid compartment, but for light induction, mediated by MgProto, a light-dependent export from the plastids to the cytoplasm must be postulated. So far, no intracellular localization of MgProto in *C. reinhardtii* cells has been reported.

While a role of MgProto and heme as signaling compounds in green algae, exemplified by *C. reinhardtii*, seems to be well established, it is still a matter of dispute whether these compounds play a corresponding role in higher plants. The surprising outcome of a genetic approach with *Arabidopsis thaliana*, performed over a number of years, was taken by J. Chory and coworkers as indirect evidence for participation of MgProto in signaling from plastids to nucleus in higher plants. The basis was photodamage of plastids by treatment with norflurazon in strong light (see above), and a search for mutants that did not respond to the signal coming from the damaged plastids. Since *LHCB* genes respond with high sensitivity to photodamage, the screen for such mutants used an *LHCB* promoter fused to suitable marker genes, integrated into the nuclear genome before mutagenesis. While the wild type was characterized by low or even no expression of *LHCB* genes after photodamage, the screened mutants showed high expression of *LHCB* genes (in this

case also of marker genes) under these conditions. Five nonallelic mutants were found and named *gun* (genomes uncoupled) mutants (Susek et al., 1993). Three mutants had defects in tetrapyrrole biosynthesis, *gun2* and *gun3* in the steps from heme to phytochromobilin, and *gun5* in the H-subunit of Mg chelatase (Mochizuki et al., 2001, see Fig. 8.2). Pigment analysis of wild type, *gun2* and *gun5* revealed less chlorophyll and chlorophyll precursors in the mutants compared to the wild type. Decisive for the hypothesis that plastid signals are mediated by MgProto was the report that, by contrast to the assumption that photooxidation should lead to bleaching of all pigments, an increase in the level of MgProto and its monomethylester was found after photooxidation of norflurazon-treated plants (Strand et al., 2003). This increase was reported to be less pronounced in the mutants than in the wild type, and the authors argued that the threshold level of MgProto for signaling was only reached in the wild type and not in the mutants.

On the other hand, two papers were published 5 years later describing a lack of increase of MgProto in norflurazon-treated *A. thaliana* plants and, furthermore, a lack of correlation between MgProto accumulation and decreased expression of *LHCB* and other photosynthesis genes (Mochizuki et al., 2008; Moulin et al., 2008). Thus, several experimental results that had been interpreted on the basis of MgProto being the effector, await now to be explained differently. Some examples may be mentioned: several mutants in tetrapyrrole synthesis prior to the step of MgProto proved to be *gun* mutants (Strand et al., 2003) while a mutant in the cyclase, located downstream of MgProto, did not show the *gun* phenotype after norflurazon treatment in the light (Strand, 2004). Also, no *gun* phenotype was found in a barley mutant with a defect in the cyclase (Rzeznicka et al., 2005; Gadjieva et al., 2005). Perhaps, the chlorophyll-deficient maize mutant described by Taylor et al. (1986) can now also be classified as a *gun* mutant. Application of dipyridyl that presumably increased the level of MgProto lead to a loss of the *gun* phenotype while additional incubation with an inhibitor of protoporphyrinogen oxidase restored the *gun* phenotype (Strand et al., 2003). A correlation of MgProto accumulation and reduced *LHCB* expression was described for an *A. thaliana* knock-out mutant in MgProto methyltransferase (Pontier et al., 2007); however, Mochizuki et al. (2008) confirmed only MgProto accumulation and did not find any reduction of *LHCB* expression in this mutant. Interestingly, expression not only of nuclear-encoded genes but also of plastid-encoded photosynthesis genes (*psbA, psbD, psaA, psaC, rbcL*) was derepressed, compared to the wild type, in the *gun5* mutant of *A. thaliana* after treatment with norflurazon and light (Ankele et al., 2007). This indicates coordinate plastid and nuclear gene expression.

The other *GUN* genes which have been investigated do not encode enzymes of chlorophyll biosynthesis. GUN4 is a plastid protein found in the stroma, thylakoids and envelope membranes; it binds protoporphyrin and MgProto and activates Mg chelatase, probably by binding to the H-subunit (Larkin et al., 2003). In this way it somehow regulates chlorophyll accumulation: *gun4* mutants develop only white to yellow or pale-green tissue depending on the growth conditions. Larkin et al. (2003) discuss the possibility that GUN4 may recruit MgProto to the envelope or promote its export from the plastid; however, so far there is no experimental evidence for this idea and the role of GUN4 in plastid-to-nucleus signaling remains to be elucidated. Interestingly, a *gun4* mutant of the cyanobacterium, *Synechocystis* sp. PCC 6803, shows reduced Mg- and Fe-chelatase activities (Wilde et al., 2004). GUN1 was at first proposed to act in a pathway different from the MgProto pathway of GUN2-5 (Strand et al., 2003) while, more recently, Koussevitzky et al. (2007) proposed that it integrates several signaling pathways from plastids to nucleus including the MgProto signaling pathway; this will be discussed below.

III. Plastid Gene Expression During Early Plastid Development

As mentioned in the Introduction, investigation on the *albostrians* mutant of *Hordeum vulgare* that lacked intact plastid ribosomes resulted in an early proposal of a plastid-derived factor that controls the expression of certain nuclear genes (Bradbeer et al., 1979). The factor was at first hypothesized to be a RNA species rather than a protein species because the group of Jürgen Feierabend had found normal expression of the same nuclear genes although plastid protein synthesis was blocked: in

this case, non-functional plastid ribosomes were induced in rye seedlings kept at the non-permissive temperature of 32°C. The plastids are free of plastid-encoded proteins and pigments; however, several plastid proteins that are encoded in the nucleus are still produced and accumulated in the white plastids (Feierabend and Schrader-Reichhardt, 1976; Feierabend and Wildner, 1978). However, the rye seedlings were kept at 22°C for the first 24 h of germination and had functional ribosomes at the beginning (in contrast to the *albostrians* mutant) before they were transferred to the non-permissive temperature.

On the other hand, the appearance of *RBCS* mRNA can be prevented if the development of proplastids into mature chloroplasts is inhibited by chloramphenicol (Oelmüller et al., 1986). Inhibitors of plastid protein synthesis like lincomycin or erythromycin decrease the expression of nuclear photosynthesis genes like *PETE* (encoding plastocyanin), *LHCB1* and *RBCS* if the inhibitors are applied within 48–72 h of germination. This inhibition is independent on the light induction of these genes as shown in mutants *lip1* of pea and *cop1-4* of *A. thaliana* that show high expression of these genes already in darkness; Sullivan and Gray (1999) concluded that production of the plastid signal must involve a product of early plastid gene expression and that signaling pathways of light induction and plastid signaling are, at least during some stages of signal transduction, distinct from one another. Differences in the regulation by plastid signals depending on the stage of plant development was shown for the *PETE* gene encoding plastocyanin (Sullivan and Gray, 2002): in 7-day-old pea and tobacco seedlings, down-regulation of the *PETE* transcript level was observed after photooxidation mediated by norflurazon and after inhibition of plastid protein synthesis mediated by lincomycin while no effect of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was found. By contrast to the results with seedlings, the transcript level of a construct containing pea *PETE* in 4-week-old transgenic tobacco plants decreased significantly, together with endogenous *LHCB1* mRNA, by DCMU treatment while the endogenous *PETE* of tobacco did not respond to this treatment.

Bräutigam et al. (2008) developed an interesting hypothesis for a correlation of plastid gene expression and regulation of *LHCB* gene expression via the LHCB precursor protein based on a previously postulated role for chlorophyllide *a* oxygenase (CAO) and chlorophyllide *b* in the regulation of LHCB protein import (Reinbothe et al., 2006). The plastid *trnE* gene encodes glutamyl-tRNA, which is essential for chlorophyll biosynthesis, and non expression of the *trnE* gene means no chlorophyllide synthesis. If the import of the LHCB precursor protein were blocked at the plastid envelope due to the absence of chlorophyllides, the precursor protein is hypothesized to inhibit transcription of nuclear *LHCB* genes via a (hitherto hypothetical) feed-back mechanism. However, the model of Reinbothe et al. (2006) has been challenged because CAO was found to act mainly at the thylakoid membrane independent of LHCB import (Hirashima et al., 2006). Further, besides the problem that this mechanism is supposed to only affect *LHCB* genes rather than a large set of nuclear genes, the hypothesis does not take into account that *trnE* must still have considerable activity because cytoplasmic and mitochondrial tetrapyrroles are produced more or less normally: in the albino tissue of the *albostrians* mutant, for example, phytochromobilin levels are 50% of the controls (see above).

IV. Signaling via the Redox State of Mature Chloroplasts

Effective photosynthesis requires permanent acclimation to changing environmental conditions, and this process has been investigated primarily with plants containing mature chloroplasts. Many algae and higher plants can respond to changing light conditions that cause an imbalance in excitation of the two photosystems, by changing the stoichiometry of photosystems I and II (PS I and PS II) and by also changing antenna size of both the photosystems: thus, less of the photosystem that is preferentially excited is formed and, in general, smaller antennae are formed under high light and larger antennae under low light. As outlined in excellent and detailed reviews, the redox state of various components of the photosynthetic machinery has been identified as the regulating parameter for this acclimation, which involves both plastid and nuclear gene expression (Pfannschmidt, 2003; Bräutigam et al., 2008; Oelze et al., 2008). Some examples for signaling of the photosynthetic redox state to the nucleus are discussed below.

In the green algae *Dunaliella tertiolecta* and *D. salina,* the transfer from high to low light resulted in increased *LHCB* transcription; this effect was related to the redox state of the plastoquinone (PQ) pool: treatment with DCMU, which kept PQ in the oxidized form, resulted in permanent high expression of *LHCB* genes while treatment with 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which kept PQ reduced, lead to lowered expression of *LHCB* genes (Escoubas et al., 1995; Maxwell et al., 1995; Durnford and Falkowski, 1997). Since application of inhibitors for protein phosphatases abolished the increase in *LHCB* transcripts after transfer from high to low light, the authors hypothesized that a phosphorylation cascade may be involved in the signal transfer from thylakoids to the nucleus. A re-investigation of the effects of inhibitors applied to *D. tertiolecta* revealed that long-term acclimation to high and low light conditions are indeed mediated by the redox state of the PQ pool while short-term acclimation probably depends mainly on the trans-thylakoidal membrane potential (Chen et al., 2004).

Similar results were obtained with the same inhibitors in higher plants. Yang et al. (2001) investigated a *Lemna persusilla* mutant deficient in the cytochrome b_6f complex that showed a constant low level of LHCII protein and high activity of the "acclimative protease" under high and low light. Incubation with DCMU restored the increase of LHCII protein in the mutant under low light; the authors, therefore, concluded that the redox state of the PQ pool affects both synthesis and degradation of LHCII. Excitation of either PS I or PS II by specific light sources that resulted in changes of the redox state of the photosynthetic apparatus was accompanied by changes in gene expression of several nuclear genes. In transgenic tobacco plants, constructs containing the promoter of plastocyanin responded under these conditions to the redox state of the PQ pool as revealed by inhibitor experiments with DCMU and DBMIB (Pfannschmidt et al., 2001). In contrast, the constructs with promoters of the PS I subunits PSAD, PSAF and of nitrate reductase are presumably regulated by other components of the photosynthetic redox chain because they responded only to inhibition by DCMU but not to inhibition by DBMIB (Pfannschmidt et al., 2001; Sherameti et al., 2002). The increase in transcript levels of *LHCB* and *RBCS*, normally found by depletion of sugar, was inhibited in *A. thaliana* by DCMU indicating that a signal from the photosynthetic electron transport chain was somehow involved (Oswald et al., 2001). Interestingly, transcript levels accumulated normally when DCMU was added 24 h after the onset of starvation; thus, the redox signal is required at the onset of the starvation. Since the redox state of PQ did not change by removal and replacement of sugar, the authors concluded that the signal must originate from a site in the electron transport chain but different from the PQ pool. Nevertheless, expression of cytosolic ascorbate peroxidase was supposed to be regulated within the first hour after high light irradiation by the redox state of the PQ pool; however, expression after prolonged irradiation that results in light stress corresponded to the elevated level of H_2O_2 (Karpinski et al., 1997, 1999; Yabuta et al., 2004), perhaps in co-operation with abscisic acid (Fryer et al., 2003). While ascorbate peroxidase responded to H_2O_2 in *A. thaliana*, a construct containing the promoter of an "early light-induced" gene (*ELIP2*) responded to the redox state of PQ as shown by opposite effects of DCMU and DBMIB (Kimura et al., 2003). In winter rye, kept under a variable temperature and light program, LHCII phosphorylation was used as a sensitive tool to monitor redox changes in chloroplasts and was found to be correlated with accumulation of *LHCB* transcripts (Pursiheimo et al., 2001). The authors concluded that the redox state of electron acceptors of PS I, and not the redox state of the PQ pool, trigger expression of *LHCB* genes in this system. Phosphorylation of LHCII by protein kinase STN7 appears to be essential for regulation of nuclear gene expression in *A. thaliana*: a *stn7* null mutant failed to regulate *LHCB* and other photosynthetic genes under changing light conditions (Bonardi et al., 2005; see also Chapter 7). Transcript levels do not necessarily indicate transcriptional regulation: Petracek et al. (1997, 1998) demonstrated that stability of ferredoxin mRNA in transgenic tobacco was enhanced during active photosynthesis; the half-life decreased in darkness or by treatment with DCMU.

Heiber et al. (2007) investigated redox-imbalanced (*rimb*) mutants of *A. thaliana* with low 2-cysteine peroxiredoxin A (2CPA) promoter activity. They found that the transcript levels not only of 2CPA but also of several other antioxidant

enzymes were decreased in spite of the presence of typical markers for oxidative stress such as high oxidation status of the ascorbate and 2CPA pools and high reduction state of the PQ pool. Comparison of gene expression patterns show that the mutation affects a redox signaling pathway different from known redox signaling cascades. A still different signal, related to the photosynthetic redox chain, was detected when expression of genes encoding enzymes for chlorophyll biosynthesis was investigated in *C. reinhardtii* (Shao et al., 2006). Light induction of these genes by phototropin was abolished when the normal function at the quinone binding Q_0 site in the cytochrome b_6f complex was lost by mutation, while normal light induction of these genes was observed in the presence of DCMU or DBMIB or in mutants with defects in PS I, PS II or in plastocyanin. It was concluded that cytochrome b_6f complex activity regulates a plastid factor that is necessary for light induction of these genes.

Chen et al. (2004) described binding of protein complexes to the promoter region of the *LHCB1* gene in *Dunaliella tertiolecta*, which under most conditions (i.e., shift from high to low light and vice versa, or application of DCMU) paralleled the level of *LHCB* transcripts. Binding of protein complexes was inhibited by thiol reagents, while phosphatases had no significant effect on in vitro binding.

Piipo et al. (2006) subjected *A. thaliana* plants to various 3 h-light treatments and compared the expression of about 6,500 randomly chosen genes with the redox state of the PQ pool, monitored by the PS II excitation pressure and by the phosphorylation state of PS II core proteins, and with the redox state in the stroma, monitored by the NADP-malate dehydrogenase activity. Only 25% of the chosen genes responded to at least one of the light treatments, and gene expression could better be correlated with the stromal redox state than with the redox state of PQ.

V. Reactive Oxygen Species

In plastids of plants that receive an excess of light, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and singlet oxygen can originate (Apel and Hirt, 2004): over-reduction of dioxygen at PS I leads to the superoxide anion, which is detoxified by superoxide dismutase to H_2O_2. The latter product is relatively stable, can penetrate biological membranes like water and, thus, at least in theory qualifies as a signal compound able to leave the plastid compartment. By contrast, singlet oxygen has a shorter life time than hydrogen peroxide and is believed to react with sensitive cellular compounds directly at the place of its formation. Singlet oxygen can be produced at PS II by energy transfer from triplet state P680 to dioxygen, and further when free porphyrins and Mg-porphyrins accumulate that act as photosensitizers in the presence of light and dioxygen. To avoid such undesirable reactions, porphyrins and Mg-porphyrins are normally protected in plants by binding to proteins that in most cases also bind carotenoids. However, if the tetrapyrrole biosynthetic pathway is interrupted or its regulation is disturbed, free porphyrins or Mg-porphyrins can accumulate (Tanaka and Tanaka, 2007).

The best known example of induction of a nuclear gene by H_2O_2 is that of cytosolic ascorbate peroxidase (*APX*) of *A. thaliana.* Induction of *APX* genes can be achieved by high light or, alternatively, by external H_2O_2 in darkness (Karpinski et al., 1997, 1999). The authors found that infiltrated catalase diminished the induction of *APX* genes under strong light. A good correlation between *APX2* expression and H_2O_2 production under varying light conditions was described by Fryer et al. (2003). As mentioned in Section IV, initial light induction of *APX2* was found to depend on the redox state of the PQ pool while the response after prolonged irradiation was attributed to increased levels of H_2O_2 (Yabuta et al., 2004). While in these examples the correlation with H_2O_2 seems to be clear, one must always consider that thylakoids produce both, singlet oxygen and H_2O_2 under excess of light (see above). Despite their simultaneous formation and their common message ("light stress") to the plant, H_2O_2 and singlet oxygen must have separate signaling pathways: Shao et al. (2007) found that promoter elements responsive to H_2O_2 were different from the elements responsive to singlet oxygen. The authors used transformants harboring constructs of the *HSP70A* promoter fused to a luciferase reporter gene. While constructs with the complete promoter were inducible by both H_2O_2 and singlet oxygen, by dissecting the promoter they found that upstream elements of the promoter region

were necessary for a response to singlet oxygen while downstream elements were essential for a response to H_2O_2.

In the *flu* mutant of *A. thaliana*, large amounts of free protochlorophyllide accumulate in darkness caused by interrupted feed-back regulation of its synthesis (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002; Goslings et al., 2004). Transfer from darkness to light leads to immediate release of singlet oxygen within the plastid compartment followed by cell death, manifested as cessation of growth in adult plants while etiolated seedlings die. Studies showed that these effects were not a result of a direct chemical reaction of singlet oxygen with structural compounds of the cell but were caused by the activation of a genetically determined cell program mediated by a gene named *EXECUTER1* (Wagner et al., 2004). This gene was found in a screen with a *flu* mutant for second-site mutation, and *A. thaliana executer1/flu* double mutants reacted much less sensitively toward singlet oxygen than the parent *flu* mutant. The authors showed that inactivation of the EXECUTER1 protein resulted in reduced sensitivity towards singlet oxygen even in wild-type plants; increasing levels of singlet oxygen were in this case produced by incubation with increasing concentration of DCMU which is known to stimulate the release of singlet oxygen from the reaction center of PS II. In the *A. thaliana* genome, a related gene, *EXECUTER2*, was detected and its role in signal transfer was investigate in detail (Lee et al., 2007). Both proteins, EXECUTER1 and EXECUTER2, are located in the thylakoids of chloroplasts; the sequences are highly homologous to each other and to the corresponding proteins of rice. Surprisingly, knockout of the *EXECUTER2* gene had no significant effect on the growth of *A. thaliana* plants: *ex2/flu* plants behaved like *flu* mutants and *ex1/ex2/flu* plants like *ex1/flu* plants. However, inspection of the transcript profiles in *flu*, *ex1/flu*, *ex2/flu* and *ex1/ex2/flu* plants revealed that EXECUTER2 either enhanced or reduced the levels of many $^1O_2$-responsive genes and thus modulates the activity of EXECUTER1. The majority of $^1O_2$-responsive genes, at least those that encode known proteins, is involved in different stress responses while genes linked to photosynthesis or the control of chloroplast development were not found in this system. H_2O_2 that serves as a plastid signal itself (see above) seems to antagonize the $^1O_2$-mediated signaling (Laloi et al., 2007).

VI. Multiple Signals or a Master Switch?

Early investigations (Oelmüller, 1989) lead to the conclusion that "the expression of those genes which were originally encoded in the plastid genome but transferred to the nucleus may still be controlled by an intracellular signal deriving from the plastids" (Oelmüller, 1989). Indirectly, the discussion implied a single signaling pathway from plastids to the nucleus. It was further postulated that the plastids had to perform development beyond the state of proplastids and had to be structurally intact in order to elicit the plastid signal; because otherwise, the expression of the investigated nuclear genes was severely reduced or even abolished. As outlined in the preceding sections, subsequent research revealed more than one plastid signal, and those signals can be classified as originating from either (1) early plastid gene expression, (2) from intermediates of chlorophyll biosynthesis or (3) from redox components related to the photosynthetic electron transport chain. Typical inhibitors for these plastid-located processes are lincomycin (for 1), norflurazon and light (for 2) and DCMU (for 3). A study of the expression of the plastocyanin-encoding gene and *LHCB* genes with all three of these inhibitors in 7-day-old seedlings of pea and transgenic tobacco plants revealed a sensitivity toward norflurazon and lincomycin, but not toward DCMU; in 4-week-old plants, however, DCMU upregulated transcription of the construct containing the pea plastocyanin promoter and downregulated transcription of the tobacco *LHCB* genes while, nevertheless, DCMU decreased the accumulation of both plastocyanin and *LHCB* transcripts (Sullivan and Gray, 2002). The authors concluded that multiple plastid signals affect transcriptional and post-transcriptional processes. Multiple signals were also found by other authors, and subsequently multiple signaling pathways were postulated by several authors (Beck, 2005; Gray, 2005; Leister, 2005; Nott et al., 2006; Pesaresi et al., 2007; Bräutigam et al., 2008; Fernandez and Strand, 2008; Oelze et al., 2008).

A new aspect arose when Koussevitzky et al. (2007) demonstrated that the chloroplast localized protein GUN1 and the transcription factor ABI4 are common to three retrograde signaling pathways. The authors used lincomycin (for pathway 1), norflurazon (for pathway 2) or strong light (for pathway 3; see Fig. 8.1 for the pathways) and

demonstrated that the treatments did not reduce expression of *LHCB* genes in *gun1* mutants in contrast to wild-type *A. thaliana* plants. The same was true for the *abi4* mutant: expression of *LHCB* genes remained high after the application of lincomycin, norflurazon or strong light while other ABA-deficient or insensitive mutants showed reduced expression of *LHCB* genes as did the wild type. The authors concluded that all plastid signals of different origin converge within the plastids at GUN1 and, although the nature of the subsequent signaling pathway is still unknown, the signal arrives in the nucleus at the transcription factor ABI4 that regulates a large number of genes via a master switch.

A master switch was also postulated, although not as the only signaling pathway, but rather as one of several signaling pathways, on the basis of transcriptome analyses (Richly et al., 2003; Biehl et al., 2005). The authors used DNA arrays of 2,661 (Richly et al., 2003) and 3,292 (Biehl et al., 2005) nuclear *A. thaliana* genes, mostly encoding plastid proteins. A master switch was deduced from these studies because the same large cluster of nuclear genes was either induced or repressed by variations of external or genetic conditions. However, besides this cluster of co-regulated genes, named a regulon, Richly et al. (2003) found two other regulons among the investigated nuclear genes encoding chloroplast proteins, and Biehl et al. (2005) refined the array analysis of these three classes of co-regulated genes to as many as 21 regulons. This finding can best be understood with the assumption of several signaling pathways and possible cross-communication between them.

Expression profiles of nuclear genes as depending on plastid signals have been investigated by several authors. Piipo et al. (2006) found that expression of a distinct set of nuclear genes, encoding thylakoid proteins and stromal proteins of the Calvin-Benson cycle, responded to changing light conditions that, in turn, changed the redox state of stromal compounds. The earlier conclusion, based on DNA array analysis, that GUN1 and GUN5 are involved in separate pathways (Strand et al. 2003) was later corrected: clustering analysis, using a new *gun1* allele, showed a very similar pattern in the DNA array between *gun1* and *gun5* mutants implicating GUN1 and GUN5 in the same signaling pathway (Koussevitzky et al., 2007). In spite of this similarity in the pattern of gene expression, Koussevitzky et al. (2007) postulated that GUN1 is involved in signaling originating from several sources (protein synthesis, redox state and MgProto level) while GUN5 was believed to be restricted to the pathway that the same authors had tried earlier to correlate with the level of MgProto.

VII. Connection with Light Signals

Based on early investigations summarized by Oelmüller (1989), three regulatory factors were postulated for expression of nuclear genes encoding chloroplast proteins, exemplified for RBCS, in the following hierarchical order: the highest rank has an endogenous factor that establishes competence for expression of this set of genes, then a signal from the plastids that is a prerequisite for its expression, and finally light that modulates gene expression via photoreceptors absorbing red or blue light. While plastid signals and light signals were at first, apart from the hierarchical order, supposed to be two independent signal types, a closer relationship was later demonstrated at least in some cases: MgProto accumulates transiently at the shift from darkness to light in tobacco and barley seedlings (Pöpperl et al., 1998) and in *C. reinhardtii* cells (Kropat et al., 2000) and, as mentioned above, cis-elements were identified in *C. reinhardtii* that were responsive to both light and MgProto leading to the proposal that light induction in this alga is mediated by MgProto (von Gromoff et al., 2006). Also in *A. thaliana*, either the same or neighbouring promoter elements respond to both light and plastid signaling (Nott et al., 2006; Koussevitzky et al., 2007). Indirect evidence pointed to light-dependent release of MgProto from the plastid compartment to the cytoplasm in *C. reinhardtii* (Kropat et al., 2000). It is still a matter of debate whether MgProto, which is formed only in the plastid compartment, is transported out of this compartment. Ankele et al. (2007) detected MgProto both in chloroplasts and cytosol of *A. thaliana* seedlings under stress conditions by confocal laser scanning spectroscopy. On the other hand, in the model of Koussevitzky et al. (2007) MgProto remains within the plastids because GUN1, which acts downstream of MgProto, has been localized in the plastids.

A most intensive cross-communication between plastid signals and light signals was detected by Ruckle et al. (2007). In a screen for new *gun* mutants of *A. thaliana*, the authors isolated new *cryptochrome* (*cry1*) alleles; further they showed that also *cry1* mutants from other laboratories did indeed exhibit defects in plastid-to-nucleus signaling. CRY1 and HY5 have been described as regulators for nuclear gene expression in response to high irradiance although this was considered to be independent of plastid signaling (Kleine et al., 2007). The key observation by Ruckle et al. (2007) was accumulation of *LHCB* and *RBCS* mRNA in *cry1* mutants when structural and functional development of plastids was inhibited by lincomycin, erythromycin or norflurazon and strong light, although the mRNAs did not accumulate to the same high level as in *gun* null mutants. In studies with double mutants, combined with application of blue, red, and far-red light, the authors showed that GUN1 acts in a signaling pathway different from that of CRY1 and that the CRY1 pathway includes the regulators COP1 and HY5, already known from studies on photomorphogenesis. The most surprising conclusion from these studies was that plastid signals convert HY5 from a positive to a negative regulator of *LHCB*. Since differences in the regulation of *RBCS* and *LHCB* were found, especially under far-red light, and since derepression of *LHCB* in the double mutant *gun1-phyA* differed from that in the double mutant *gun1-phyB*, a complex network between plastid signals and signal pathways of various photoreceptors was postulated, which remains to be elucidated in detail.

VIII. Unsolved Questions, Outlook and Concluding Remarks

While it is evident that plastid signals originate from several sources within the plastids, the pathways of transmittance to the nucleus remain to be elucidated. Unclear, is also the relationship of signaling in carotenoid-free seedlings with that in the *flu* mutant: in both cases reactive oxygen species, produced by light activation of protochlorophyllide, are believed to be the primary source, leading in carotenoid-free seedlings to impairment of plastid development, destruction of thylakoid membranes, increase in the level of MgProto, and affecting primarily expression of nuclear photosynthesis genes. Plant development, even to the stage of flowering, was reported to be unimpaired under conditions of heterotrophic growth (Oelmüller, 1989). In contrast, singlet oxygen produced in the *flu* mutant activates nuclear stress response genes, mediated by EXECUTER proteins, while photosynthesis-related genes are less or not at all affected; visible consequences are cessation of plant growth, cell death and seedling death (Wagner et al., 2004; Lee et al., 2007).

The original view that plastid signals transmit the specific requirements of plastids to the nucleus leading to responses specific for the plastids seems now to be too simple. Pesaresi et al. (2006) investigated expression of nuclear photosynthetic genes in *A. thaliana* mutants defective in either plastid or mitochondrial protein synthesis. The single mutants *prpl-11* and *mrpl-11*, defective in the ribosomal protein L11 of plastids and mitochondria, respectively, showed only a marginal decrease in expression of the nuclear genes while transcript levels of the investigated nuclear photosynthetic genes were reduced by up to 80% in leaves of the double mutant, *prpl-11/mrpl-11*. A significant reduction in the expression of nuclear photosynthetic genes was also found in leaky mutants of the *PRORS1* gene encoding prolyl-tRNA synthetase 1 that acts in both plastids and mitochondria. Pesaresi et al. (2006) concluded that plastid and mitochondrial protein synthesis contribute synergistically to the transcriptional regulation of nuclear photosynthesis genes and that a defect in plastid protein synthesis can in part be compensated by signaling from mitochondrial protein synthesis. An interorganellar cross-communication, probably coordinating functional activity of mitochondria and plastids, was also proposed by Emanuel et al. (2005) for the *albostrians* mutant of barley: in this plastid ribosome-deficient mutant, transcript levels not only of RpoTp, the plastid nuclear-encoded phage-type RNA polymerase, but also of RpoTm, the mitochondrial counterpart, were higher than in wild-type leaves.

In summary, an interorganellar signaling network must be assumed to be acting in addition to the network of several plastid signals and signals from cytosolic photoreceptors. As mentioned in this chapter, various hypotheses have been articulated to describe how signals mentioned above

are transmitted from the plastids to the nucleus; however, even if genetic studies have identified single factors involved in such pathways, not even one of the signaling pathways has been completely elucidated. A major question that remains to be answered is how the signal is transmitted across the plastid envelope membrane. Pesaresi et al. (2007) stated that "mutants are of limited use for dissecting interorganellar signaling" because compensatory effects may mask the primary defect and can also trigger other signaling pathways. However, further progress can be expected when methods of Genetics, Biochemistry and Biophysics are further developed, refined and combined.

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References

Ankele E, Kindgren P, Pesquet E and Strand Å (2007) In vivo visualization of Mg-protoporphyrin IX, a coordinator of photosynthetic gene expression in the nucleus and the chloroplast. Plant Cell 19: 1964–1979

Apel K and Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399

Baier M and Dietz K-J (2005) Chloroplasts as source and target of cellular redox regulation: a discussion on chloroplast redox signals in the context of plant physiology. J Exp Bot 56: 1449–1462

Beck CF (2005) Signaling pathways from the chloroplast to the nucleus. Planta 222: 743–756

Biehl A, Richly E, Noutsos C, Salamini F and Leister D (2005) Analysis of 101 nuclear transcriptomas reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. Gene 344: 33–41

Bonardi V, Pesaresi P, Becker T, Schleiff E, Wagner R, Pfannschmidt T, Jahns P and Leister D (2005) Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. Nature 437: 1179–1182

Bradbeer JW, Atkinson YE, Börner T and Hagemann R (1979) Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid-synthesized RNA. Nature 279: 816–817

Bräutigam K, Dietzel L and Pfannschmidt T (2008) Plastid-nucleus communication: anterograde and retrograde signaling in the development and function of plastids. In: Bock R (ed) Topics in Current Genetics, Vol 19: Cell and Molecular Biology of Plastids, pp 409–456. Springer, Berlin/Heidelberg

Chen YB, Durnford DG, Koblizek M and Falkowski PG (2004) Plastid regulation of *Lhcb1* transcription in the chlorophyte alga *Dunaliella tertiolecta*. Plant Physiol 136: 3737–3750

Durnford DG and Falkowski PG (1997) Chloroplast redox regulation of nuclear gene transcription during photoacclimation. Photosynth Res 53: 229–241

Emanuel C, Weihe A, Graner A, Hess WR and Börner T (2005) Chloroplast development affects expression of phage-type RNA polymerases in barley leaves. Plant J 38: 460–472

Escoubas JM, Lomas M, La Roche J and Falkowski PG (1995) Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci USA 92: 10237–10241

Feierabend J and Schrader-Reichhardt U (1976) Biochemical differentiation of plastids and other organelles in rye leaves with a high-temperature-induced deficiency of plastid ribosomes. Planta 129: 133–145

Feierabend J and Wildner J (1978) Formation of the small in the absence of the large subunit of RuBP carboxylase in 70S ribosome-deficient rye leaves. Arch Biochem Biophys 186: 283–291

Fernandez AP and Strand Å (2008) Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. Curr Opinion Plant Biol 11: 509–513

Fey V, Wagner R, Bräutigam K and Pfannschmidt T (2005) Photosynthetic redox control of nuclear gene expression. J Exp Bot 56: 1491–1498

Fryer MJ, Ball L, Oxborough K, Karpinski S, Mullineaux PM and Baker NR (2003) Control of ascorbate peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. Plant J 33: 691–705

Gadjieva R, Axelsson E, Olsson U and Hansson, M (2005) Analysis of gun phenotype in barley magnesium chelatase and Mg protoporphyrin IX monomethyl ester cyclase mutants. Plant Physiol Biochem 43: 901–908

Goslings D, Meskauskiene R, Kim C, Lee KP, Nater M and Apel K (2004) Concurrent interactions of heme and FLU with Glut RNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. Plant J 40: 957–967

Gray JC (2005) Regulation of nuclear gene expression by plastid signals. Annu Plant Rev 13: 237–266

Heiber I, Ströher E, Raatz B, Busse I, Kahmann U, Bevan M, Dietz K-J and Baier M (2007) The *redox imbalanced* mutants of Arabidopsis differentiate signaling pathways for redox regulation of chloroplast antioxidant enzymes. Plant Physiol 143: 1774–1788

Hess WR, Schendel R, Börner T and Rüdiger W (1991) Reduction of mRNA level for two nuclear encoded light regulated genes in the barley mutant *albostrians* is not correlated with phytochrome content and activity. J Plant Physiol 138: 292–298

Hess WR, Müller A, Nagy F and Börner T (1994) Ribosome-deficient plastids affect transcription of light-induced nuclear genes: genetic evidence for a plastid-derived signal. Mol Gen Genet 242: 305–312

Hirashima M, Satoh S, Tanaka R and Tanaka A (2006) Pigment shuffling in antenna systems achieved by expressing prokaryotic chlorophyllide *a* oxygenase in *Arabidopsis*. J Biol Chem 281: 15385–15393

Johanningmeier U (1988) Possible control of transcript levels by chlorophyll precursors in *Chlamydomonas*. Eur J Biochem 177: 417–424

Johanningmeier U and Howell SH (1984) Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii.* J Biol Chem 259: 13541–13549

Karpinski S, Escobar C, Karpinska B, Creissen G and Mullineaux P *(*1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell 9: 627–640

Karpinski, S, Reynolds H, Karpinska B, Wingsle G, Creissen G and Mullineaux P (1999) Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. Science 284: 654–657

Kimura M, Manabe K, Abe T, Yoshida S, Matsui M and Yamamoto YY (2003) Analysis of hydrogen peroxide-independent expression of the high-light-inducible ELIP2 gene with the aid of the ELIP2 promoter-luciferase fusion. Photochem Photobiol 77: 668–674

Kittsteiner U, Brunner H and Rüdiger W (1991a) The greening process in cress seedlings II. Complexing agents and 5-aminolevulinate inhibit accumulation of cab-mRNA coding for the light-harvesting chlorophyll *a*/*b* protein. Physiol Plant 81: 190–196

Kittsteiner U, Mostowska A and Rüdiger W (1991b) The greening process in cress seedlings I. Pigment accumulation and ultrastructure after application of 5-aminolevulinate and complexing agents. Physiol Plant 81: 139–147

Kleine T, Kindgren P, Benedict C, Hendrickson L and Strand A (2007) Genome-wide gene expression analysis reveals a critical role for crytochrome 1 in the response of Arabidopsis to high irradiance. Plant Physiol 144: 1391–1406

Kropat J, Oster U, Rüdiger W and Beck CF (1997) Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-hock genes. Proc Natl Acad Sci USA 94:14168–14172

Kropat J, Pöpperl G, Rüdiger W and Beck CF (1999) Identification of Mg-protoporphyrin IX as a chloroplast signal that mediates the expression of nuclear genes. In: Wagner E, Normann J, Greppin H, Hackstein JHP, Hermann RG, Kowallik KV, Schenk HEA, Seckbach J (eds) From symbiosis to eukaryotism. Endocytobiol VII, pp 341–348. University of Geneva, Geneva

Kropat J, Oster U, Rüdiger W and Beck CF (2000) Chloroplast signaling in the light induction of nuclear *HSP70* genes requires the accumulation of chlorophyll precursors and their accessibility to cytoplasm/nucleus. Plant J 24: 523–531

Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim J, Mittler R and Chory J (2007) Signals from chloroplasts converge to regulate nuclear gene expression. Science 316: 715–719

La Rocca N, Rascio N, Oster U and Rüdiger W (2001) Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of Lhc and RbcS genes. Planta 213: 101–108

La Rocca N, Rascio N, Oster U and Rüdiger W (2007) Inhibition of lycopene cyclase results in accumulation of chlorophyll precursors. Planta 225: 1019–1029

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I and Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104: 672–677

Larkin RM, Alonso JM, Ecker JR and Chory J (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling. Science 299: 902–906

Lee KP, Kim C, Landgraf F and Apel K (2007) EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. Proc Natl Acad Sci USA104: 10270–10275

Leister D (2005) Genomics-based dissection of the cross-talk of chloroplasts with the nucleus and mitochondria in Arabidopsis. Gene 354: 110–116

Maxwell DP, Laudenbach DE and Huner NPA (1995) Redox regulation of light-harvesting complex II and cab mRNA abundance in *Dunaliella salina*. Plant Physiol 109: 787–795

Meskauskiene R and Apel K (2002) Interaction of FLU, a negative regulator of tetrapyrrole biosynthesis, with the glutamyl-tRNA reductase requires the tetratricopeptide repeat domain of FLU. FEBS Lett 532: 27–30

Meskauskiene R, Nater M, Goslings D, Kessler F, op den Camp R and Apel K (2001) FLU: A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 98: 12826–12831

Mochizuki N, Brusslan JA, Larkin R, Nagatani A and Chory J (2001) *Arabidopsis* genomes uncoupled 5 (GUN 5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc Natl Acad Sci USA 98: 2053–2058

Mochizuki N, Tanaka R, Tanaka A, Masuda T and Nagatani A (2008) The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in *Arabidopsis*. Proc Natl Acad Sci USA 105: 15184–15189

Moulin M, McCormac MC, Terry MJ and Smith AG (2008) Tetrapyrrole profiling in *Arabidopsis* seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 105: 15178–15183

Nott A, Jung H-S, Koussevitzky S and Chory J (2006) Plastid-to-nucleus retrograde signaling- Annu Rev Plant Biol 57: 739–759

Oelmüller R (1989) Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. Photochem Photobiol 49:229–239

Oelmüller R, Dietrich G, Link G and Mohr H (1986) Regulatory factors involved in gene expression (subunits of ribulose-1,5-bisphosphate carboxylase) in mustard (*Sinapis alba* L.) cotyledons. Planta 169: 260–266

Oelze ML, Kandlbinder A and Dietz K-J (2008) Redox regulation and overreduction control in the photosynthesizing cell: complexity in redox regulatory networks. Biochim Biophys Acta 1780: 1261–1272

Oster U, Brunner H and Rüdiger W (1996) The greening process in cress seedlings. V. Possible interference of chlorophyll precursors, accumulated after thujaplicin treatment, with light-regulated expression of Lhc genes. J Photochem Photobiol B 36: 255–261

Oswald O, Martin T, Dominy PJ and Graham IA (2001) Plastid redox state and sugars: Interactive regulators of nuclear-encoded photosynthetic gene expression. Proc Natl Acad Sci USA 98: 2047–2052

Pesaresi P, Masiero S, Eubel H, Braun HP, Bhushan S, Glaser E, Salamini F and Leister D (2006) Nuclear photosynthetic gene expression is synergistically modulated by rates of protein synthesis in chloroplasts and mitochondria. Plant Cell 18: 970–991

Pesaresi P, Schneider A, Kleine T and Leister D (2007) Interorganellar communication. Curr Opinion Plant Biol 10: 1–7

Petracek ME, Dickey LF and Thompson WF (1997) Light-regulated changes in abundance and polyribosome association of ferredoxin are dependent on photosynthesis. Plant Cell 9: 2291–2300

Petracek ME, Dickey LF, Nguyen TT, Gatz G, Sowinski DA, Allen GC and Thompson WF (1998) Ferredoxin-1 mRNA is destabilized by changes in photosynthetic electron transport. Proc Natl Acad Sci USA 95: 9009–9013

Pfannschmidt T (2003) Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33–41

Pfannschmidt T, Schütze K, Brost M and Oelmüller R (2001) A novel mechanism of nuclear photosynthesis gene regulation by redox signals from the chloroplast during photosystem stoichiometry adjustment. J Biol Chem 276: 36125–36130

Piipo M, Allahverdiyeva Y, Paakkarinen V, Suoranta UM, Battchikova N and Aro EM (2006) Chloroplast-mediated regulation of nuclear genes in *Arabidopsis thaliana* in the absence of light stress. Physiol Genomics 25: 142–152

Pontier D, Albrieux C, Joyard J, Lagrange T and Block M (2007) Knock-out of the magnesium protophorphyrin IX methyltransferase gene in Arabidopsis. Effects on chloroplast development and on chloroplast-to-nucleus signaling. J Biol Chem 282: 2297–2304

Pöpperl G, Oster U and Rüdiger W (1998) Light-dependent increase in chlorophyll precursors during the day-night cycle in tobacco and barley seedlings. J Plant Physiol 153: 40–45

Pursiheimo S, Mulo P, Rintamäki E and Aro EM (2001) Coregulation of light-harvesting complex II phosphorylation and *Lhcb* accumulation in winter rye. Plant J 26: 317–327

Reinbothe C, Bartsch S, Eggink LL, Hoober K, Bruslan J, Andrade-Paz R, Monnet J and Reinbothe S (2006) A role foe chlorophyllide *a* oxygenase in the regulated import and stabilization of light-harvesting chlorophyll *a*/*b* proteins. Proc Natl Acad Sci USA 103: 4777–4782

Richly E, Dietzmann A, Biehl A, Kurth J, Laloi C, Apel K, Salamini F and Leister D (2003) Covariations in the nuclear chloroplast transcriptome reveal a regulatory master-switch. EMBO Rep 4: 491–498

Ruckle ME, DeMarco SM and Larkin RM (2007) Plastid signals remodel light signaling networks and are essential for efficient chloroplast biogenesis in *Arabidopsis*. Plant Cell 19: 3944–3960

Rzeznicka K, Walker CJ, Westergren T, Kannangara CG, von Wettstein D, Merchant S, Gough SP and Hansson, M. (2005) Xantha-l encodes a membrane subunit of the aerobic Mg-protoporphyrin IX monomethyl ester cyclase involved in chlorophyll biosynthesis. Proc Natl Acad Sci USA 102: 5886–5891

Shao N, Vallon O, Dent R, Niyogi KK and Beck CF (2006) Defects in the cytochrome b_6f complex prevent light-induced expression of nuclear genes involved in chlorophyll biosynthesis. Plant Physiol 141: 1128–1137

Shao N, Krieger-Liszkay A, Schroda M and Beck CF (2007) A reporter system for the individual detection of hydrogen peroxide and singlet oxygen: its use for the assay of reactive oxygen species produced in vivo. Plant J 50: 475–487

Sherameti I, Sopory SK, Trebicka A, Pfannschmidt T and Oelmüller R (2002) Photosynthetic electron transport determines nitrate reductase gene expression and activity in higher plants. J Biol Chem 277: 46594–46600

Strand Å (2004) Plastid-to-nucleus signaling. Curr Opinion Plant Biol 7: 621–625

Strand Å, Asami T, Alonso J Ecker JR and Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature 421: 79–83

Sullivan JA and Gray JC (1999) Plastid translation is required for the expression of nuclear photosynthesis genes in the dark and in the roots of the pea *lip1* mutant. Plant Cell 11: 901–910

Sullivan JA and Gray JC (2002) Multiple plastid signals regulate the expression of the pea plastocyanin gene in pea and transgenic tobacco plants. Plant J 32: 763–774

Susek RE, Ausubel FM and Chory J (1993) Signal transduction mutants in Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell 74: 787–799

Tanaka R and Tanaka A (2007) Tetrapyrrole biosynthesis in higher plants. Annu Rev Plant Biol 58: 321–346

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu Rev Plant Physiol Plant Mol Biol 40: 211–233

Taylor WC, Burgess DG and Mayfield SP (1986) The use of carotenoid deficiencies to study nuclear-chloroplast regulatory interactions. In: Randall D (ed) Current Topics in Plant Biochemistry and Physiology, Vol 5, pp 117–127. The Interdisciplinary Plant Biochemistry and Physiology Program, University of Missouri, Columbia

Vasileuskaya Z, Oster U and Beck C (2004) Involvement of tetrapyrroles in inter-organellar signaling in plants and algae. Photosynth Res 82: 289–299

Vasileuskaya Z, Oster U and Beck C (2005) Mg-Protoporphyrin IX and heme control *HEMA*, the gene encoding the first specific step of tetrapyrrole biosynthesis, in *Chlamydomonas reinhardtii.* Eukaryotic Cell 4: 1620–1628

von Gromoff ED, Schroda M, Oster U and Beck CF (2006) Identification of a plastid response element that acts as an enhancer within the *Chlamydomonas HSP70*A. Nucleic Acids Res 34: 4767–4779

von Gromoff ED, Alawady A, Meinecke L, Grimm B and Beck CF (2008) Heme, a plastid-derived regulator of nuclear gene expression in *Chlamydomonas*. Plant Cell 20: 552–567

Wagner D, Przybyla D, op den Camp R, Kim C, Landgraf F, Lee KP, Würsch M, Laloi C, Nater M, Hideg E and Apel K (2004) The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. Science 306: 1183–1185

Wilde A, Mikolajczyk S, Alawady A, Lokstein H and Grimm B (2004) The *gun4* gene is essential for cyanobacterial porphyrin metabolism. FEBS Lett 571: 119–123

Yabuta Y, Maruta T, Yoshimura K, Ishikawa T and Shigeoka S (2004) Two distinct redox signaling pathways for cytosolic APX induction under photooxidative stress. Plant Cell Physiol 45: 1586–1594

Yang DH, Andersson B, Aro EM and Ohad I (2001) The redox state of the plastoquinone pool controls the level of the light-harvesting chlorophyll a/b binding protein complex II (LHII) during photoacclimation. Photosynth Res 68: 163–174

Chapter 9

Role of the Envelope Membranes in Chloroplast Glycerolipid Biosynthesis

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Summary

All plastid membranes are characterized by the presence of large amounts of galactolipids. The galactolipid content of thylakoids is especially high since galactolipids represent up to 80% of the membrane glycerolipids, out of which monogalactosyldiacylglycerol (MGDG) constitutes the main part (50%). In its first part, this chapter describes the structure of the main glycerolipids present in plastids, particularly of galactolipids and details their subcellular localization. Functional analysis of mutants deleted of specific lipids and structural data obtained from Photosystem I and Photosystem II crystallization are presented to characterize the specific role of each glycerolipid. In the second part, this chapter summarizes our current

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understanding of the role of the chloroplast envelope in glycerolipid biogenesis and points out the emerging lipid trafficking mechanisms that envelope membranes take part in. The building up of eukaryotic and prokaryotic lipid structures proceeds from at least two distinct pathways requiring different trafficking of lipids. Whereas synthesis of prokaryotic glycerolipids is entirely realized in the envelope, synthesis of eukaryotic glycerolipids relies on the transfer of precursors from the endoplasmic reticulum to the envelope. The final assembly of glycerolipids is made in the envelope membranes prior to transfer to thylakoids membranes. A special focus is given to current results concerning MGDG and digalactosyldiacylglycerol (DGDG) synthesis in plants grown in phosphate-deficient conditions.

I. Introduction

The chloroplast membrane system is made up of a limiting two-membrane envelope and a sophisticated network of thylakoid membranes devoted to the photosynthetic process. Chloroplast membranes are essentially enriched in glycoglycerolipids: these are the two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG). This is in contrast to other plant cell membranes where phospholipids are the major lipid constituents. Phosphatidylethanolamine (PE) is totally excluded from chloroplast membranes but some specific phospholipids are, however, present in chloroplast membranes. A C16:1-trans fatty acid-containing phosphatidylglycerol (PG) molecular species is specific to chloroplast membranes. Phosphatidylcholine (PC) is also present in chloroplasts but only in the outer leaflet of the outer envelope membrane. An overview of the complex metabolic pathway that leads to the biosynthesis of the chloroplast lipid compounds emphasizes the striking importance of envelope membranes and the necessity of regulated trafficking of lipid intermediates between cell compartments.

Fatty acids synthesized in the plastid stroma are either directly metabolized into the chloroplast glycerolipids within the envelope or exported across the envelope to the endoplasmic reticulum (ER) where they are incorporated into major cell phospholipids, especially into PC. Part of the glycolipids necessary for chloroplast membrane structure derives from these PC molecules through diacylglycerol (DAG) and their synthesis requires import of lipid intermediate(s) from the ER to the envelope. The final galactosylation or sulfoquinovosylation of DAG is realized within the chloroplast envelope before exporting of lipids to thylakoids.

The discovery that in plants deprived of phosphate (Pi), DGDG can specifically increase and replace PC in non plastidic membranes opened a new area of research dealing with the ability of the envelope to synthesize these molecules and export them to particular membranes of the cell in response to Pi starvation.

This chapter summarizes our current understanding of the role of the chloroplast envelope in glycerolipid biogenesis and points out the emerging lipid trafficking mechanisms that are found associated with envelope membranes. Complementary details are also available in volume 30 of this book series (see Wada and Murata (2009)).

II. Glycerolipid Composition of Chloroplast Membrane

A. Galactolipids

All plastid membranes are characterized by the presence of large amounts of galactolipids (Table 9.1). The galactolipid content of thylakoids

Abbreviations: ACCase – Acetyl CoA carboxylase; ACP – Acyl carrier protein; DAG – Diacylglycerol; DGD – DGDG synthase; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; GGGT – Galactolipid-galactolipid galactosyltransferase; K_m – Michaelis-Menton constant; LHCII – Light-harvesting complex of photosystem II; LysoPC – Lysophosphatidylcholine; MCS – Membrane contact sites; MGD – MGDG synthase; MGDG – Monogalactosyldiacylglycerol; MurG – UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PI – Phosphatidylinositol; PS I – Photosystem I; PS II – Photosystem II; SQD – Enzyme involved in sulfolipid synthesis; SQDG – Sulfoquinovosyldiacylglycerol; UDP-gal – Uridine diphosphate galactose

Table 9.1. Glycerolipid composition of spinach chloroplast membranes as reported by Douce and Joyard (1996)

	Outer envelope membrane	Inner envelope membrane	Total envelope membranes	Thylakoids
Total polar lipids (mg/mg protein)	2.5–3	1	1.2–1.5	0.6–0.8
Polar lipids (% of total)				
MGDG	17	55	32	57
DGDG	29	29	30	27
SQDG	6	5	6	7
PC	32	0	20	0
PG	10	9	9	7
PI	5	1	4	1
PE	0	0	0	0

and of the inner envelope membrane is fairly similar and especially high since galactolipids represent up to 80% of membrane glycerolipids, out of which MGDG constitutes the main part (50%). Considering that huge thylakoid areas are required for photosynthesis, the high enrichment in galactolipids instead of phospholipids can be viewed as a way to enable cells to build up these membranes with a minimal phosphate requirement. The basic structures of MGDG and DGDG are similar but numerous different characteristics correlate with specific features of each of these galactolipids in the chloroplast membranes.

1. Monogalactosyldiacylglycerol

MGDG and DGDG contain respectively, one and two galactose molecules attached to the *sn*-3 position of the glycerol backbone (Fig. 9.1). The single galactose in MGDG and the first one in DGDG are linked to DAG via a $\beta 1 \rightarrow 3$-glycosidic bond (Carter et al., 1956). At the *sn*-1 and *sn*-2 positions of the glycerol backbone, MGDG contains mostly polyunsaturated fatty acids; in some plant species up to 95% of the total fatty acid of MGDG is linolenic acid (18:3). However, this average value does not tell us about the local MGDG enrichment in the membranes. For instance, MGDG associated with the Photosystem II (PS II) reaction center complex have been found to be highly enriched in 14:0, 16:0 and 18:0 fatty acids (Murata et al., 1990); and also see reviews by Kern and Guskov (2011) and Mizusawa and Wada (2011).

The most abundant molecular species of MGDG have C18 fatty acids at both *sn*-1 and *sn*-2 positions of the glycerol backbone like PC (Heinz, 1977). This structure with C18 at the *sn*-2 position is referred to as a eukaryotic structure. Plants such as pea and cucumber, having almost only C18:3 in MGDG are called "18:3 plants". In "16:3 plants", such as spinach or *Arabidopsis thaliana*, part of the MGDG molecular species contain C16 fatty acids at the *sn*-2 position instead of C18 fatty acids (Heinz, 1977). This structure is referred to as a prokaryotic structure because it is characteristic for cyanobacterial glycerolipids. Together, plant species vary in their specific ratio of prokaryotic versus eukaryotic structures in MGDG. Among Angiosperms, dicots are either 16:3 or 18:3 plants whereas monocots are mostly 18:3 plants suggesting that loss of the prokaryotic pathway occurred several times independently and at different rates (Mongrand et al., 1998).

Globally MGDG presents a cone-like shape due to the small development of its head group compared to the volume of the unsaturated fatty acid chains. Pure MGDG in water does not form bilayers in contrast to DGDG or PC. It preferentially organizes in inverted hexagonal (H_{II}) phase. However, in the thylakoid membranes this type of organization is not normally observed. It has been suggested that the MGDG cone shape can act to pack large protein complexes into biological membranes and through lipid-protein interactions prevent MGDG adopting non-bilayer structures (Gounaris and Barber, 1983). Indeed when MGDG and PS II light-harvesting complex (LHCII) proteins were incubated in vitro, the formation of lamellar structures was observed (Simidjiev et al., 2000). MGDG occupies the inner leaflet of the highly curved margins of the thylakoid membrane, and it may exert a lateral packing pressure on the membrane proteins and thereby facilitate the embedding of proteins. In support of this concept, analyses of the *mgd1* mutants, strongly reduced in MGDG content, have shown that mutant plants have a yellow-green

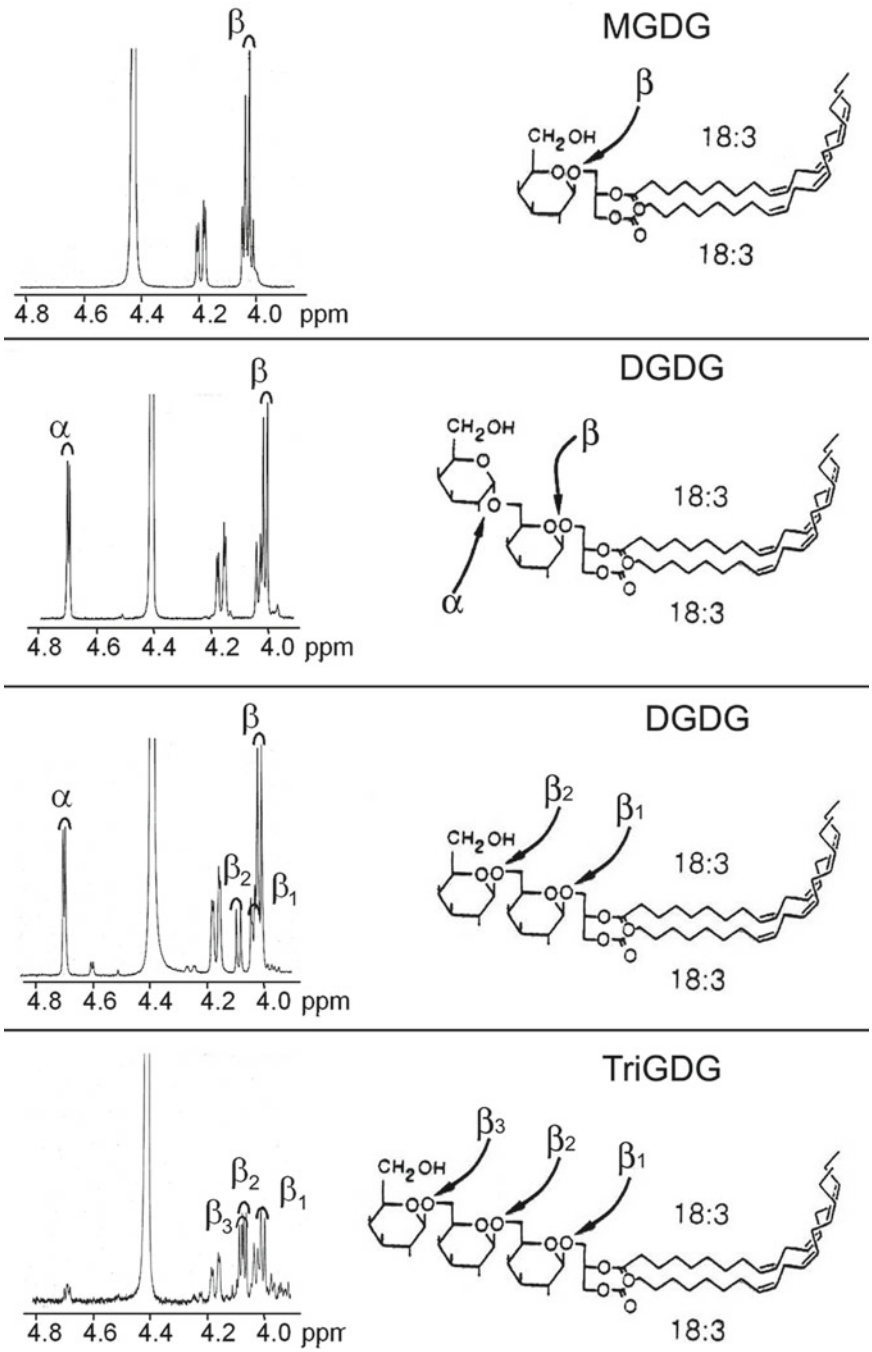

Fig. 9.1. Structure of galactolipids present in plants. β MGDG, α-β DGDG, β-β DGDG, and β-β-β TriGDG. α and β conformation of the anomeric carbon of each galactose is analyzed by ^{1}H-NMR on fractions containing β MGDG, α-β DGDG, a mixture of α-β DGDG and β-β DGDG, or β-β-β TriGDG. α-conformation is characterized by a doublet peak around 4.7 ppm and β-conformation by a doublet peak around 4.0 ppm.

phenotype and striking defects in chloroplast ultrastructure (Jarvis et al., 2000). Complete suppression of MGD1 in *A. thaliana* induces a lack of formation of MGDG and DGDG and an impairment of photosynthetic growth with an arrest of embryo development (Kobayashi et al., 2007).

This indicates a unique role for MGDG in the structural organization of plastidic membranes. The association of MGDG with thylakoid proteins was shown to be essential to maintain their functional properties (Trémolières et al., 1994). The analysis of the location and specific binding position of different lipids in the PS I complex indicated that lipids including MGDG are integral to and functionally important in the PS I complex (Jordan et al., 2001).

2. Digalactosyldiacylglycerol

Considering the anchoring of the second galactose present in DGDG, two different glycosidic bonds have been reported in plant DGDG: either Gal (α1-6) or Gal (β1-6) (Kojima et al., 1989). Only the first one is considered to be present in standard conditions, whereas the formation of the second one is likely related to stress conditions or deregulation of the galactolipid synthesis pathway (Sakaki et al., 1990; Xu et al., 2003).

In a given plant the ratio of eukaryotic to prokaryotic molecular species is different in MGDG and DGDG. For instance, although half of the spinach MGDG has a prokaryotic structure, this holds true for only 10–15% of DGDG (Bishop et al., 1985). In addition, C16 fatty acids in DGDG are more saturated than in MGDG (Siebertz et al., 1979).

In the aqueous phase, DGDG molecules organize in bilayers and DGDG is expected to be important for proper assembly of chloroplast membranes. Indeed DGDG is present in all chloroplast membranes at a high proportion (about 30% of total polar lipids) and deficiency of DGDG (90% reduction of the DGDG content in the *dgd1* mutant) was shown to affect thylakoid ultrastructure and photosynthetic capability (Dörmann et al., 1995). The mutant exhibited a decreased PS II/PS I ratio and an increase in the amount of peripheral light-harvesting complexes of PS II relative to both the inner antenna complexes and the PS II reaction center complexes (Härtel et al., 1997). Importance of DGDG for the structural and functional stability of the water-oxidizing complex is likely since DGDG deficiency was shown to cause a change in the Arrhenius plot of the temperature dependence of electron transfer from Q_A^- to Q_B, the primary and secondary plastoquinone electron acceptors of PS II, respectively (Reifarth et al., 1997). The significance of DGDG was also studied on purified photosystems. DGDG was required for the formation of 2D and 3D crystals of LHCII trimers (Nussberger et al., 1993; Flachmann and Kühlbrandt, 1996) and removal of DGDG from PS II-enriched samples inhibited O_2 evolution, an effect that was reversed by subsequent re-supplementation with the lipid (Gounaris et al., 1983). The degree of trimerization of LHCII and the photosynthetic quantum yield of *dgd1* plants complemented with glucose containing glycerolipids were only partially restored indicating specific interactions of the galactolipid head group with photosynthetic protein complexes (Holzl et al., 2006). A small fraction of thylakoid DGDG molecules was inferred to be specifically bound to PS II as an essential constituent for its functional competence (Steffen et al., 2005). By mutant analysis, it was finally shown that DGDG plays important roles in PS II through the binding of extrinsic proteins required for stabilization of the oxygen-evolving complex (Sakurai et al., 2007).

DGDG is likely to play specific roles in the chloroplast envelope as well. For instance, DGDG may play a role in protein import since the *dgd1* mutant was shown to be defective in protein import into chloroplasts (Chen and Li, 1998).

DGDG acts also in the adaptation of the plant to Pi starvation, replacing phospholipids mainly in non-plastidial membranes (Härtel et al., 2000; Andersson et al., 2003; Jouhet et al., 2004) and likely in plastidial membranes. Since DGDG is synthesized only in the outer envelope membrane, envelope DGDG can therefore be considered as a key element in this adaptation (see also Sections IV.D and IV.E). The *pho1* mutant of *A. thaliana* deficient in phosphate content in the aerial part (Poirier et al., 1991) has been shown to respond to phosphate deficiency in the leaves by decreasing the amount of PG, PC and PE and to concomitantly increase DGDG and SQDG (Härtel et al., 1998). In the *dgd1/pho1* double mutant, a significant DGDG accumulation induced by Pi deprivation could not restore photosynthetic activity, indicating that the molecular DGDG species synthesized upon phosphate deprivation in leaves cannot substitute for the DGDG species present under normal nutrient availability. It was suggested that depending on the environmental growth conditions different pools of DGDG species exist in

plants of which one is not associated with the photosynthetic apparatus (Härtel et al., 2001).

3. Tri and Tetragalactolipids

Trace amounts of trigalactosyldiacylglycerol (Tri-GDG) and tetragalactosyldiacylglycerol (Tetra-GDG) were found in isolated chloroplast fractions resulting from the activation of an outer envelope galactolipid-galactosyltransferase (Van Besouw and Wintermans, 1978). These oligogalactolipids were also reported in plant tissues i.e., in Adzuki bean (*Vigna angularis*) (Kojima et al., 1989) and in the *tgd A. thaliana* mutants likely deficient in phosphatidic acid transport in the chloroplast envelope (Xu et al., 2003; Awai et al., 2006; Lu et al., 2007). Various structures were resolved by NMR corresponding to Gal (β1 → 6) derivatives of MGDG (Xu et al., 2003) and DGDG (Kojima et al., 1989).

B. Sulfolipids

The sulfolipid sulfoquinovosyldiacylglycerol (SQDG) is a glycolipid present in small amounts in all plastidial membranes and only in plastidial membranes. Unlike galactolipids, it is anionic at physiological pH because of its 6-deoxy-6-sulfonate-glucose (sulfoquinovose) head group.

In a 16:3 plant such as spinach, a higher proportion of SQDG has a prokaryotic structure as compared to MGDG, whereas in wheat, an 18:3 plant, SQDG is exclusively eukaryotic (Bishop et al., 1985). In addition, high proportions of 16:0/16:0 molecular species have been reported in spinach. In this plant, SQDG contains a higher level of C16 fatty acid than either MGDG or DGDG and this fatty acid is fully saturated.

Like galactolipids, SQDG is accessible to specific antibodies on the cytosolic face of isolated intact chloroplasts (Billecocq et al., 1972). Consistent with its localization on the chloroplast surface, SQDG was demonstrated to interact with an annexin in a Ca^{2+}-dependent manner on the outer surface of chloroplast suggesting a role of SQDG in the binding of this protein to the envelope and eventually in a Ca^{2+}-regulated process (Seigneurin-Berny et al., 2000).

SQDG might be essential for plants only in specific conditions. With a complete lack of sulfolipid, the *sqd2 A. thaliana* mutant did not show any obvious signs of altered growth and photosynthetic parameters were affected only mildly under normal laboratory growth conditions (Yu et al., 2002). However, the s*qd2* mutant showed reduced growth under phosphate-limited growth conditions (see for details Section IV.E). Along with PG, sulfolipid contributes to maintaining a negatively charged lipid-water interface, which is required for the proper functioning of photosynthetic membranes (Yu et al., 2002; Yu and Benning, 2003). Lipid analyses of chlorophyll-protein complexes have shown than SQDG is present in the PS II core complex and in LHCII but absent from PS I in *Chlamydomonas reinhardtii* (Sato et al., 2003).

C. Phospholipids

1. Phosphatidylglycerol

Phosphatidylglycerol (PG) is a minor constituent of both plastidial and extra plastidial membranes. Chloroplast PG is unique because it has a prokaryotic structure and moreover it contains a unique trans Δ3- hexadecenoic acid at the *sn*-2 position of the glycerol backbone (Dubacq and Trémolières, 1983).

Despite the fact that PG is only a minor plastid membrane constituent, multiple independent lines of evidence suggest that it plays a critical role in the proper functioning of photosynthetic membranes associated with cold acclimation. For instance, fatty acid unsaturation of PG in thylakoid membranes stabilizes the photosynthetic machinery against low-temperature photoinhibition by accelerating the recovery of the PS II protein complex (Moon et al., 1995). A number of studies on cyanobacteria have revealed that PG is required to preserve the native conformation of both the PS I and the PS II complexes but that PG is less tightly bound to PS II than to PS I (for a review see Frentzen, 2004). Two mutants of *C. reinhardtii*, *mf1* and *mf2*, characterized by a marked reduction in their PG content together with a complete loss in their $16{:}1_{trans}$ fatty acid-containing form, also lost their PS II activity. A role of PG in the coupled process of cotranslational insertion and assembly of PS II core subunits has been proposed (Pineau et al., 2004). PG could also contribute to the cotranslational insertion of D1 in the thylakoid membranes (Pineau

et al., 2004). In *A. thaliana* mutants affected in the biosynthesis of chloroplast PG (for instance in the *pgp-1* mutant), the development of chloroplasts is severely affected (Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003). PG contributes to the synthesis and/or stability of the PS I complex for maintenance of the cellular content of chlorophyll, and to construction of the PS I trimer from the monomer at least through stabilization of the trimerized conformation (Jordan et al., 2001; Domonkos et al., 2004; Sato et al., 2004). PG is also involved in the formation of trimers of LHCII (Nussberger et al., 1993) and in the dimerization of the PS II reaction center (Kruse et al., 2000).

2. Phosphatidylcholine

Phosphatidylcholine (PC) is only a minor constituent of plastid membranes but a major constituent of the outer envelope. In chloroplasts, PC is concentrated in the outer leaflet of the outer envelope membrane and is absent from the inner envelope membrane and from the thylakoids (Dorne et al., 1990). Intriguing questions, which remain to be elucidated, are how and why PC accumulates specifically in the cytosolic leaflet of the outer envelope membrane. Phospholipid asymmetry is a common feature of biological membranes and is particularly studied in erythrocyte plasma membrane where asymmetry perturbation has been related to various changes of cell surface properties (Daleke, 2003). In plants, it is interesting that most non plastidial cellular membranes are enriched in PC and that furthermore, in mitochondria, the outer membrane contains much more PC than the inner membrane (Bligny and Douce, 1980). The physiological significance of PC accumulation in the outer envelope might also be related to the involvement of PC as an intermediate in chloroplast polar lipid synthesis.

III. Biosynthesis of Phosphatidic Acid and Diacylglycerol

Plastid envelope membranes are the site of assembly of fatty acids, glycerol and polar head groups. However, in vitro kinetics of acetate incorporation into chloroplast lipids have demonstrated that isolated chloroplasts can synthesize glycerolipids containing almost exclusively a C18/C16 DAG backbone, but are unable to catalyze the formation of phosphatidic acid and DAG containing only C18 fatty acids. Taking into account the last results concerning lipid transfer from and to chloroplasts, we discuss below that the building up of eukaryotic and prokaryotic structures proceeds from at least two distinct pathways, both involving envelope membranes. The genes potentially involved in the biosynthesis steps are listed in Table 9.2.

A. Synthesis of Fatty Acids in the Plastid Stroma

The major site of de novo synthesis of fatty acids in the plant cell is the plastid stroma. It is not the unique site of fatty acid synthesis in plant cells, since plant mitochondria are also able to synthesize C18 and C16 fatty acids although in a much lower rate than chloroplasts (Gueguen et al., 2000). In plastids, fatty acid synthesis depends on the supply of carbon substrate from the cytosol. A wide range of metabolites is used for fatty acid synthesis in plastids, including glucose-6-phosphate, triose phosphate, malate, pyruvate and phosphoenolpyruvate (Kubis et al., 2004). Precursors are metabolized into acetyl-CoA inside the stroma. The synthesis of malonyl-CoA is catalyzed by acetyl-CoA carboxylase (ACCase) in an ATP-dependent step. Most of the plastid ACCase is of type II, a multisubunit complex analogous to that found in prokaryotes. Type I ACCase, a large multifunctional enzyme analogous to that found in yeast and mammals occurs also in some plastids (Rawsthorne, 2002). Type II ACCase is strongly associated with the chloroplast envelope through non-ionic interactions of an unidentified integral membrane protein, to the carboxyltransferase subunit (Thelen and Ohlrogge, 2002). The presence of α and β subunits of ACCase in envelope preparations was demonstrated by western blot experiments (Thelen and Ohlrogge, 2002) and by proteomic studies of envelope membranes from *A. thaliana* chloroplasts (Ferro et al., 2003). Since acetyl-CoA carboxylase activity is a stromal activity, these two proteins should be present at the stroma side of the inner envelope membrane, anchoring the ACCase complex to the membrane. Interestingly the β subunit of ACCase is chloroplast encoded. One possible

Table 9.2. Arabidopsis thaliana gene candidates for enzymes potentially involved in plastid lipid biosynthesis

Description	EC	Locus	Protein name	Evidence	TM	Localization
Fatty acid synthesis						
Homomeric acetyl-CoA carboxylase (Type I)	6.4.1.2	At1g36180	ACC2	Sequence similarity	0	P
Acetyl-CoA carboxylase, α-carboxyltransferase (Type II)	6.4.1.2	At2g38040	CAC3	Sequence similarity	0	P (S, IEM)
Acetyl-CoA carboxylase, β-carboxyltransferase (Type II)		AtCg00500	ACCD	Sequence similarity	0	P (S, IEM)
Acetyl-CoA carboxylase, biotin carboxylase (Type II)	6.4.1.2	At5g35360	CAC2	Protein expression	0	P
Acetyl-CoA carboxylase, biotin carboxyl carrier protein (Type II)	6.4.1.2	At5g16390	BCCP1	Protein expression	0	P
Acetyl-CoA carboxylase, biotin carboxyl carrier protein (Type II)	6.4.1.2	At5g15530	BCCP2	Protein expression	0	P
Malonyl-CoA : ACP malonyltransferase	2.3.1.39	At2g30200		Sequence similarity	0	P
Acyl carrier protein (FAS)		At5g27200		Sequence similarity	0	P
Acyl carrier protein (FAS)		At1g54580	ACP2	Sequence similarity	0	P
Acyl carrier protein (FAS)		At1g54630	ACP3	Sequence similarity	0	P
Acyl carrier protein (FAS)		At3g05020	ACP1	Sequence similarity	0	P
Acyl carrier protein (FAS)		At4g25050		Sequence similarity	0	P
Acyl carrier protein (FAS)		At3g11470		Sequence similarity	0	P
Ketoacyl-ACP synthase III (FAS)	2.3.1.41	At1g62640	KAS III	Sequence similarity	0	P
Ketoacyl-ACP synthase I (FAS)	2.3.1.41	At5g46290	KAS I	Sequence similarity	0	P
Ketoacyl-ACP synthase II (FAS)	2.3.1.41	At1g74960	KAS II/ FAB1	Sequence similarity	0	P
Ketoacyl-ACP reductase	1.1.1.100	At1g24360		Sequence similarity	0	P
Hydroxyacyl-ACP dehydrase	4.2.1.*	At2g22230		Sequence similarity	0	P
Hydroxyacyl-ACP dehydrase	4.2.1.*	At5g10160		Sequence similarity	0	P
Enoyl-ACP reductase	1.3.1.9	At2g05990		EMS mutant characterization	0	P
Fatty acid desaturation						
Stearoyl-ACP desaturase	1.14.19.2	At2g43710	FAB2/SSI2	Protein expression	0	P
Stearoyl-ACP desaturase	1.14.19.2	At3g02610		Sequence similarity	0	P
Stearoyl-ACP desaturase	1.14.19.2	At3g02620		Sequence similarity	0	P
Stearoyl-ACP desaturase	1.14.19.2	At3g02630		Sequence similarity	0	P
Stearoyl-ACP desaturase	1.14.19.2	At5g16230		Sequence similarity	0	P
Stearoyl-ACP desaturase	1.14.19.2	At5g16240		Sequence similarity	0	P
Oleate desaturase	1.14.99.*	At4g30950	FAD6/FD6C	EMS mutant characterization, functional complementation	2	P
Linoleate desaturase	1.14.99.*	At3g11170	FAD7/FD3C	EMS mutant characterization, complementation	3	P
Linoleate desaturase	1.14.99.*	At5g05580	FAD8	EMS mutant characterization, functional complementation	3	P
Monogalactosyldiacylglycerol desaturase (palmitate-specific)	1.14.99.*	At3g15850	FAD5	Sequence similarity	2	P

Omega-6 oleate desaturase/delta-12 desaturase/	1.14.99.*	At3g12120	FAD2	T-DNA mutant characterization, functional complementation	6	– (ER)
Omega-3 linoleate desaturase	1.14.99.*	At2g29980	FAD3	EMS mutant characterization, complementation	3	– (ER)
DAG synthesis						
Glycerol-phosphate acyltransferase	2.3.1.15	At1g32200	ATS1/ACT1	Protein expression	0	P
2-Lysophosphatidic acid acyltransferase	2.3.1.51	At4g30580	ATS2	Protein expression	2	P (E)
Phosphatidic acid phosphatase	3.1.3.4	At5g03080	LPPγ	Rescue of yeast mutant	3	P
Phosphatidic acid phosphatase	3.1.3.4	At3g50920	LPPε1	Rescue of yeast mutant	3	P
Phosphatidic acid phosphatase	3.1.3.4	At5g66450	LPPε2	Rescue of yeast mutant	3	P
MGDG synthesis						
Monogalactosyldiacylglycerol synthase	2.4.1.46	At4g31780	MGD1	T-DNA mutant characterization, protein expression	0	P (IEM)
Monogalactosyldiacylglycerol synthase	2.4.1.46	At2g11810	MGD3	Protein expression	0	P (OEM)
Monogalactosyldiacylglycerol synthase	2.4.1.46	At5g20410	MGD2	Protein expression	0	P (OEM)
DGDG synthesis						
Digalactosyldiacylglycerol synthase	2.4.1.184	At3g11670	DGD1	EMS mutant characterization, complementation, protein expression	0	P (OEM)
Digalactosyldiacylglycerol synthase	2.4.1.184	At4g00550	DGD2	Protein expression	0	P (OEM)
Sulfolipid synthesis						
UDP-sulfoquinovose synthase	3.13.1.1	At4g33030	SQD1	Protein expression	0	P
UDP-sulfoquinovose:DAG sulfoquinovosyltransferase	2.4.1.*	At5g01220	SQD2	T-DNA mutant characterization, complementation	0	P (IEM)
PG synthesis						
CDP-diacylglycerol synthetase	2.7.7.41	At2g45150		Sequence similarity	6	P (IEM)
CDP-diacylglycerol synthetase	2.7.7.41	At3g60620		Sequence similarity	6	P (IEM)
CDP-diacylglycerol synthetase	2.7.7.41	At4g26770		Sequence similarity	8	P (IEM)
Phosphatidylglycerol-phosphate synthase	2.7.8.5	At2g39290	PGP1	EMS mutant characterization, complementation, protein expression	1	P (IEM)
Phosphatidylglycerol-phosphate synthase	2.7.8.5	At3g55030	PGP2/ PGS1	Protein expression	3	P (E)
Fatty acid exchange and lipid transfer						
Acyl-ACP thioesterase FatA type	3.1.2.14	At3g25110	FATA1	Protein expression	0	P
Acyl-ACP thioesterase FatA type	3.1.2.14	At4g13050	FATA2	Sequence similarity	0	P
Acyl-ACP thioesterase FatB type	3.1.2.14	At1g08510	FATB	Protein expression	0	P
Acyl-CoA thioesterase	3.1.2.1	At5g48370		Sequence similarity	0	P
Acyl-CoA synthetase	6.2.1.3	At1g77590	LACS9	Protein expression	0	P (E)
Acyl-CoA synthetase	6.2.1.3	At3g23790		Sequence similarity	0	P
Acyl-CoA synthetase	6.2.1.3	At4g14070		Sequence similarity	0	P (E)

(continued)

Table 9.2. (continued)

Description	EC	Locus	Protein name	Evidence	TM	Localization
Acyl-CoA synthetase	6.2.1.3	At1g49430	LACS2	Protein expression	0	–
Acyl-CoA synthetase	6.2.1.3	At1g64400	LACS3	Protein expression	0	–
Acyl-CoA synthetase	6.2.1.3	At2g04350	LACS8	Protein expression	2	–
Acyl-CoA synthetase	6.2.1.3	At2g47240	LACS1	Protein expression	0	–
Acyl-CoA synthetase	6.2.1.3	At4g11030	LACS5	Protein expression	0	–
Acyl-CoA synthetase	6.2.1.3	At4g23850	LACS4	Protein expression	0	–
Permease-like protein of outer chloroplast envelope		At1g19800	TGD1	EMS mutant characterization, complementation, protein expression	5	P (E)
Permease-like protein of outer chloroplast envelope		At3g20320	TGD2	EMS mutant characterization, complementation, protein expression	1	P (E)
Permease-like protein of outer chloroplast envelope		At1g65410	TGD3	T-DNA mutant characterization, complementation	0	P (E)
ABC acyl transporter		At1g54350		Sequence similarity	4	P

Genes involved in the metabolic pathway required to form eukaryotic DAG from phosphatidylcholine were mostly omitted considering that they are unidentified up to date. Gene references were collected from the *A. thaliana* Lipid Genes Databases established by Beisson et al. (2003). Plastid localization of proteins according to TargetP (Emanuelsson et al., 2000) is indicated with *P*. Detailed localization obtained from biological data as referred in the review text are given in brackets with *S* for stroma, *E* for envelope, *IEM* for inner-envelope membrane, *OEM* for outer-envelope membrane, and *ER* for endoplasmic reticulum. When enzymatic activity has been defined, proteins are referred to by their Enzyme Commission number (EC). Prediction of transmembrane spanning domains TM was done according to Sonnhammer et al. (1998)

*last number non identified

function of an envelope-associated type II ACCase might be to perceive cytosolic demand for fatty acids. Malonyl-CoA:ACP malonyltransferase subsequently catalyzes the transfer of the malonyl moiety to acyl carrier protein (ACP), generating malonyl-ACP that is the substrate for sequential two-carbon elongations catalyzed by a series of 3 ketoacyl-ACP synthase condensing enzymes. Fatty acids are produced predominantly as C18:1-ACP and C16:0-ACP. They are metabolic substrates for numerous pathways (Rawsthorne, 2002) such as membrane glycerolipid biosyntheses in the plastid envelope as well as in the endoplasmic reticulum production of storage oil, i.e., triacylglycerol, biosynthesis of wax after fatty acid elongation (Kunst and Samuels, 2003) or phyto-oxylipins after oxidative transformation (for review, see Blée, 2002). An acyl-ACP thioesterase, localized in the inner-envelope membrane, can hydrolyze acyl-ACP and release free fatty acid, subsequently incorporated into glycerolipids at the endoplasmic reticulum (Eukaryotic pathway). The free fatty acids released from the inner envelope are channelled to the outer envelope and reactivated there to acyl-CoA (Koo et al., 2004). Long chain acyl-CoA synthetase activity has indeed been detected in the outer envelope (Block et al., 1983a) and at least 2 different acyl-CoA synthetase homolog proteins have been located in the chloroplast envelope (Ferro et al., 2003; Schnurr et al., 2002) (Table 9.2). The critical importance of plastid fatty acid production for plant survival makes the fatty acid synthesis a target for herbicides, such as cerulenin (an inhibitor of type II ACCase) or triclosan (an inhibitor of enoyl ACP reductases). Importance for the structure of cell membranes is illustrated by the *fab2* mutation of *A. thaliana*. The *fab2* mutant, characterized by a defective plastid 18:0-ACP desaturase, possesses a severe dwarf phenotype and many cell types in the mutant fail to expand (Lightner et al., 1994). Our view of the metabolic and regulatory scheme that leads to fatty acid is still not fully understood, since partial activation or inactivation of genes responsible for fatty acid biosynthesis, such as type II ACCase, lead to alterations in both the bulk fatty acid composition and in the C18/C16 fatty acid ratio (Thelen and Ohlrogge, 2002).

B. Phosphatidic Acid Biosynthesis in the Envelope Membranes

As an alternative to export, fatty acid-ACPs remain in the plastid and are transferred to glycerol-3-phosphate or to lysophosphatidic acid to feed the plastid glycerolipid biosynthetic pathway. The first enzyme of the pathway is a soluble glycerol-3-phosphate acyltransferase, which produces lysophosphatidic acid. This enzyme is closely associated with the envelope (Joyard and Douce, 1977) and catalyzes the transfer of preferentially 18:1 from 18:1-ACP to exclusively the *sn*-1 position of glycerol (Frentzen et al., 1983). In fact, several forms of this enzyme have been reported in chloroplasts likely different in specificity for acyl chains (Nishida et al., 1997, 2000). The impairment of one of the enzymes (ACT1 also named ATS1) led to the almost full loss of the prokaryotic pathway for glycolipid synthesis and the replacement of prokaryotic glycolipids by eukaryotic glycolipids without any changes for growth in standard conditions (Kunst et al., 1988). Interestingly, although chloroplast PG is exclusively prokaryotic (Dubacq and Trémolières, 1983; Fritz et al., 2007), PG was only mildly affected (Kunst et al., 1988).

Lysophosphatidic acid is further acylated to form phosphatidic acid by the action of a 1-acylglycerol-3-phosphate acyltransferase present in the envelope membranes of chloroplasts (Joyard and Douce, 1977) and non-green plastids (Alban et al., 1989). In spinach chloroplasts, 1-acylglycerol-3-phosphate acyltransferase activity is detected in both the outer and inner envelope membranes (Block et al., 1983b). It directs preferentially 16:0 to the available *sn*-2 position unlike non-plastidial 1-acylglycerol-3-phosphate acyltransferases that have a better affinity for C18 acyl chains (Frentzen et al., 1983). A 16:0-specific plastidial form of the enzyme has been identified in *Brassica napus* (BAT2) (Bourgis et al., 1999) and in *A. thaliana* (ATS2) (Yu et al., 2004). The protein ATS2 was identified by proteomics in both spinach and *A. thaliana* envelope membranes (Ferro et al., 2002, 2003). Disruption of the *ATS2* gene in *A. thaliana* caused embryo lethality, at a developmental stage when chloroplasts begin to form (Yu et al., 2004).

C. Dual Origin of Diacylglycerol Molecular Species

A phosphatidic acid phosphatase located on the inner-envelope membrane converts phosphatidic acid to DAG (Joyard and Douce, 1977, 1979; Block et al., 1983b). Several other phosphatidic acid phosphatases were reported in the cell. The chloroplast enzyme is clearly different from all other phosphatidic acid phosphatases since it is membrane bound, has a clear alkaline optimum pH and is inhibited by cations such as Mg^{2+} (Joyard and Douce, 1977, 1979). As Mg^{2+} concentration in chloroplasts varies from 1 to 6 mM (Leegood et al., 1985), the sensitivity of phosphatidic acid phosphatase to Mg^{2+} suggests a control of the enzyme according to photosynthetic activity. Furthermore, phosphatidic acid phosphatase activity is retro-inhibited by DAG (Malherbe et al., 1992). Together, inhibition of the enzyme might lead to accumulation of phosphatidic acid and therefore favor PG synthesis. The enzyme activity was clearly reported in 16:3 plants. In contrast, chloroplasts from 18:3 plants do not exhibit any phosphatidic acid phosphatase activity (Andrews et al., 1985) and, in chloroplasts of 18:3 plants, PG is the only product of the prokaryotic pathway. Three different genes for *A. thaliana* chloroplast phosphatidic acid phosphatase were identified by Nakamura et al. (2007). Whereas double knock out mutant *lppε1lppε2* showed no significant changes in lipid composition, *lppγ* homozygous mutant was isolated only under ectopic overexpression of LPPγ, suggesting that loss of LPPγ may cause a lethal effect on plant viability (Nakamura et al., 2007).

The process described above does not account for the synthesis of eukaryotic DAG. Indeed, the specificity of the envelope acyltransferases does not allow the formation of phosphatidic acid and DAG containing C18 fatty acids at both *sn*-1 and *sn*-2 positions of glycerol (C18/C18), despite the fact that 18:1 is the main fatty acid produced by chloroplasts. In vivo kinetics of acetate and glycerol incorporation into chloroplast lipids have suggested that PC could provide the DAG backbone for plastid eukaryotic glycerolipids (Heinz, 1977; Slack et al., 1977). Furthermore, desaturated PC molecular species are probably important in this process since the *A. thaliana fad2* mutant lacking desaturation of PC 18:1 fatty acid in the endoplasmic reticulum has shown a decrease in eukaryotic MGDG molecular species with a parallel increase in prokaryotic species (Okuley et al., 1994). Although PC is present in the outer-envelope membrane, its de novo synthesis occurs in the ER. Hence a net import of some PC species from ER into chloroplasts is necessary.

Phosphatidylcholine (PC) was proposed to be transported via phospholipid transfer (PLT) proteins (Kader, 1996); however, this looks now very unlikely in view of the properties of PLT proteins (expression, localization). A second hypothesis was that LysoPC rather than PC might be transferred. The transfer between ER and chloroplasts would involve (a) a partial hydrolysis of PC in the ER, (b) a partition of the resulting LysoPC between the ER membrane, the cytosol aqueous phase and the envelope followed by (c) acylation of LysoPC by a plastidial LysoPC acyltransferase (Bessoule et al., 1995). Indeed, LysoPC is transiently formed in the ER for instance during the exchange of fatty acids between 18:1-CoA and unsaturated fatty acid esterified at the *sn*-2 position of PC (Stymne and Stobart, 1984). In addition, LysoPC is generated by phospholipases A2 that are very common in most membranes. The presence in the chloroplast envelope of a specific LysoPC acyltransferase generating PC has been verified (Bessoule et al., 1995). A set of specific phospholipases would then be required in the chloroplast envelope to transform PC (or LysoPC) into eukaryotic MGDG. These lipases have never been detected in the envelope membranes. Cytosolic lipases could however function in close association with the envelope (Andersson et al., 2004). This would ultimately suggest a transfer of PC hydrolysis products rather than of PC itself.

A transfer of phosphatidic acid (PA) was proposed. Three different envelope proteins, TGD1, TGD2 and TGD3, likely contribute to the transport of PA in the envelope membranes (Xu et al., 2003; Awai et al., 2006; Lu et al., 2007). Their deletion greatly disturbs chloroplast lipid biosynthesis, particularly eukaryotic MGDG biosynthesis. This indicates that PA is transported into chloroplasts and is necessary for the eukaryotic MGDG biosynthesis pathway. Some observations remain however unexplained in this scheme. Phosphatidic acid phosphatase activity is not detected in the chloroplast envelope of every plant (Andrews et al., 1985) although a

phosphatidic acid phosphatase is necessary for transformation of PA into DAG leading to MGDG synthesis. In absence of phosphatidic acid phosphatase, accumulation of eukaryotic PA inside the plastid should lead to formation of eukaryotic PG which is usually not present in chloroplasts (Fritz et al., 2007). Characterization of the phosphatidic acid phosphatase(s) functioning in the chloroplast envelope will be essential to understand the full process.

A transfer of DAG from ER to chloroplasts is also possibly involved in eukaryotic galactolipid formation (Williams et al., 2000). In plant cell suspensions, phosphate starvation induces increased synthesis of eukaryotic DGDG and, synchronously, an increased proportion of DAG with the same fatty acid composition as PC was observed (Jouhet et al., 2003). This observation supports the idea that, under phosphate deprivation, DAG is imported into chloroplasts.

The hydrolysis of PC can be mediated either directly by a phospholipase C (PLC) or in a two-step production, by a phospholipase D (PLD) and a phosphatidic acid phosphatase. The identification of the PC phospholipases and their localization appear as clues for the understanding of the transfer mechanism. The only phospholipase C that was shown to be related to galactolipid formation was NPC4 but this plasma membrane protein has a low expression level in leaves and is likely involved in catabolism of plasma membrane PC during phosphate deprivation (Nakamura et al., 2005). Similarly, two phospholipases D, PLDζ1 and PLDζ2, were shown to play some role in galactolipid formation under phosphate deprivation (Misson et al., 2005; Li et al., 2006; Cruz-Ramirez et al., 2006) but the localization of PLDζ2 in the leaf tonoplast rather suggests an indirect role of the enzyme in galactolipid formation probably through PA signaling (Yamaryo et al., 2008).

The mechanism of lipid trafficking between ER and chloroplasts is intriguing. ER and the chloroplast envelope can be very close to each other. Although vesicles have never been observed between ER and the chloroplast outer membrane, it has been suggested that lipid could transit through the secretory pathway. Some data indicate that targeting of some chloroplast proteins can involve a vesicular compartment between ER and chloroplasts (Villarejo et al., 2005) but treatment of plant cells with drugs inhibiting such vesicle trafficking does not affect galactolipid formation (Mérigout et al., 2002) and there are no indications for transport of lipids through this compartment. When considering mechanisms of lipid transfer in animal and yeast cells, it seems possible that contacts between membranes may play a role (Hanada et al., 2003; Levine, 2004). Anna Stina Sandeluis and coworkers explored this possibility, working on identification of a peculiar set of ER membranes that is specifically associated with chloroplasts (Sandelius and Andersson, 2003). However, Andersson et al. (2007) used confocal microscopy in combination with a laser scalpel and optical tweezers to visualize and optically manipulate the ER-chloroplast interface, and analyzed the relationship between ER and chloroplast envelope. The ER appears as an extensive network enclosing the chloroplasts, with ER branch end points localized at the chloroplast surfaces establishing membrane contact sites (MCS). Whether these MCS are involved in lipid transfers necessary for MGDG formation remains an open question.

IV. Biosynthesis of Glycerolipids

A. Phosphatidylglycerol Biosynthesis

In chloroplasts, PG synthesis occurs in the inner-envelope membrane where CDP-diacylglycerol synthetase, phosphatidylglycerol-phosphate synthase and phosphatidylglycerol-phosphate phosphatase were detected (Andrews and Mudd, 1985). PG synthesis differs from glycolipid synthesis by the fact that it does not utilize DAG. Plastid PG from 16:3 and 18:3 plants has the same structure as prokaryotic phosphatidic acid synthesized by the envelope membrane. In *A. thaliana*, two related genes encode phosphatidylglycerol-phosphate synthase: *PGP1* and *PGP2* (Muller and Frentzen, 2001; Frentzen, 2004). Functional expression studies in yeast provided evidence that the PGP2 protein is a microsomal PGPS while the PGP1 protein is synthesized as a precursor protein that can be imported into yeast mitochondria and processed to a mature form. However, proteomic analyses of *A. thaliana* chloroplast envelope membranes (Ferro et al., 2003) led to the identification of both PGP1 and PGP2 proteins. Furthermore, in silico

analyses (Ferro et al., 2002) also suggested that a putative CDP-diacylglycerol synthetase could be present in envelope membranes. This enzyme could be the first step committed to chloroplast PG synthesis. Xu et al. (2002) demonstrated the importance of PGP1 in chloroplast phosphatidylglycerol synthesis: an *A. thaliana* mutant, impaired in the *PGP1* gene, has an overall PG content reduced by 30% and shows an 80% reduction in plastidial enzyme activity. Furthermore, Babiychuk et al. (2003) demonstrated that although the *PGP1* gene encoded a precursor polypeptide that was targeted in vivo to both plastids and mitochondria, the PGP1 protein was essential for chloroplast differentiation and for the biosynthesis of plastidial PG, but was dispensable for mitochondrial function. Mitochondria can presumably also import PG from ER.

B. Sulfolipid Biosynthesis

A first distinctive step in sulfolipid formation is the synthesis of UDP-sulfoquinovosyl, the sulfolipid head group donor, catalyzed by SQD1 in *A. thaliana* (Sanda et al., 2001). The mechanism of the reaction has been described by Essigmann et al. (1999) and Mulichak et al. (1999). DAG molecules formed de novo in the inner-envelope membrane constitute a pool of receptor substrates, shared by galactolipid and sulfolipid synthesis (Joyard et al., 1986). In *A. thaliana*, SQD2 is the enzyme that transfers sulfoquinovose onto DAG (Yu et al., 2002). The activity is concentrated in the inner-envelope membranes (Tietje and Heinz, 1998). Competition experiments between MGDG synthase and sulfolipid synthase were carried out in isolated envelope membranes supplied with UDP-gal and UDP-sulfoquinovose, loaded with 16:0/16:0 and/or 18:1/16:0 DAGs (Seifert and Heinz, 1992). Both DAG species could be used by MGDG synthase and sulfolipid synthase, but 16:0/16:0 was incorporated with a much higher efficiency into sulfolipid than into MGDG. This observation shows that the enzyme specificity for DAG molecular species may be responsible for the unique DAG structure of sulfolipid in the envelope membrane.

C. Monogalactosyldiacylglycerol Biosynthesis

The envelope of chloroplasts as well as of non-green plastids is characterized by the presence of a 1,2-DAG 3-β-galactosyltransferase (or MGDG synthase) which transfers galactose from UDP-galactose to DAG (Douce, 1974). MGDG synthase is of key importance in plastid biogenesis as well as extra-plastidial membrane biogenesis under phosphate deprivation (see Section IV.E), since it is the turning point for galactolipid biosynthesis. To date, no herbicide is known to inhibit this particular target (Nishiyama et al., 2003). The biochemical properties of higher plant MGDG synthase were analyzed mostly in fractions derived from spinach chloroplast envelope membranes. Using a delipidated envelope fraction inserted in mixed micelles containing a mixture of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent, PG and DAG, Maréchal et al. (1994a) showed that the MGDG synthase activity from the spinach chloroplast envelope was able to use several DAG molecular species but with different affinity. The prokaryotic DAG species synthesized within the chloroplast i.e., 18:1/16:0 was a rather good substrate (K_m18:1/16:0 = 0.029-mol fraction), better than 16:0/18:1 (K_m16:0/18:1 = 0.042-mol fraction). The highest affinity was for dilinoleoylglycerol (K_m18:2/18:2 = 0.0089-mol fraction) as compared to any other species of DAG analyzed. The relevance of these results was confirmed further in envelope lipid vesicles (Maréchal et al., 1994b).

Shimojima et al. (1997) reported the first identification of a MGDG synthase and several homologous genes were then identified (Miège et al., 1999; Awai et al., 2001). From the sequence analysis, MGDG synthase proteins can be classified in two families: type A with MGD1 and type B with MGD2 and MGD3 in *A. thaliana*. The two families differ by (1) the presence of a cleavable transit peptide for type A absent in type B, (2) the localization in the inner envelope membrane for type A whereas type B enzymes are presumably associated with the outer envelope, (3) the high expression of type A in standard conditions and in the photosynthetic tissues and (4) the expression enhancement of type B in Pi-starving conditions (Awai et al., 2001). A detailed analysis of recombinant spinach MGD1 has shown similar characteristics as MGDG synthase purified from chloroplast envelopes (Maréchal et al., 1995; Miège et al., 1999) and MGD1 was identified in a proteomic study of envelope membranes from spinach chloroplasts (Ferro et al., 2002). MGD1 catalyzes the synthesis of both prokaryotic and eukaryotic MGDG molecular

species in vitro, with a higher affinity for eukaryotic 18:2/18:2 DAG. The redox state in chloroplasts appears to affect the catalytic activity of MGD1 (Yamaryo et al., 2003; Benning and Ohta, 2005). In an *A. thaliana* mutant containing an insertion in the *MGD1* gene and where *MGD1* mRNA abundance is reduced by 75%, the abundance of MGDG in mature leaves is reduced by 42% compared with wild type to the benefit of other plastidial and non plastidial lipids (Jarvis et al., 2000). MGD1 is the predominant MGDG synthase in leaves and contributes essentially to development of chloroplast membranes and furthermore of the plant since complete suppression of MGD1 induces in *A. thaliana* a lack of formation of MGDG and DGDG and an impairment of photosynthetic growth with an arrest of embryo development (Kobayashi et al., 2007).

The role of type B MGDG synthases is less evident. Their expression is observed in restricted parts such as leaf tips or floral tissues (Kobayashi et al., 2004). Since they appear early and are strongly induced during phosphate deprivation, they may be acting primarily to supply MGDG as a precursor for DGDG synthesis (Awai et al., 2001; Kobayashi et al., 2004).

Although recombinant MGDG synthases can be purified to homogeneity in sufficient quantity, no crystal has yet been obtained for structural resolution (Nishiyama et al., 2003). In the absence of direct structure, topological and structural information could be deduced from enzymological analyses. First of all, non-processivity of MGDG synthases could be only assessed from completely pure enzyme fractions (Nishiyama et al., 2003). In mixed micelles containing DAG, MGDG synthase activity proved to be a sequential random bireactant system, in which the binding of one substrate did not change the specificity for the co-substrate (Maréchal et al., 1994a, 1995). Cysteine residues are present in the vicinity of the 1,2-sn-DAG binding site and are essential for the galactosylation activity, since the protection of –SH (or –S-) groups against oxidation or N-ethylmaleimide, by dithiothreitol (DTT) or 1,2-sn-DAG, is essential to prevent loss of activity (Maréchal et al., 1995; Miège et al., 1999). Inactivation of chloroplast envelope MGDG synthase by a hydrophobic chelating agent, *ortho*-phenanthroline, blocked by DAG, suggests that at least one bivalent cation is likely associated with the mature enzyme; addition of Zn^{2+} allowed partial restoration of galactosylation activity in *ortho*-phenanthroline treated enzymes or in the refolding procedures of denatured MGDG synthases (Maréchal et al., 1995; Nishiyama et al., 2003). The association of apo-MGDG synthase with a metal and the nature of the metal that is involved however remain to be confirmed.

The strength of the association of *Spinacia oleracea* MGD1 with the envelope membrane was analyzed using high salt and sodium hydroxide and proved to be consistent with a monotopic enzyme associated with one leaflet of the inner envelope membrane, a structure which may involve amphipathic α-helices (Miège et al., 1999). Indeed, despite the lack of hydrophobic transmembrane domains, MGD1 could be solubilized from envelope membranes by chloroform/methanol (Ferro et al., 2002). Eventually, inactivation kinetics after γ-ray irradiation suggested that the native as well as the recombinant *S. oleracea* MGD1 are likely to be active as a dimer (Miège et al., 1999). Organization as a dimer was later confirmed by cross-linking experiments (Nishiyama et al., 2003). Interestingly, the structure of MGDG synthase as a monotopic homodimer, with separate sites for its substrates might be analogous to that described for its closest homologue, i.e., MurG (UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase), a bacterial peptidoglycan synthesizing enzyme whose crystal structure has been resolved (Hu et al., 2003). MurG was used as a template for structure prediction of the MGDG synthase soMGD1. Together with site-directed mutagenesis, this approach revealed residues that are essential for catalysis (Botté et al., 2005). Figure 9.2 summarizes the main functional and structural features accumulated on MGDG synthases.

D. Digalactosyldiacylglycerol Biosynthesis

The characterization of the *A. thaliana dgd1* mutant severely impaired in DGDG synthesis led to the first identification of a DGDG synthase gene (Dörmann et al., 1995). The *dgd1* mutant is strongly deficient in photosynthesis (Dörmann et al., 1995; Härtel et al., 1997) and protein import into chloroplasts (Chen and Li, 1998). By similarity to *DGD1*, a second gene (*DGD2*) was identified in the *A. thaliana* genome (Dörmann et al., 1999). The *DGD2* gene is not essential for

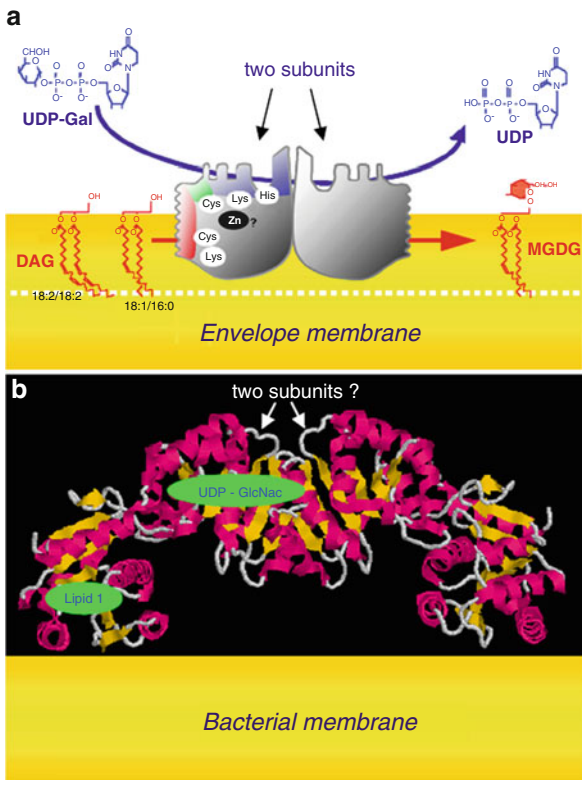

Fig. 9.2. (**a**) Schematic topological, structural and functional view of MGDG synthase. Enzymological and biochemical evidence support MGDG synthase as being a monotopic homodimer. Two independent binding sites for UDP-Gal and DAG are indicated. Key amino acids that react with inactivating agents are indicated. Association with a metal (probably Zn^{2+}) is supported by inactivation by *ortho*-phenanthroline, and renaturation experiments. (**b**) Structure of MurG. The closest homologue of MGDG synthases, MurG, is an enzyme transferring GlcNac on bacterial lipid 1 from a UDP-GlcNac donor. Two independent binding sites for UDP-GlcNac and Lipid 1 are indicated. Its crystal structure contains two proteins per crystal unit. Association with envelope involves a monotopic embedding.

DGDG synthesis under optimal growth conditions (Kelly et al., 2003). The DGD1 protein is composed of 2 distinct domains: an N-terminal domain of unknown function and a C-terminal glycosyltransferase domain. DGD2 protein has only the glycosyltransferase domain. Both DGD1 and DGD2 catalyze DGDG synthesis by transfer of galactose from UDP-galactose to MGDG (Kelly and Dörmann, 2002; Kelly et al., 2003). Analyses of the *dgd1 and dgd2* null mutants indicated that in vivo DGD1 preferentially acts on MGDG molecular species with 18-carbon fatty acids in both *sn*-positions of DAG where DGD2 seems to act preferentially on MGDG with 16-carbon fatty acids in the *sn-1* position and 18-carbon fatty acids in the *sn-2* position (Kelly et al., 2003). Both enzymes presumably are localized on the outer membrane of the chloroplast envelope (Froehlich et al., 2001; Kelly et al., 2003), but an additional mitochondrial localization is possible for DGD1 since the protein was detected in a proteome survey of mitochondrial membranes (Heazlewood et al., 2004). In fact, the only DGDG-forming activity clearly described in isolated plastids is the galactolipid:galactolipid galactosyltransferase (Dorne et al., 1982). This enzyme is present in the outer-envelope membrane and catalyzes the exchange of galactose between galactolipids with the production of DAG. One can easily differentiate this enzyme from DGD1 and DGD2 due to the formation of oligogalactolipids TriGDG and TetraGDG and the absence of UDP-galactose requirement. However, this protein remains unknown and its role is still enigmatic. It is likely that it does not contribute to net DGDG synthesis in plants, because the *dgd1:dgd2* double mutant contained only trace amounts of DGDG (Kelly et al., 2003). It may be considered in galactolipid catabolism instead of anabolism since its activity leads to degradation of MGDG into DAG. Moreover, there are some indications that this enzyme is stimulated under some stress conditions such as ozone treatment (Sakaki et al., 1990), when cell compartmentation is disturbed, in isolated chloroplast envelope (Dorne et al., 1982) or when lipid transfer between ER and chloroplast is affected (Xu et al., 2003), and in protoplast preparations (J. Jouhet and M.A. Block, unpublished).

The difference between MGDG and DGDG fatty acid composition suggests that some MGDG molecular species are selectively used for DGDG synthesis. For instance, in 16:3 plants such as *A. thaliana*, DGDG structure is mainly eukaryotic when half of MGDG derives from prokaryotic DAG. Selective affinity of DGD1 and DGD2 for some MGDG molecules is possible (see above). Specific association of DGD1 or DGD2 with MGD2 or MGD3 that mainly produce eukaryotic MGDG is another possibility. Localization of all these enzymes in the outer envelope membrane slightly favors this hypothesis.

E. Adaptation to Phosphate Deprivation

Phosphorus is an essential macro element for plant growth and development but, in most soils, it is moderately available due to its adsorption properties (Raghothama, 1999, 2000). Plant cells have developed safety mechanisms circumventing its shortage, including decrease of their P_i consumption and mobilization of their P_i reserve. Phospholipids are a main form of cellular P_i reserve and their content markedly declines in plants during P_i starvation (Rebeillé, 1983). At the same time, the glycolipid content increases, notably SQDG (Essigman et al., 1998) and DGDG (Härtel et al., 1998).

In *A. thaliana* grown on a medium with reduced amounts of phosphate as well as in the *pho1* mutant of *A. thaliana* an increase in sulfolipid with a concomitant decrease in phosphatidylglycerol is observed. Furthermore, during phosphate starvation, the expression of *SQD1* and *SQD2*, two essential genes for SQDG synthesis, is stimulated (Essigman et al., 1998; Yu et al., 2002) and in the *sqd2* knock out mutants, PG content doesn't decrease during Pi deprivation (Yu and Benning, 2003). Yu and Benning (2003) also made a series of interesting observations based on the use of single and double mutants affected in the biosynthesis of both sulfolipid (*sqd2 mutant*) and phosphatidylglycerol (*pgp-1 mutant*). The *pgp-1 A. thaliana* mutant is leaky and can still synthesize part of its PG. In standard conditions, it has a compromised photosynthetic capacity (see Section II.C). A reduction in Pi availability lowers the amount of PG, which in turn raises the abundance of sulfolipid. Compared to the single mutant, the double mutant (*sqd2*, *pgp-1*) presents a series of major perturbations: namely a 30% reduction of leaf surface, a reduction in the pigment (carotenoid, chlorophyll *a* and *b*) content, a reduction in the electron flux through PS II, and a change in the redox potential of PS II reaction centers, to a more reduced state. These results demonstrate that these two anionic lipids play an essential role in the correct functioning of the photosynthetic apparatus. Since SQDG and PG are anionic and have the same bilayer forming capability, it was proposed that sulfolipid can function as a substitute for some part of thylakoid PG under phosphate-limited growth conditions (Härtel et al., 1998; Essigmann et al., 1998; Yang et al., 2004).

Increase in DGDG content during Pi starvation is related to stimulated expression of both *MGD* and *DGD* genes. The expression of *MGD2* and *MGD3* is highly increased whereas *MGD1* expression is stable (Awai et al., 2001; Kobayashi et al., 2004). However, there is no increase in the amount of MGDG, but only a change in MGDG structure: MGDG is enriched in its eukaryotic moiety (Klaus et al., 2002) which is consistent with enhanced activity of MGD2 and MGD3 if we consider the MGD2 and MGD3 preference for eukaryotic DAG (Awai et al., 2001). Nevertheless, the C16/C18 ratio in MGDG keeps constant (Kelly et al., 2003) indicating that MGDG is preferentially formed from C16/C18 DAG rather than from C18/C16. On the other hand, the amount of DGDG strongly increases. Both *DGD1* and *DGD2* genes are over expressed during phosphate deprivation indicating that DGD1 and DGD2 are implicated in DGDG synthesis under Pi deprivation but a more important role of DGD2 was also evoked (Klaus et al., 2002; Kelly et al., 2003; Benning and Ohta, 2005). Interestingly, during phosphate deprivation, newly synthesised DGDG comes from prokaryotic and eukaryotic pathways in the same proportion as in the phosphate sufficient condition: on average, 80% of eukaryotic DGDG and 20% of prokaryotic DGDG (Klaus et al., 2002). The constant ratio of prokaryotic/eukaryotic form of DGDG suggests that a galactosylation of MGDG formed by MGD1 is still possible. Considering the eukaryotic form of DGDG, since the C16/C18 ratio increases in DGDG (Klaus et al., 2002), it indicates that C16 is incorporated in the *sn-1* position and that C18/C18 DAG is replaced by a C16/C18 moiety. The increase in C16/C18 moiety in MGDG and DGDG is likely provided by PC itself, probably coming from PE and PG degradation as indicated, when following C16 fatty acid content of each lipid in a time course experiment of Pi deprivation (Jouhet et al., 2003). By comparison with SQDG and PG, DGDG is expected to replace PC, because both lipids are neutral and bilayer forming lipids. Since the DGDG increase is very high when compared to the amount of plastid PC (Dorne et al., 1985), DGDG was proposed to be exported to extraplastidial membranes although, in standard conditions, DGDG is restricted to plastidial membranes (Härtel et al., 2000; Härtel and Benning, 2000). Indeed, reports indicate that

DGDG is present in the plasma membrane (Andersson et al., 2003; Russo et al., 2007), tonoplast (Andersson et al., 2005) and mitochondria under Pi starvation (Jouhet et al., 2004). Furthermore, data indicate that a transfer of DGDG occurs from plastid envelope to mitochondria and that contact between plastid and mitochondria play some role in the transfer (Jouhet et al., 2004). The galactosyltransferases involved in the synthesis of DGDG induced by Pi-deprivation (MGD2, MGD3, DGD1 and DGD2) are presumably located in the outer-envelope membrane as suggested for DGD1, by western blotting of an outer-envelope fraction (Froehlich et al., 2001), and more generally for all these proteins, by the observation that recombinant proteins are imported into chloroplasts and subsequently susceptible to protease digestion on the chloroplast surface (Awai et al., 2001; Froehlich et al., 2001; Kelly et al., 2003). Synthesis of DGDG by isolated mitochondria was dependent on envelope contamination that was hard to get rid of particularly when working on mitochondria from Pi-deprived cells, supporting the hypothesis that synthesis and transfer are closely linked (Jouhet et al., 2004). Contamination of isolated mitochondria by envelope membranes could additionally be the reason why DGD1 was detected in a proteome survey of mitochondria (Heazlewood et al., 2004). The coupling of MGDG and DGDG synthesis with transfer to non-plastidial membranes is presently a challenging question. A comprehensive survey of global gene expression during Pi deprivation revealed a number of responsive genes in lipid metabolism (Misson et al., 2005). Some of them were already known to be sensitive to Pi deprivation and have been described in this chapter (*MGD2*, *MGD3*, *DGD1*, *DGD2*, *SQD1*, *SQD2*). Some others such as *NPC4* or *PLDζ2* are important for the degradation of phospholipids and the formation of galactolipids (Nakamura et al., 2005; Li et al., 2006; Cruz-Ramirez et al., 2006; Yamaryo et al., 2008), but their exact role remains unclear.

F. Transport of Chloroplast Lipids from Envelope to Thylakoids

Glycerolipids synthesized in the envelope are selectively exported from the inner envelope membrane to the thylakoids (Rawyler et al., 1995). The detailed mechanism of lipid transfer is still unclear but there are some indications for vesicular transport. The budding of vesicles from the inner envelope has been documented by electron microscopy (Carde et al., 1982; Morré et al., 1991a). Bio-informatic studies have also suggested that a machinery similar to the cytosolic vesicular pathway is present in chloroplasts (Andersson and Sandelius, 2004). The plastid vesicular transfer is dependent on ATP and stromal proteins (Morré et al., 1991b; Räntfors et al., 2000). Some stromal proteins have been identified: an NSF (N-ethylmaleimide Sensitive Factor)-like protein (Hugueney et al., 1995), a dynamin-like protein (Park et al., 1998) and a vesicle-inducing protein (VIPP1) (Kroll et al., 2001). In the *vipp1* deletion mutant, thylakoid membrane formation is abolished indicating that *VIPP*1 is essential for the maintenance of thylakoids by a vesicular transport pathway.

G. Fatty Acid Desaturation

Palmitic acid (16:0) and oleic acid (*cis*-9-18:1) are desaturated when they are esterified to polar lipids. Desaturation is carried out by membrane desaturases of the chloroplast and of the ER (Ohlrogge et al., 1991). In higher plant chloroplasts, desaturation results in incorporation of double bonds at a few preferential positions and always in the same order. In PG, a *trans* desaturation of palmitic acid occurs on C3 (as numbered from the fatty acid carboxyl group resulting in a Δ3 desaturated fatty acid; or omega-13 fatty acid desaturation when numbered from the other end of the fatty acid) whereas in glycolipids, palmitic acid is desaturated first on C7 (omega-9 fatty acid desaturation), then on C10 (omega-6 desaturation) and finally on C13 (omega-3 desaturation) positions with *cis* configuration. In all glycerolipids, oleic acid is desaturated first on C12 (omega-6 desaturation) and then on C15 (omega-3 desaturation) positions with *cis* configuration. Investigation of membrane desaturases by traditional biochemical approaches has been limited because their solubilization and purification have proven very difficult (Schmidt and Heinz, 1993). However, desaturation of MGDG was achieved by isolated chloroplasts and pure envelope membranes have been used as a source of enzymes for desaturation of oleic acid to linoleic acid (Heinz

and Roughan, 1983; Schmidt and Heinz, 1990). Desaturation is dependent on electron transport elements (Andrews et al., 1989) and preliminary evidence for the involvement of a ferredoxin: NADPH oxidoreductase has been presented (Schmidt and Heinz, 1990). In fact, envelope membranes very likely contain the enzymatic machinery necessary for fatty acid desaturation including electron carriers (Schmidt et al., 1994; Jäger-Vottero et al., 1997). Using EPR spectroscopy, Jäger-Vottero et al. (1997) characterized, in spinach chloroplast envelope membranes, EPR signals corresponding to putative components of an electron transfer chain. None of the envelope components responsible for such signals have been identified to date, but the identification of a putative quinone oxidoreductase (At4g13010; Miras et al., 2002) in envelope membranes from spinach chloroplasts may provide a first clue towards characterization of members of an envelope electron transfer chain. Interestingly, Ferro et al. (2003) also identified a putative flavin-containing oxidoreductase (At3g09580).

Our understanding of chloroplast desaturases has considerably benefited from the pioneering characterization of *A. thaliana* mutants, each deficient in a specific desaturation step (Ohlrogge et al., 1991; Ohlrogge and Browse, 1995). Five loci, i.e. *FAD4*, *FAD5*, *FAD6*, *FAD7* and *FAD8*, affect chloroplast lipid desaturation. Two of these desaturases are highly substrate specific. The *FAD4* locus drives insertion of a Δ3-*trans* double bond into the 16:0 esterified to *sn*-2 glycerol position of PG, but this gene is still not identified. The gene product belonging to the *fad5* mutant is responsible for the synthesis of Δ7 16:1 on MGDG and possibly on DGDG. The 16(18):1 desaturase (omega-6 fatty acid desaturase) is dependent on the *FAD6* gene, whereas two isozymes necessary for 16(18):2 desaturation (omega-3 desaturases) are encoded by *FAD7* and *FAD8*. Proteomic analyses of chloroplast envelope membranes led to the identification of FD3C (FAD7) and FD6C (or FAD6).

The genes *FAD2* and *FAD3* encode ER desaturases. The proteins have been characterized as oleate and linoleate PC desaturases, but it is possible that they act on other phospholipids as well. In addition, synthesis and desaturation of eukaryotic galactolipids depends on FAD2 desaturase, but may be at least partially bypassed by FAD6 plastid desaturase (Klaus et al., 2002). FAD3 is not necessary for desaturation of eukaryotic galactolipids except in some tissues such as roots when phosphate is missing (Härtel et al., 2000; Klaus et al., 2002).

Specialized roles have been demonstrated for desaturated fatty acids including production of jasmonic acid, which is a key mediator of several processes as diverse as wound signaling (Blée and Joyard, 1996). Moreover, polyunsaturation is essential for maintaining cellular function and plant viability at low temperature and under salt stress (Wada et al., 1990; Ohlrogge and Browse, 1995). However with the exception of the *fab2* mutant of *A. thaliana* (blocked in the desaturation of 18:0; see above), most of the single-gene desaturase mutants do not show any visible phenotype at normal growth temperatures (25°C) or following several days of exposure to low temperatures (Somerville and Browse, 1996). In order to block possible compensation by alternative pathways, double and triple mutants were constructed (McConn and Browse, 1998). The *fad2*, *fad6* double mutant can not desaturate 18:1 and 16:1. Very interestingly, the double mutant is not capable of autotrophic growth but, on sucrose media, the plant develops and chloroplasts contain thylakoids although with a highly reduced chlorophyll content. This supports the idea that fatty acid desaturation is important for general plant cell metabolism and can be achieved by several parallel pathways that can be compensated by each other. Moreover, photosynthesis appears as a process critically affected by the absence of polyunsaturated lipids. Other data indicate a role for specific unsaturated lipids in thylakoid functioning. For instance, the rate of damage and repair of the D1 protein at low temperature was much slower in transgenic tobacco plants in which the proportion of saturated plastid PG was increased by expression of an isoform of glycerol-3-phosphate acyltransferase which prefers saturated fatty acyl substrates (Moon et al., 1995).

V. Conclusions

The plastid envelope is a major metabolic site for the biosynthesis of all glycerolipids necessary to build thylakoid membranes. During the last several years, major progress resulted in the identification

of most enzymes required for the synthesis reactions. One can expect that the last unknown steps such as those involved into getting from PC to eukaryotic galactolipids will soon be resolved taking advantage of the identification of a wide variety of mutants affected in galactolipid composition and of the opening of a new research field dealing with lipid response of plants during phosphate deprivation (Moellering and Benning, 2011). The identification of the galactolipid-galactolipid galactosyltransferase would help to resolve the puzzling question of the role of this enzyme.

Characterization of the structure, the mechanisms and the partnership of these enzymes will be needed to understand how the syntheses of the different lipids are coupled and regulated to maintain membrane lipid homeostasis on the one hand and to adapt the membrane lipid composition to environment stresses, such as phosphate deprivation, on the other hand. The coupling between synthesis in the envelope and transport of specific precursor or produced lipids from ER to envelope, or between envelope membranes, or from envelope to thylakoids, or even from envelope to non-plastidic membranes are other fields that remain totally obscure despite the recent identification of some proteins involved in transport processes. It is now very likely that the transfer from inner envelope to thylakoids occurs through vesicles but nothing is known about the envelope domains that might be capable of vesicle budding, and about interactions with chloroplast protein import. Future challenges include elucidation of the molecular mechanisms involved in the synthesis and transfer of DGDG from plastid envelope to non-plastidic membranes. One will need to take into account that envelope membranes are a highly dynamic system capable of rapid movements as indicated by reports of transient tubular projections named stromules and also that, although not established, interaction with the cell cytoskeleton is likely to play some role in the lipid synthesis machinery.

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References

Alban C, Joyard J and Douce R (1989) Comparison of glycerolipid biosynthesis in non-green plastids from sycamore (*Acer pseudoplatanus*) cells and cauliflower (*Brassica oleracea*) buds. Biochem J 259: 775–83

Andersson MX and AS Sandelius (2004) A chloroplast-localized vesicular transport system: a bio-informatics approach. BMC Genomics 5: 40–40

Andersson MX, Stridh MH, Larsson KE, Liljenberg C and Sandelius AS (2003) Phosphate-deficient oat replaces a major portion of the plasma membrane phospholipids with the galactolipid digalactosyldiacylglycerol. FEBS Lett 537: 128–32

Andersson MX, Kjellberg JM and Sandelius AS (2004) The involvement of cytosolic lipases in converting phosphatidyl choline to substrate for galactolipid synthesis in the chloroplast envelope. Biochim Biophys Acta 1684: 46–53

Andersson MX, Larsson KE, Tjellstrom H, Liljenberg C and Sandelius AS (2005) Phosphate-limited oat. The plasma membrane and the tonoplast as major targets for phospholipid-to-glycolipid replacement and stimulation of phospholipases in the plasma membrane. J Biol Chem 280: 27578–27586

Andersson MX, Goksor M and Sandelius AS (2007) Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. J Biol Chem 282: 1170–1174

Andrews J and Mudd JB (1985) Phosphatidylglycerol synthesis in pea chloroplasts. Pathways and localization. Plant Physiol 79: 259–265

Andrews J, Ohlrogge JB and Keegstra K (1985) Final step of phosphatidic acid synthesis in pea chloroplasts occurs in the inner envelope membrane. Plant Physiol 78: 459–465

Andrews J, Schmidt H and Heinz E (1989) Interference of electron inhibitors with desaturation of monogalactosyldiacylglycerol in intact chloroplasts. Arch Biochem Biophys 270: 611–622

Awai K, Maréchal E, Block MA, Brun D, Masuda T, Shimada H, Takamiya K, Ohta H and Joyard J (2001) Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 98: 10960–10965

Awai K, Xu C, Lu B and Benning C (2006) Lipid trafficking between the endoplasmic reticulum and the chloroplast. Biochem Soc Trans 34: 395–398

Babiychuk E., Muller F, Eubel H, Braun HP, Frentzen M and Kushnir S (2003) *Arabidopsis* phosphatidylglycerophosphate synthase 1 is essential for chloroplast differentiation, but is dispensable for mitochondrial function. Plant J 33: 899–909

Beisson F, Koo AJ, Ruuska S, Schwender J, Pollard M, Thelen JJ, Paddock T, Salas JJ, Savage L, Milcamps A, Mhaske VB, Cho Y and Ohlrogge JB (2003) Arabidopsis

genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol 132: 681–697

Benning C and Ohta H (2005) Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. J Biol Chem 280: 2397–2400

Bessoule JJ, Testet E and Cassagne C (1995) Synthesis of phosphatidylcholine in the chloroplast envelope after import of lysophosphatidylcholine from endoplasmic reticulum membranes. Eur J Biochem 228: 490–497

Billecocq A, Douce R and Faure M (1972) Structure des membranes biologiques : Localisation des galactosyldiglycérides dans les chloroplastes au moyen des anticorps spécifiques. C R Acad Sci Paris 275: 1135–1137

Bishop DG, Sparace SA and Mudd JB (1985) Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: the origin of the diacylglycerol moiety. Arch Biochem Biophys 240: 851–858

Blée E (2002) Impact of phyto-oxylipins in plant defense. *Trends Plant Sci* 7: 315–22

Blée E and Joyard J (1996) Envelope membranes from spinach chloroplasts are a site of metabolism of fatty acid hydroperoxides. Plant Physiol 110: 445–454

Bligny R and Douce R (1980) A precise localization of cardiolipin in plant cells. Biochim Biophys Acta 617: 254–263

Block MA, Dorne AJ, Joyard J and Douce R (1983a) The acyl-CoA synthetase and the acyl-CoA thioesterase are located respectively on the outer and on the inner membrane of the chloroplast envelope. FEBS Lett 153 : 377–381

Block MA, Dorne AJ, Joyard J and Douce R (1983b) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. II. Biochemical characterization. J Biol Chem 258: 13281–13286

Botté C, Jeanneau C, Snajdrova L, Bastien O, Imberty A, Breton C, Maréchal E (2005) Molecular modelling and site directed mutagenesis of plant chloroplast MGDG synthase reveal critical residues for activity. J Biol Chem 280: 34691–34701

Bourgis F, Kader JC, Barret P, Renard M, Robinson D, Robinson C, Delseny M and Roscoe TJ (1999) A plastidial lysophosphatidic acid acyltransferase from oilseed rape. Plant Physiol 120: 913–922

Carde JP, Joyard J and Douce R (1982) Electron microscopic studies of envelope membranes from spinach plastids. Biol Cell 44: 315–324

Carter HE, McCluer RH and Slifer ED (1956) Lipids of wheat flour. I. Characterization of galactosylglycerol components. J Am Chem Soc 78: 3735–3738

Chen LJ and Li HM (1998) A mutant deficient in the plastid lipid DGD is defective in protein import into chloroplasts. Plant J 16: 33–39

Cruz Ramirez A, Oropeza Aburto A, Razo Hernandez F, Ramirez Chavez E and Herrera Estrella L (2006) Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in *Arabidopsis* roots. Proc Natl Acad Sci USA 103: 6765–6770

Daleke DL (2003) Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res 44: 233–242

Domonkos I, Malec P, Sallai A, Kovacs L, Itoh K, Shen G, Ughy B, Bogos B, Sakurai I, Kis M, Strzalka K, Wada H, Itoh S, Farkas T and Gombos Z (2004) Phosphatidylglycerol is essential for oligomerization of photosystem I reaction center. Plant Physiol 134: 1471–1478

Dörmann P, Hoffmann-Benning S, Balbo I and Benning C (1995) Isolation and characterization of an Arabidopsis mutant deficient in the thylakoid lipid digalactosyl diacylglycerol. Plant Cell 7: 1801–1810

Dörmann P, Balbo I and Benning C (1999) *Arabidopsis* galactolipid biosynthesis and lipid trafficking mediated by DGD1. Science 284: 2181–2184

Dorne AJ, Block MA, Joyard J and Douce R (1982) The galactolipid: galactolipid galactosyltransferase is located on the outer surface of the outer chloroplast envelope. FEBS Lett 145: 30–34

Dorne AJ, Joyard J, Block MA and Douce R (1985) Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. J Cell Biol 100: 1690–1697

Dorne AJ, Joyard J and Douce R (1990) Do thylakoids really contain phosphatidylcholine? Proc Natl Acad Sci USA 87: 71–74

Douce R (1974) Site of galactolipid synthesis in spinach chloroplasts. Science 183: 852–853

Douce R and Joyard J (1996) Biosynthesis of thylakoid membrane lipids. In: Ort DR and Yocum CF (eds) Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, Vol 4, pp 69–101. Kluwer Academic Publishers, Dordrecht

Dubacq JP and Trémolières A (1983) Occurrence and function of phosphatidyl glycerol containing 3-trans-hexadecenoic acid in photosynthetic lamellae. Physiol Veg 2: 293–312

Emanuelsson O, Nielsen H, Brunak S and von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005–1016

Essigmann B, Güler S, Narang RA, Linke D and Benning C (1998) Phosphate availability affects the thylakoid lipid composition and the expression of SQD1, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 95: 1950–1955

Essigmann B, Hespenheide BM, Kuhn LA and Benning C (1999) Prediction of the active-site structure and NAD^+ binding in SQD1, a protein essential for sulfolipid biosynthesis in *Arabidopsis*. Arch Biochem Biophys 369: 30–41

Ferro M, Salvi D, Riviere-Rolland H, Vermat T, Seigneurin-Berny D, Grunwald D, Garin J, Joyard J and Rolland N (2002) Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. Proc Natl Acad Sci USA 99: 11487–11492

Ferro M, Salvi D, Brugiere S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J and Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana.* Mol Cell Proteomics 2: 325–345

Flachmann R and Kuhlbrandt W (1996). Crystallization and identification of an assembly defect of recombinant antenna complexes produced in transgenic tobacco plants. Proc Natl Acad Sci USA 93: 14966–14971

Frentzen M (2004) Phosphatidylglycerol and sulfoquinovosyldiacylglycerol: anionic membrane lipids and phosphate regulation. Curr Opin Plant Biol 7: 270–276

Frentzen M., Heinz E, McKeon TA and Stumpf PK (1983) Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. Eur J Biochem 129: 629–636

Fritz M, Lokstein H, Hackenberg D, Welti R, Roth M, Zahringer U, Fulda M, Hellmeyer W, Ott C, Wolter FP and Heinz E (2007) Channeling of eukaryotic diacylglycerol into the biosynthesis of plastidial phosphatidylglycerol. J Biol Chem 282: 4613–4625

Froehlich JE, Benning C and Dörmann P (2001) The digalactosyldiacylglycerol (DGDG) synthase DGD1 is inserted into the outer envelope membrane of chloroplasts in a manner independent of the general import pathway and does not depend on direct interaction with monogalactosyldiacylglycerol synthase for DGDG biosynthesis. J Biol Chem 276: 31806–31812

Gounaris K and Barber J (1983) Monogalactosyldiacylglycerol: the most abundant polar lipid in nature. Trends Biochem Sc 8: 378–381

Gounaris K, Whitford D and Barber J (1983) The effect of thylakoid lipids on an oxygen-evolving Photosystem II preparation. FEBS Lett 163: 230–234

Gueguen V, Macherel D, Jaquinod M, Douce R and Bourguignon J (2000) Fatty acid and lipoic acid biosynthesis in higher plant mitochondria. *J Biol Chem* 275: 5016–5025

Hagio M, Sakurai I, Sato S, Kato T, Tabata S and Wada H (2002) Phosphatidylglycerol is essential for the development of thylakoid membranes in *Arabidopsis thaliana*. Plant Cell Physiol 43: 1456–1464

Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M and Nishijima M (2003) Molecular machinery for non-vesicular trafficking of ceramide. Nature 426: 803–809

Härtel H and Benning C (2000) Can digalactosyldiacylglycerol substitute for phosphatidylcholine upon phosphate deprivation in leaves and roots of *Arabidopsis*? Biochem Soc Trans 28: 729–732

Härtel H, Lokstein H, Dörmann P, Grimm B and Benning C (1997) Changes in the composition of the photosynthetic apparatus in the galactolipid-deficient dgd1 mutant of *Arabidopsis thaliana*. Plant Physiol 115: 1175–1184

Härtel H, Essigmann B, Lokstein H, Hoffmann-Benning S, Peters-Kottig M and Benning C (1998) The phospholipid-deficient pho1 mutant of *Arabidopsis thaliana* is affected in the organization, but not in the light acclimation, of the thylakoid membrane. Biochim Biophys Acta 1415: 205–218

Härtel H, Dörmann P and Benning C (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. Proc Natl Acad Sci USA 97: 10649–10654

Härtel H, Dörmann P and Benning C (2001) Galactolipids not associated with the photosynthetic apparatus in phosphate-deprived plants. J Photochem Photobiol 61: 46–51

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J and Millar AH (2004) Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell 16: 241–256

Heinz E (1977) Enzymatic reactions in galactolipid biosynthesis. In: Tevini M and Lichtenthaler HK (eds) Lipids and Lipid Polymers, pp 102–120. Springer Verlag, Berlin

Heinz E and Roughan PG (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol 72: 273–279

Holzl G, Witt S, Kelly AA, Zahringer U, Warnecke D, Dörmann P and Heinz E (2006) Functional differences between galactolipids and glucolipids revealed in photosynthesis of higher plants. Proc Natl Acad Sci USA 103: 7512–7517

Hu Y, Chen L, Ha S, Gross B, Falcone B, Walker D, Mokhtarzadeh M and Walker S (2003) Crystal structure of the MurG:UDP-GlcNAc complex reveals common structural principles of a superfamily of glycosyltransferases. Proc Natl Acad Sci USA 100: 845–849

Hugueney P, Bouvier F, Badillo A, d'Harlingue A, Kuntz M and Camara B (1995) Identification of a plastid protein involved in vesicle fusion and/or membrane protein translocation. Proc Natl Acad Sci USA 92: 5630–5634

Jäger-Vottero P, Dorne AJ, Jordanov J, Douce R and Joyard J (1997) Redox chains in chloroplast envelope membranes: Spectroscopic evidence for the presence of electron carriers, including iron-sulfur centers. Proc Natl Acad Sci USA 94: 1597–1602

Jarvis P, Dörmann P, Peto CA, Lutes J, Benning C and Chory J (2000) Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis* MGD synthase 1 mutant. Proc Natl Acad Sci USA 97: 8175–8179

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W and Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 A resolution. Nature 411: 909–917

Jouhet J, Maréchal E, Baldan B, Bligny R, Joyard J and Block MA (2004) Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. J Cell Biol 167: 863–874

Jouhet J, Maréchal E, Bligny R, Joyard J and Block MA (2003) Transient increase of phosphatidylcholine in plant cells in response to phosphate deprivation. FEBS Lett 544: 63–68

Joyard J and Douce R (1977) Site of synthesis of phosphatidic acid and DAG in spinach chloroplasts. Biochim Biophys Acta 486: 273–285

Joyard J and Douce R (1979) Characterization of phosphatidate phosphohydrolase activity associated with chloroplast envelope membranes. FEBS Lett 102: 147–150

Joyard J, Blée E and Douce R (1986) Sulfolipid synthesis from $^{35}SO_4^{2-}$ and [1-^{14}C]-acetate in isolated intact spinach chloroplasts. Biochim Biophys Acta 879: 78–87

Kader JC (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 627–654

Kelly AA and Dörmann P (2002) DGD2, an *Arabidopsis* gene encoding a UDP-galactose-dependent digalactosyldiacylglycerol synthase is expressed during growth under phosphate-limiting conditions. J Biol Chem 277: 1166–1173

Kelly AA, Froehlich JE and Dörmann P (2003) Disruption of the two digalactosyldiacylglycerol synthase genes DGD1 and DGD2 in Arabidopsis reveals the existence of an additional enzyme of galactolipid synthesis. Plant Cell 15: 2694–2706

Kern J and Guskov A (2011) Lipids in photosystem II: Multifunctional cofactors. J Photochem Photobiol B: Biol 104: 19–34

Klaus D, Härtel H, Fitzpatrick LM, Froehlich JE, Hubert J, Benning C and Dörmann P (2002) Digalactosyldiacylglycerol synthesis in chloroplasts of the Arabidopsis *dgd1* mutant. Plant Physiol 128: 885–895

Kobayashi K, Awai K, Takamiya K and Ohta H (2004) Arabidopsis type B monogalactosyldiacylglycerol synthase genes are expressed during pollen tube growth and induced by phosphate starvation. Plant Physiol 134: 640–648

Kobayashi K, Kondo M, Fukuda H, Nishimura M and Ohta H (2007) Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis. Proc Natl Acad Sci USA 104: 17216–17221

Kojima M, Seki K, Ohnishi M, Ito S and Fujino Y (1989) Structure of novel glycerolipids in Adzuki bean (*Vigna angularis*) seeds. Biochem Cell Biol 68: 59–64

Koo AJK, Ohlrogge JB and Pollard M (2004) On the export of fatty acids from the chloroplast. J Biol Chem 279: 16101–16110

Kroll D, Meierhoff K, Bechtold N, Kinoshita M, Westphal S, Vothknecht UC, Soll J and Westhoff P (2001) VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. Proc Natl Acad Sci USA 98: 4238–4242

Kruse O, Hankamer B, Konczak C, Gerle C, Morris E, Radunz A, Schmid GH and Barber J (2000). Phosphatidylglycerol is involved in the dimerization of photosystem II. J Biol Chem 275: 6509–6514

Kubis SE, Pike MJ, Hill LM and Rawsthorne S (2004) The import of phosphoenolpyruvate by plastids from developing embryos of oilseed rape, *Brassica napus* (L.), and its potential as a substrate for fatty acid synthesis. J Exp Botany 55: 1455–1462

Kunst L and Samuels AL (2003) Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res 42: 51–80

Kunst L, Browse J and Somerville CR (1988) Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci USA 85: 4143–4147

Leegood RC, Walker DA and Foyer CH (1985) Regulation of the Benson Calvin cycle. In: Barber J and Barber NR (eds), Photosynthetic mechanism and the environment, pp 190–258 Elsevier Science Publishers, Amsterdam

Levine T (2004) Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions. Trends Cell Biol 14: 483–490

Li MY, Welti R and Wang XM (2006) Quantitative profiling of Arabidopsis polar glycerolipids in response to phosphorus starvation. Roles of Phospholipases D zeta 1 and D zeta 2 in phosphatidylcholine hydrolysis and digalactosyldiacylglycerol accumulation in phosphorus-starved plants. Plant Physiol 142: 750–761

Lightner J, Wu J and Browse J (1994) A mutant of *Arabidopsis* with increased levels of stearic acid. Plant Physiol 106: 1443–1451

Lu B, Xu C, Awai K, Jones AD and Benning C (2007) A small ATPase protein of *Arabidopsis*, TGD3, involved in chloroplast lipid import. J Biol Chem 282: 35945–35953

Malherbe A, Block MA, Joyard J and Douce R (1992) Feedback inhibition of phosphatidate phosphatase from spinach chloroplast envelope membranes by diacylglycerol. J Biol Chem 267: 23546–23553

Maréchal E, Block MA, Joyard J and Douce R (1994a) Kinetic properties of monogalactosyldiacylglycerol synthase from spinach chloroplast envelope membranes. J Biol Chem 269: 5788–5798

Maréchal E, Block MA, Joyard J and Douce R (1994b) Comparison of the kinetic properties of MGDG synthase in mixed micelles and in envelope membranes from spinach chloroplast. FEBS Lett 352: 307–310

Maréchal E, Miège C, Block MA, Douce R and Joyard J (1995) The catalytic site of monogalactosyldiacylglycerol synthase from spinach chloroplast envelope membranes. Biochemical analysis of the structure and of the metal content. J Biol Chem 270: 5714–5722

McConn M and Browse J (1998) Polyunsaturated membranes are required for photosynthetic competence in a mutant of *Arabidopsis*. Plant J 15: 521–530

Merigout P, Kepes F, Perret AM, Satiat-Jeunemaitre B and Moreau P (2002) Effects of brefeldin A and nordihydroguaiaretic acid on endomembrane dynamics and lipid synthesis in plant cells. FEBS Lett 518: 88–92

Miège C, Maréchal E, Shimojima M, Awai K, Block MA, Ohta H, Takamiya K, Douce R and Joyard J (1999) Biochemical and topological properties of type A MGDG synthase, a spinach chloroplast envelope enzyme cata-

lyzing the synthesis of both prokaryotic and eukaryotic MGDG. Eur J Biochem 265: 990–1001

Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J and Rolland N (2002) Non-canonical transit peptide for import into the chloroplast. J Biol Chem 277: 47770–47778

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R,Ortet P, Creff A, Somerville S, Rolland N, Doumas P, Nacry P, Herrerra-Estrella L, Nussaume L and Thibaud MC (2005) A wide transcriptional analysis using the *Arabidopsis thaliana* whole genome Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA 102: 11934–11939

Mizusawa N and Wada H (2011) The role of lipids in photosystem II. Biochim Biophys Acta doi:10.1016/j.bbabio.2011.04.008

Moellering ER and Benning C (2011) Galactoglycerolipid metabolism under stress: a time for remodeling. Trends Plant Sci 16: 98–107

Mongrand S, Bessoule JJ, Cabantous F and Cassagne C (1998) The C16:3/C18:3 fatty acid balance in photosynthetic tissues from 468 plant species. Phytochem 49: 1049–1064

Moon BY, Higashi S, Gombos Z and Murata N (1995) Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants. Proc Natl Acad Sci USA 92: 6219–6223

Morré DJ, Seldén G, Sundqvist S and Sandelius AS (1991a) Stromal low temperature compartment derived from the inner membrane of the envelope. Plant Physiol 97: 1558–1564

Morré DJ, Morre JT, Morre SR, Sundqvist C and Sandelius AS (1991b) Chloroplast biogenesis. Cell-free transfer of envelope monogalactosylglycerides to thylakoids. Biochim Biophys Acta 1070: 437–445

Mulichak AM, Theisen MJ, Essigmann B, Benning C and Garavito RM (1999) Crystal structure of SQD1, an enzyme involved in the biosynthesis of the plant sulfolipid headgroup donor UDP-sulfoquinovose. Proc Natl Acad Sci USA 96: 13097–13102

Muller F and Frentzen M (2001) Phosphatidylglycerophosphate synthases from *Arabidopsis thaliana*. FEBS Lett 509: 298–302

Murata N, Higashi S-I and Fujimura Y (1990) Glycerolipids in various preparations of photosystem II from spinach chloroplasts. Biochim Biophys Acta 1019: 261–268

Nakamura Y, Awai K, Masuda T, Yoshioka Y, Takamiya K and Ohta H (2005) A novel phosphatidylcholine-hydrolyzing phospholipase C induced by phosphate starvation in *Arabidopsis*. J Biol Chem 280: 7469–7476

Nakamura Y, Tsuchiya M and Ohta H (2007) Plastidic phosphatidic acid phosphatases identified in a distinct subfamily of lipid phosphate phosphatases with prokaryotic origin. J Biol Chem 282: 29013–29021

Nishida I, Frentzen M, Ishizaki O and Murata N (1997) Purification of isomeric forms of acyl-(acyl-carrier-protein): glycerol-3-phosphate acyltransferase from greening squash cotyledons. Plant Cell Physiol 28: 1071–1079

Nishida I, Sugiura M, Enju A and Nakamura M (2000) A second gene for acyl-(acyl-carrier-protein): glycerol-3-phosphate acyltransferase in squash, *Cucurbita moschata* cv. Shirogikuza, codes for an oleate-selective isozyme: molecular cloning and protein purification studies. Plant Cell Physiol 41: 1381–1391

Nishiyama Y, Hardre-Lienard H, Miras S, Miège C, Block MA, Revah F, Joyard J and Maréchal E (2003) Refolding from denatured inclusion bodies, purification to homogeneity and simplified assay of MGDG synthases from land plants. Prot Expr Purif 31: 79–87

Nussberger S, Dorr K, Wang DN and Kuhlbrandt W (1993) Lipid-protein interactions in crystals of plant light-harvesting complex. J Mol Biol 234: 347–356

Ohlrogge JB and Browse J (1995) Lipid biosynthesis. Plant Cell 7: 957–970

Ohlrogge JB, Browse J and Somerville CR (1991) The genetics of plant lipids. Biochim Biophys Acta 1082: 1–26

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E and Browse J (1994) Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6: 147–158

Park JM, Cho JH, Kang SG, Jang HJ, Pih KT, Piao HL, Cho MJ and Hwang I (1998) A dynamin-like protein in *Arabidopsis thaliana* is involved in biogenesis of thylakoid membranes. EMBO J 17: 859–867

Pineau B, Girard-Bascou J, Eberhard S, Choquet Y, Trémolières A, Gerard-Hirne C, Bennardo-Connan A, Decottignies P, Gillet S and Wollman FA (2004) A single mutation that causes phosphatidylglycerol deficiency impairs synthesis of photosystem II cores in *Chlamydomonas reinhardtii*. Eur J Biochem 271: 329–338

Poirier Y, Thoma S, Somerville C and Schiefelbein J (1991) A mutant of *A rabidopsis* deficient in xylem loading of phosphate. Plant Physiol 97: 1087–1093

Raghothama KG (1999) Phosphate acquisition. Annu Rev Plant Physiol Plant Mol Biol 50: 665–693

Raghothama KG (2000) Phosphate transport and signaling. Curr Opin Plant Biol 3: 182–187

Räntfors M, Everstsson I, Kjellberg JM and Sandelius AS (2000) Intraplastidial lipid trafficking: Regulation of galactolipid release from isolated chloroplast envelope. Physiol Plant 110: 262–270

Rawsthorne S (2002) Carbon flux and fatty acid synthesis in plants. Prog Lipid Res 41: 182–196

Rawyler A, Meylan-Bettex M and Siegenthaler PA (1995) (Galacto) lipid export from envelope to thylakoid membranes in intact chloroplasts. II. A general process with a key role for the envelope in the establishment of lipid asymmetry in thylakoid membranes. Biochim Biophys Acta 1233: 123–133

Rebeillé F (1983) Quelques observations sur le métabolisme du phosphate chez les cellules d'érable sycomore. In : PhD thesis. Université J. Fourier, Grenoble

Reifarth F, Christen G, Seeliger AG, Dörmann P, Benning C and Renger G (1997) Modification of the water oxidizing complex in leaves of the *dgd1* mutant of *Arabidopsis thaliana* deficient in the galactolipid digalactosyldiacylglycerol. Biochemistry 36: 11769–11776

Russo MA, Quartacci MF, Izzo R, Belligno A and Navari-Izzo F (2007) Long- and short-term phosphate deprivation in bean roots: plasma membrane lipid alterations and transient stimulation of phospholipases. Phytochemistry 68: 1564–1571

Sakaki T, Kondo N and Yamada M (1990) Pathway for the synthesis of triacylglycerol from monogalactosyldiacylglycerols in ozone-fumigated spinach leaves. Plant Physiol 94: 773–780

Sakurai I, Mizusawa N, Ohashi S, Kobayashi M and Wada H (2007) Effects of the lack of phosphatidylglycerol on the donor side of photosystem II. Plant Physiol 144: 1336–1346

Sanda S, Leustek T, Theisen MJ, Garavito RM and Benning C (2001) Recombinant Arabidopsis SQD1 converts udpglucose and sulfite to the sulfolipid head group precursor UDP-sulfoquinovose *in vitro*. J Biol Chem 276: 3941–3946

Sandelius AS and Andersson MX (2003) Isolation and characterization of an endoplasmic reticulum fraction associated with chloroplasts isolated from garden pea (*Pisum sativum*). In: 1st European Symposium on Plant Lipids Aachen abstract booklet

Sato N, Aoki M, Maru Y, Sonoike K, Minoda A and Tsuzuki M (2003) Involvement of sulfoquinovosyl diacylglycerol in the structural integrity and heat-tolerance of photosystem II. Planta 217: 245–251

Sato N, Suda K and Tsuzuki M (2004) Responsibility of phosphatidylglycerol for biogenesis of the PS I complex. Biochim Biophys Acta 1658: 235–243

Schmidt H and Heinz E (1990) Involvement of ferredoxin in desaturation of lipid-bound oleate in chloroplasts. Plant Physiol 94: 214–220

Schmidt H and Heinz E (1993) Direct desaturation of intact galactolipids by a desaturase solubilized from spinach (*Spinacia oleracea*) chloroplast envelopes. Biochem J 289: 777–782

Schmidt H, Dresselhaus T, Buck F and Heinz E (1994) Purification and PCR-based cDNA cloning of a plastidial n-6 desaturase. Plant Mol Biol 26: 631–642

Schnurr JA, Shochey JM, de Boer GJ and Browse JA (2002) Fatty acid export from the chloroplast. Molecular characterization of a major plastidial acyl-coenzyme A synthetase from Arabidopsis. Plant Physiol 129, 1700–1709

Seifert U and Heinz E (1992) Enzymatic characteristics of UDP-sulfoquinovose: DAG sulfoquinovosyltransferase from chloroplast envelopes. Botanica Acta 105: 197–205

Seigneurin-Berny D, Rolland N, Dorne AJ and Joyard J (2000) Sulfolipid is a potential candidate for annexin binding to the outer surface of chloroplast. Biochem Biophys Res Commun 272: 519–524

Shimojima M, Ohta H, Iwamatsu A, Masuda T, Shioi Y and Takamiya K (1997) Cloning of the gene for monogalactosyldiacylglycerol synthase and its evolutionary origin. Proc Natl Acad Sci USA 94: 333–337

Siebertz HP, Heinz E, Lindscheid M, Joyard J and Douce R (1979) Characterization of lipids from chloroplast envelopes. Eur J Biochem 101: 429–438

Simidjiev I, Stoylova S, Amenitsch H, Javorfi T, Mustardy L, Laggner P, Holzenburg A and Garab G (2000) Self-assembly of large, ordered lamellae from non-bilayer lipids and integral membrane proteins *in vitro*. Proc Natl Acad Sci USA 97: 1473–1476

Slack CR, Roughan PG and Balasingham N (1977) Labelling studies in vivo on the metabolism of the acyl and glycerol moieties of the glycerolipids in the developing maize leaf. Biochem J 162: 289–296

Somerville C and Browse J (1996) Dissecting desaturation: Plants prove advantageous. Trends Cell Biol 6: 148–153

Sonnhammer EL, von Heijne G and Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 6: 175–182

Steffen R, Kelly AA, Huyer J, Dörmann P and Renger G (2005) Investigations on the reaction pattern of photosystem II in leaves from Arabidopsis thaliana wild type plants and mutants with genetically modified lipid content. Biochemistry 44: 3134–3142

Stymne S and Stobart AK (1984) Evidence for the reversibility of the acyl-CoA:lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius L.*) cotyledons and rat liver. Biochem J 223: 305–314

Thelen JJ and Ohlrogge JB (2002) The multisubunit acetyl-CoA carboxylase is strongly associated with the chloroplast envelope through non-ionic interactions to the carboxyltransferase subunits. Arch Biochem Biophys 400: 245–257

Tietje C and Heinz E (1998) Uridine-diphospho-sulfoquinovose: diacylglycerol sulfoquinovosyltransferase activity is concentrated in the inner membrane of chloroplast envelopes. Planta 206: 72–78

Trémolières A, Dainese P and Bassi R (1994) Heterogenous lipid distribution among chlorophyll-binding proteins of photosystem II in maize mesophyll chloroplasts. Eur J Biochem 221: 721–730

Van Besouw A and Wintermans JF (1978) Galactolipid formation in chloroplast envelopes. I. Evidence for two mechanisms in galactosylation. Biochim Biophys Acta 529: 44–53

Villarejo A, Buren S, Larsson S, Dejardin A, Monne M, Rudhe C, Karlsson J, Jansson S, Lerouge P, Rolland N, von Heijne G, Grebe M, Bako L and Samuelsson G (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nature Cell Biology 7: 1124–1131

Wada H and Murata N (eds) (2009) Lipids in Photosynthesis: Essential and Regulatory Functions, Advances in Photosynthesis and Respiration, Vol 30. Springer, Dordrecht

Wada H, Gombos Z and Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature 347: 200–203

Williams JP, Imperial V, Khan MU and Hodson JN (2000) The role of phosphatidylcholine in fatty acid exchange and desaturation in *Brassica napus* L leaves. Biochem J 349: 127–133

Xu C, Härtel H, Wada H, Hagio M, Yu B, Eakin C and Benning C (2002) The pgp1 mutant locus of Arabidopsis encodes a phosphatidylglycerolphosphate synthase with impaired activity. Plant Physiol 129: 594–604

Xu CC, Fan JL, Riekhof W, Froehlich JE and Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in *Arabidopsis*. EMBO J 22: 2370–2379

Yamaryo Y, Kanai D, Awai K, Shimojima M, Masuda T, Shimada H, Takamiya K and Ohta H (2003) Light and cytokinin play a co-operative role in MGDG synthesis in greening cucumber cotyledons. Plant Cell Physiol 44: 844–855

Yamaryo Y, Dubots E, Albrieux C, Baldan B and. Block M A (2008) Phosphate availability affects the tonoplast localization of PLD 2, an *Arabidopsis thaliana* phospholipase D. FEBS Lett 582: 685–690

Yang W, Liu S, Feng FY, Hou HT, Jiang GZ, Xu YN and Kuang TY (2004) Effects of phosphate deficiency on the lipid composition in cucumber thylakoid membranes and PSII particles. Plant Sci 166: 1575–1579

Yu B and Benning C (2003) Anionic lipids are required for chloroplast structure and function in *Arabidopsis*. Plant J 36: 762–770

Yu B, Xu C and Benning C (2002) *Arabidopsis* disrupted in SQD2 encoding sulfolipid synthase is impaired in phosphate-limited growth. Proc Natl Acad Sci USA 99: 5732–5737

Yu B, Wakao S, Fan J and Benning C (2004) Loss of plastidic lysophosphatidic acid acyltransferase causes embryolethality in *Arabidopsis*. Plant Cell Physiol 45: 503–510

Chapter 10

Leaf Senescence and Transformation of Chloroplasts to Gerontoplasts

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Summary

Senescence of green leaves brings about several structural and functional alterations in the cells. The process causes modifications in mitochondrial cristae, condensation of the nucleus, shrinkage of chloroplasts and extensive alteration of thylakoid structure. Senescence-induced changes in chloroplasts are extensive. These changes during senescence result in transdifferentiation of a chloroplast into a gerontoplast, a plastid form with unique structural features and physiology. During leaf senescence, the cells lose essential macromolecules including proteins, lipids and nucleic acids. The stroma proteins and lipids of the thylakoid membrane are the major targets for degradation. In addition to macromolecular degradation, the process causes loss in the photosynthetic pigments, namely chlorophylls and carotenoids. The enzymes that participate in chlorophyll degradation and their regulation are now known. However, the mechanism of degradation of carotenoids still remains a mystery. Macromolecular degradation and mobilization of the breakdown products that participate in the nutrient recycling mechanism are mediated by up-regulation of senescence-related genes. These genes are known as senescence-associated genes (SAGs), many of which have been cloned and characterized. Data are now available on signaling systems associated with expression of SAGs. Down-regulation of photosynthetic genes, cellular sugar-sensing mechanisms, phytohormones and reactive oxygen species are likely to play major roles in the signal transduction pathway in the initiation, progression and termination of the senescence process. Leaf senescence is directly related to plant productivity and therefore its implication in the area of agricultural biotechnology is important. However, its biotechnological application will be possible only when some of the outstanding fundamental questions relating to the process are addressed.

I. What We Know

In this section, we provide some basic information about what we know about the general features of leaf senescence, its molecular biology, and its signaling; and about the gerontoplasts, their physiology, and their metabolism of proteins, pigments, lipids and nucleic acids.

A. We Live to Die and Die to Live

Life is a process that perpetuates. Death is a phenomenon of degeneration of physical form. Life flows from degenerated form to regenerated form. This is reflected in leaf senescence in plants. A leaf grows green, expands, carries on photosynthesis, turns yellow, decays and finally falls only to nourish life in the other plants, and in other parts of the plant through a nutrient recycling process (Biswal et al., 2003; Ougham et al., 2005; Lim et al., 2007).

B. General Features of Leaf Senescence

Yellowing is the visible feature, which marks the onset of leaf senescence. The change in color is associated with loss of green pigments, chlorophylls (Chls). In addition to loss of pigments, leaf senescence causes condensation of the nucleus, changes in mitochondrial cristae, size shrinkage of chloroplasts, as well as degradation of proteins, lipids and other cellular molecules. These events occur in all mesophyll cells (Krupinska and Humbeck, 2004). The chloroplast is the first organelle to show the symptoms of senescence and is the last one to retain some level of organization when all other organelles are completely damaged (Biswal and Biswal, 1988). The changes in the photosynthetic organelle are extensive and sequential. The transformation of mature chloroplasts of green leaves to gerontoplasts (senescing chloroplasts) during leaf senescence is the focus of this chapter, which describes the formation of

Abbreviations: Chl – Chlorophyll; FCC – Fluorescent chlorophyll catabolite; pFCC – Primary fluorescent chlorophyll catabolite; LHC – Light-harvesting complex; mFCC – Modified fluorescence chlorophyll catabolite; NCC – Non-fluorescent chlorophyll catabolite; PAO – Pheophorbide *a* oxygenase; PCD – Programmed cell death; RCC – Red chlorophyll catabolite; ROS – Reactive oxygen species; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; SAG – Senescence associated gene; SDG – Senescence down-regulated gene

gerontoplasts, their structural features and their physiological role for mobilizing nutrients to other growing organs of the plant (Biswal et al., 2003).

1. Gerontoplasts: Dying for Death but Not Dead

The term gerontoplast was first introduced by Sitte (1977) for the plastid form with unique features that develop during leaf senescence. Gerontoplasts during senescence exhibit extensive structural modifications of the thylakoid membranes which are reported to unstack at the initial stages. This is followed by a gradual loss of membranes with concomitant formation of a large number of plastoglobuli with lipophilic materials. The envelope of the plastid, however, remains intact (Fig. 10.1) (Biswal and Biswal, 1988; Biswal, 2005).

Formation of gerontoplasts is synchronized with a functional shift of the leaf from a photosynthetic organ to the organ responsible for mobilization of nutrients during senescence. Gerontoplasts in senescing leaves export nutrients, primarily nitrogen, to other growing and reproductive parts of the plant. Thus, senescing leaves actively prepare for their death. In a way we like to compare it to an old person writing a will before death to distribute his/her properties to deserving relatives or organizations.

Structurally gerontoplasts are plastids with some of the components, different from that of proplastids, chloroplasts and chromoplasts though all the plastid forms exhibit a basal level of common structural features (Smart, 1994). Chromoplasts may develop from proplastids, amyloplasts, or young chloroplasts and are related to growth. Gerontoplasts develop only from fully mature chloroplasts without any further growth and they gradually lose biosynthetic potential (Fig. 10.1) (Thomas et al., 2001, 2003).

Chlorosis and transition of color from green to yellow in senescing leaves may indicate variation in the developing status of gerontoplasts. However, it is difficult to define the onset of leaf senescence or initiation of gerontoplast formation. Equally fuzzy is its end point i.e., death when necrosis takes over followed by collapse of the entire organelle (Biswal et al., 2003; Thomas et al., 2003).

Gerontoplasts exhibits protein turnover and show characteristic physiology. Observation that the gerontoplasts efficiently import some of the nuclear-encoded proteins shows their envelope membrane is capable of selective transport (Kawakami and Watanabe, 1993). Gerontoplasts have the potential (Fig. 10.2) and vitality for reversion back to chloroplasts during regreening of senescent leaves (see the review by Biswal et al., 1983; and by Smart, 1994). The level of cytokinins has been reported to play a key role in this reversion process (Smart, 1994; Thomas, 1994; Zavaleta-Mancera et al., 1999a, b). Active transport across the envelope and the capability to redifferentiate has led to classification of gerontoplasts as a plastid form. It is a dying entity, but it is not a dead one.

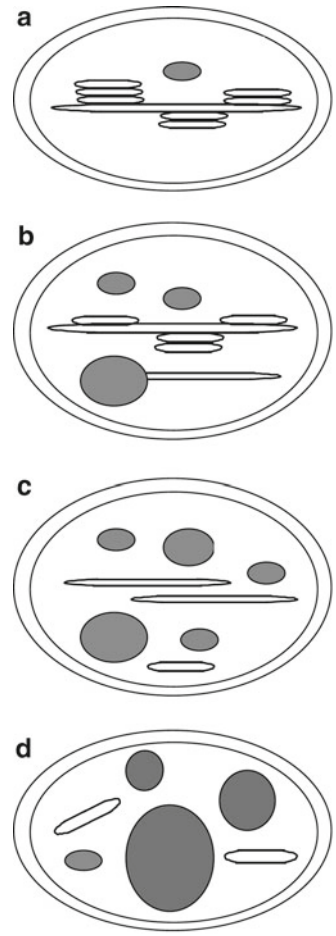

Fig. 10.1. Schematic diagram of stages of chloroplast to gerontoplast transformation: (**a**) Ultrastructure of thylakoid membrane of a chloroplast of green leaves; (**b**) Leaf-senescence-induced initiation of destacking of membranes and accumulation of plastoglobuli; (**c**) Complete destacking, appearance of parallel destacked membranes accompanied by an increased number of plastoglobuli; (**d**) Disappearance of thylakoid membrane with concomitant appearance of large size plastoglobuli.

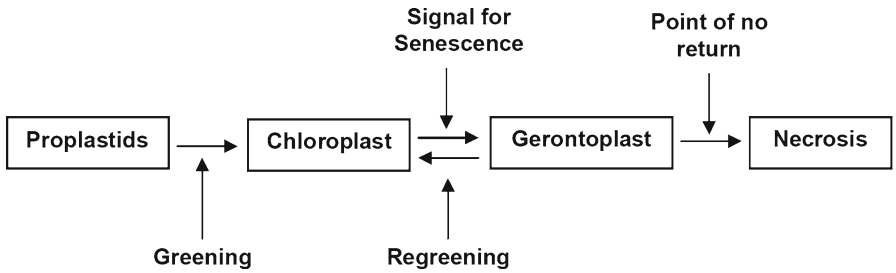

Fig. 10.2. Various stages in chloroplast development, formation of gerontoplasts and programmed cell death of mesophyll cells.

2. Molecular Biology of Senescence

Leaf senescence is a genetically controlled developmental program. The process is directly or indirectly controlled by several genes. The genes that are down-regulated during senescence are known as senescence down-regulated genes (SDGs) and the genes that are up-regulated during the process are known as senescence-associated genes (SAGs). SDGs are mainly associated with anabolic activities and SAGs primarily execute the senescence program and, therefore, mediate the metabolic activities associated with the process. A number of SAGs have been isolated, cloned and characterized (Buchanan-Wollaston et al., 2005). These SAGs are expected to participate in various functions such as macromolecular degradation, nutrient recycling, stress adaptation and signal transduction (Gepstein, 2004; Lim et al., 2007). Table 10.1 summarizes the types of SAGs and their possible functions. The down-regulation of photosynthetic genes (i.e., the SDGs) with simultaneous up-regulation of SAGs may suggest a close link between the formation of gerontoplasts and expression of SAGs during leaf senescence (Fig. 10.3).

Several studies have been made in the area of leaf senescence by using *stay-green* mutants to characterize the function of genes involved in the senescence process. A number of *stay-green* mutants have been characterized (Thomas and Stoddart, 1975; Biswal et al., 1994; Fang et al., 1998; Oh et al., 2000; Thomas and Howarth, 2000; Reyes-Arribas et al., 2001; Cha et al., 2002; Efrati et al., 2005; Jiang et al., 2007; Kusuba et al., 2007; Park et al., 2007; Sato et al., 2007). Results from these mutants also indicate the participation of certain genes in the process of leaf senescence and transformation of chloroplasts to gerontoplasts.

A *stay-green* mutant, non-yellow coloring 1 (*nyc1*), which impairs Chl degradation in rice leaves exhibits protection against degradation of pigment-binding light-harvesting complexes (LHCs) and grana structure (Kusuba et al., 2007). NYC1 has been identified to be a short chain dehydogenase/reductase, which may play an important role in the degradation of LHCs and the thylakoid membrane during leaf senescence (Kusuba et al., 2007).

3. Physiology of Gerontoplasts

Formation of gerontoplasts and senescence of leaves are marked by a decline in photosynthesis. This decline in photosynthesis may initiate a signal for transdifferentiation of mature chloroplasts to gerontoplasts. The physiology of gerontoplasts is directed at the degradation of macromolecules and subsequent mobilization of nutrients to other parts of the plant during leaf senescence (Matile, 1992; Smart, 1994; Thomas, 1994; Biswal, 1997, 2005; Biswal and Biswal, 1999; Biswal et al., 2003; Lim et al., 2007). The metabolism of gerontoplasts is mostly catabolic in nature. The disorganization of cellular organization, including that of the photosynthetic organelle, is carried out by enzymes such as proteases, lipases, and hydrolases – the enzymes that are involved in various other catabolic events. The enhancement of activity of these enzymes involves up-regulation of the genes coding for them. The evidence that the entire process of senescence is controlled by up- and down-regulation of genes establishes that gerontoplasts are active functional plastids with well-defined physiology and they are an important form of plastids during developmental processes (Biswal, 1999; Biswal et al., 2003; Krupinska, 2006; Lim et al., 2007).

Table 10.1. Classification of senescence associated genes

Senescence associated genes	Senescence related metabolism	References
Homologs of genes for Chl degradation	Chl degradation	Jiang et al. (2007), Park et al. (2007)
Homologs of genes for serine protease Homologs of genes for cysteine proteases and aspartic proteases Homologs of genes for ubiquitin-related genes, Glutamine synthatase and aspargine synthatase	Protein degradation and Nitrogen mobilization	See the book by Biswal et al. (2003), Gepstein (2004), Guo et al. (2004), Biswal (2005), Lim et al. (2007)
Homologs of genes for β-glucosidase, glucanases, pyruvate-O phosphate dikinase, and β-galactosidase	Carbohydrate metabolism	Callard et al. (1996), Biswal et al. (2003), Chrost et al. (2004, 2007), Fujiki et al. (2005)
Homologs of genes for phospholipase –D, phosphoenol pyruvate carboxy kinase, NAD malate dehydrogenase, isocitrate lyase, and malate synthase	Lipid metabolism and mobilization	Thompson et al. (2000), Gepstein (2004), Guo et al. (2004), Biswal (2005)
Homologs of genes for xanthine dehydrogenase	Nucleic acid degradation	Brychkova et al. (2008)
Homologs of genes for PR like proteins, various metallotheonines	Defense metabolism and PR	Gepstein (2004), Espinozoa et al. (2007), Lim et al. (2007), Liu et al. (2008)
Homologs of genes for Zinc finger proteins, transcription factors of the WRKY, NAC, AP2, MYB, HB, TCP and GRAS families	Transcriptional regulation	Guo et al. (2004), Buchanan-Wollaston et al. (2005), Guo and Gan (2006), Uauy et al. (2006), Gresersen and Holm (2007), Lim et al. (2007), Ulker et al. (2007)
Homologs of genes for Receptor-like kinases, components of MAP kinase signal cascades, G-proteins, phosphatases and phospholipases, calcium-binding proteins, calcium-dependent protein kinases	Signal Transduction	Guo et al. (2004), Li et al. (2006), Ouelhadj et al. (2007)

C. Gerontoplast Metabolism: Its Role in Modulating Senescence in Leaves and Green Plants

Metabolism in gerontoplasts reflects coordinated degradation of pigments and proteins (Biswal, 1995, 1997; Biswal et al., 2003; Lim et al., 2007). The loss of pigments and proteins of thylakoid membrane is associated with the loss and changes in the composition of lipids (Biswal et al., 2003; Lin and Wu, 2004).

1. Loss of Proteins

Literature on the loss of various chloroplast proteins during senescence is abundant (Biswal et al., 2003; Krupinska, 2006; Park et al., 2007). Gerontoplasts during senescence significantly lose the proteins of the stroma (Makino et al., 1983; Crafts-Brandner et al., 1990; Matile, 1992; Smart, 1994; Biswal, 1997; Dangl et al., 2000) as well as of the thylakoids (Woolhouse, 1987; Matile, 1992; Lin and Wu, 2004). The soluble proteins of the stroma, predominantly Rubisco, are the easy targets of degradation compared to the proteins of thylakoid membranes (see Biswal and Biswal, 1988). The prominent proteins prone to senescence-induced degradation include phosphoenol pyruvate carboxylase (PEPC), large subunit (LSU) of Rubisco and LHCs (Fischer et al., 1998).

Proteolysis is catalyzed by the proteases present in the system or synthesized de novo during senescence. Some of the genes up-regulated during senescence are known to code for different kinds of proteases (Hensel et al., 1993; Lohman et al., 1994; Drake et al., 1996; Buchanan-Wollaston, 1997; Griffiths et al., 1997; Biswal, 1999; Parrott et al., 2005; Martinez et al., 2007). Cysteine proteases, induced during senescence, may have a major role in nitrogen mobilization from the

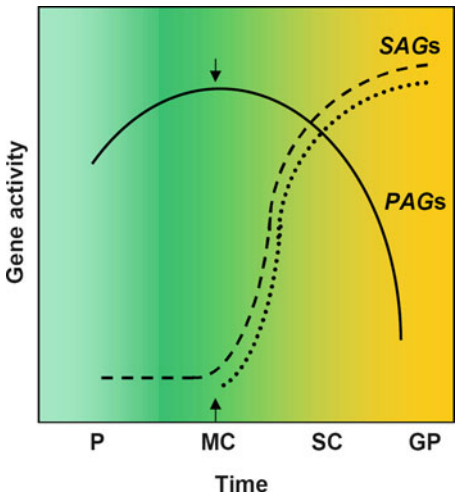

Fig. 10.3. A model showing correlation between expression of photosynthesis-associated genes (*PAGs*) and senescence-associated genes (*SAGs*). The *downward arrow* (↓) indicates down-regulation of (PAGs) and *upward arrow* (↑) indicates up-regulation of SAGs; (——) PAGs; (-----) SAGs nonspecific to the process; (……) SAGs specific to the process. *P* proplastid, *MC* mature chloroplast, *SC* senescing chloroplast, *GP* gerontoplast (Modified from Gan and Amasino (1997); Biswal (1999)).

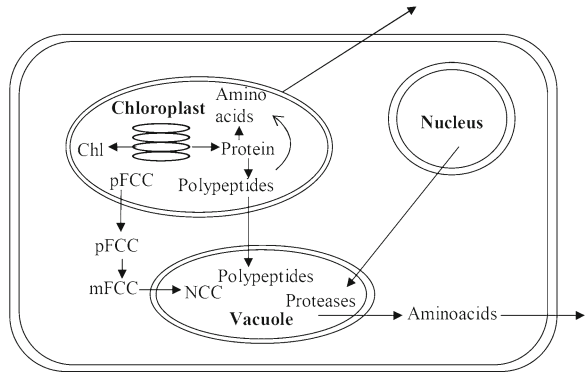

Fig. 10.4. Schematic diagram of Chl and protein degradation in chloroplasts and vacuoles and transport of amino acids outside the cell. *Chl* chlorophyll, *pFCC* primary fluorescent chlorophyll catabolite, *mFCC* modified chlorophyll fluorescence catabolite, *NCC* non-fluorescent chlorophyll catabolite (Modified from Hörtensteiner and Feller (2002)).

senescing tissue (Fischer et al., 1998; Biswal et al., 2003).

Proteases are localized in gerontoplasts and the cytoplasm (Dangl et al., 2000). Disappearance of chloroplast constituents faster than the number of chloroplasts during leaf senescence indicates location of proteases in gerontoplasts (Thayer et al., 1987). Plastid location of the proteases is further supported by the degradation of proteins during in vitro aging of isolated chloroplasts (Panigrahi and Biswal, 1979; Misra and Biswal, 1982). Some of the plastidic proteases are ATP dependent, hence energy requiring. Though the existence of ATP-dependent proteases within the chloroplasts was known since 1984 (Liu and Jagendorf, 1984; Malek et al., 1984), characterization of some of these proteases was carried out about a decade later (Adam, 1996). Energy requiring proteolytic systems associated with the degradation of the photosystem II (PS II) reaction center D1 protein has been well studied (Andersson and Aro, 1997; Spetea et al., 1999; Lindahl et al., 2000). Clip proteases (Clp) have been the most extensively investigated, these are ATP-dependent proteases localized in chloroplasts (Lin and Wu, 2004). Interestingly, initial characterization of Clp proteases was done in *E. coli* (Goldberg, 1992; Gottesman and Maurizi, 1992; Maurizi, 1992; Dangl et al., 2000). These proteases consist of two subunits: the proteolytic subunit, ClpP and ATPase-like regulatory subunit ClpA. Later, plant systems were found to contain homologous genes for both ClpA and ClpP, expressed during leaf senescence of *Arabidopsis thaliana* (Nakabayashi et al., 1999).

Other proteases expressed during senescence are aspartic proteases, ubiquitin, and F-box proteins (Buchanan-Wollaston et al., 2003). These proteins are located in vacuoles and are not in contact with the plastid proteins (Otegui et al., 2005). Therefore, a non-enzymatic degradation of stromal proteins namely, Rubisco and glutamine synthetase have been suggested in a number of reports (Roulin and Feller, 1998; Ishida et al., 1999, 2002). A probable mechanism suggested is that the initiation of degradation of proteins by ROS generated during senescence may occur in the plastid. The breakdown products of proteins may be released to cytoplasm and the final proteolysis may be done by vacuolar proteases (Biswal et al., 2003; Buchanan-Wollaston et al., 2003) (Fig. 10.4). The vacuolar involvement in senescence-related protein degradation with particular reference to cysteine proteases has been worked out by Martinez et al. (2007). The precise regulatory mechanism of protein degradation is not yet clearly understood.

2. Loss of Pigments

Formation of gerontoplasts during leaf senescence is marked by the loss of all the major photosynthetic pigments, Chl *a*, Chl *b*, carotenes and xanthophylls, with a faster rate of loss of Chls (see Biswal and Biswal, 1984, 1990; Smart, 1994; Dangl et al., 2000). The loss of LHCs precedes the leaching of Chls and carotenoids from them. Free Chls pose a threat for the formation of toxic free radicals. Therefore, faster degradation of Chls in gerontoplasts is a kind of detoxification process (Dangl et al., 2000; Biswal et al., 2003). Chl catabolism is carried out by specific enzymes involving several steps (see the reviews by Matile, 1992; Smart, 1994; Matile et al., 1999; Dangl et al., 2000; Hortensteiner, 1999, 2006). Initial steps of Chl degradation involve several enzymes: chlorophyllase, Mg-dechelatase, pheophorbide *a* oxygenase (PAO) and red chlorophyll catabolite reductase (RCC-reductase). These enzymes are well characterized and their role in a series of reactions involved in the conversion of Chl to red chlorophyll catabolite (RCC) is known.

A key step in Chl catabolism is conversion of pheophorbide *a* to RCC by PAO. RCC reductase, the product of the *accelerated cell death 2 (Acd2),* gene converts RCC to primary fluorescent chlorophyll catabolite (pFCC) (Pruzinska et al., 2007), which is transported to the cytoplasm by a catabolite transporter and then by an ATP-binding cassette transporter (ABC transporter) to vacuoles where they are converted to non-fluorescent Chl catabolites (NCCs). Possibly, NCC is converted to monopyrroles in vacuoles (Buchanan-Wollaston et al., 2003; Hortensteiner, 2006) (Fig. 10.4).

The regulatory mechanisms governing enzyme action on Chl degradation at various steps during leaf senescence is yet to be clearly understood (Biswal, 2005). However, gene induction by senescence signals and modulation of PAO, the most important enzyme of Chl degradation pathway, has been worked out (Jiang et al., 2007). The involvement of genes in activating the pigment degradation pathway through translational or post translational regulation of Chl degrading enzymes cannot be ruled out as revealed by mutational studies (Sato et al., 2007).

The yellow pigment carotenoids are relatively stable and are present in the senescent leaf until necrosis begins. Degradation of the carotenoids is speculated to be enzymatic in nature but it is still a grey area (see Biswal et al., 2003). Chemical changes in carotenoids have been reported. For example, formation of xanthophyll acyl esters (Cardini, 1983; Tevini and Steinmuller, 1985; Britton and Young, 1989; Biswal et al., 1994) with a concomitant decrease in the concentration of free xanthophylls has clearly been demonstrated during leaf senescence of many plant species (see Biswal, 1995). The epoxidation of β-carotene is known to be associated with senescence. The qualitative change in the composition of carotenoids during senescence in *Festuca pratensis* leaves reveals the formation of carotenoid esters and also the appearance of β-carotene epoxide (Biswal et al., 1994). Protein degradation and thylakoid disorganization result in a change in the location of carotenoids from thylakoids to plastoglobuli (see Biswal et al., 2003). Delayed degradation of carotenoids may be a plant's strategy to utilize their anti-oxidant properties modulating oxidative damage during senescence.

3. Loss of Lipids

Loss of lipids in gerontoplasts is a result/cause of structural disorganization of membranes by enzyme-regulated processes. Proteomic analysis suggests involvement of lipase, acyl hydrolase, phosphatidic acid phosphatase, lipoxygenase, and phospholipase-D in senescence- induced loss of lipids (He and Gan, 2002; Buchanan-Wollaston et al., 2003; Lin and Wu, 2004).

Galactolipids of thylakoid membranes namely monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) degrade to polyunsaturated fatty acids (Thomas, 1986; Smart, 1994). However, senescence-induced activity of galactolipase has not been observed thus far. The alternative pathway of galactolipid degradation may be through gluconeogenesis (see Buchanan-Wollaston, 1997; Buchanan-Wollaston et al., 2003). Fatty acids, probably, become an important source of energy in carbohydrate-depleted senescent leaves. Senescence-enhanced expression of genes associated with peroxisomal β-oxidation, glyoxylate pathway, and gluconeogenesis supports the above proposition of degradation of lipids to provide energy to the senescent leaves (Graham and Eastmond, 2002; Buchanan-Wollaston et al., 2003).

4. Loss of Nucleic Acids

Nucleic acids are known to be degraded by many senescence-induced nucleases and other related enzymes (Buchanan-Wollaston et al., 2003; Brychkova et al., 2008). Total RNA level declines rapidly along the time course of senescence. DNA perhaps "conducts the orchestra" of senescence till the last event of dismantling of gerontoplasts during leaf senescence: mobilization of nutrients and then finally, so-to-say, the orchestra strikes the last note leading to yellowing of leaves, followed by necrosis. We note that mitochondrial and nuclear DNAs are reported to remain relatively stable compared to plastid DNA during senescence (Lim and Nam, 2005).

D. Senescence Signaling System

The signals that initiate chloroplast to gerontoplast transformation during leaf senescence are not yet understood. The process of initiation, however, is coupled to initiation of leaf senescence. What happens during leaf senescence is easier to follow than what makes the leaf to senesce. Signaling systems of leaf senescence have been studied in several laboratories but the conclusions are still in a grey zone. The picture of interplay between several enzymes, hormones, and up- and down-regulation of genes associated with onset and control of senescence still remain hazy (Guo et al., 2004; Lim et al., 2007).

1. Hormones

Cytokinin has been known to be a key hormone in signaling senescence (Fig. 10.5). The response to the hormone depends on cell type, environment, and developmental stage and interplay with other plant signaling pathways (Dangl et al., 2000; Müller and Sheen, 2007). Design of a *stay-green* tobacco mutant which expresses a cytokinin biosynthesis gene encoding isopentenyl transferase (*IPT*) fused to the promoter of a senescence-associated gene (*SAG12*), elegantly demonstrates the role of this hormone in controling senescence (Gan and Amasino, 1995). The mutant stays green by delaying senescence, and thus photosynthetic decline, consequently maintaining green color (Wingler et al., 1998). Over expression of the farnesyl diphosphate synthase (*FPS*) gene induces

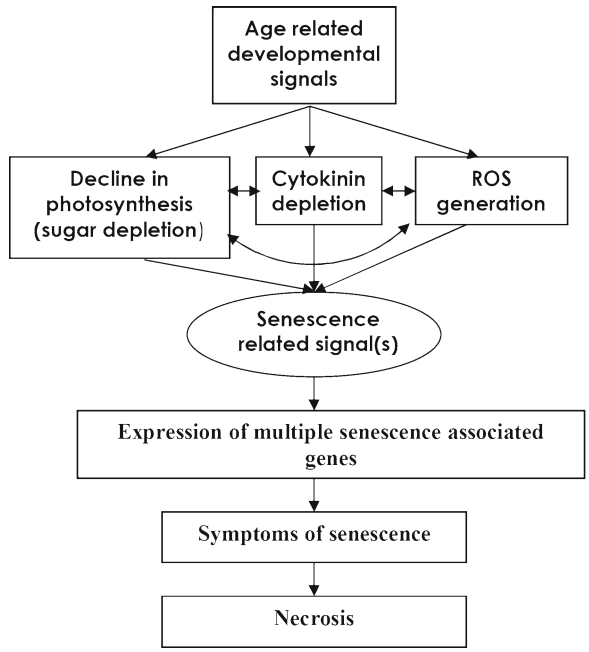

Fig. 10.5. A model of signaling and progress of leaf senescence and programmed cell death of mesophyll cells of leaf (Modified from Biswal and Biswal (1999)).

premature senescence in *A. thaliana*. The farnesyl diphosphate synthase (FPS) is associated with reduction in the levels of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the substrates for cytokinin biosynthesis, hence reduction in the level of cytokinin (Masferrer et al., 2002). Thus involvement of cytokinin in senescence has been cross checked.

2. Sugars

Decline in photosynthesis leading to the depletion of carbohydrate levels is considered as the beginning of the senescence process. This condition is similar to what is observed in starvation. The level of sugar is believed to be sensed by a sugar-sensing mechanism and a decline in the level is likely to send a signal for senescence. But how does sugar signaling operate in plants during induction of senescence? It is quite likely that the sugar level reaches its maximum when leaves are fully mature. The high level of sugar sends a signal for down regulation of photosynthetic genes; consequently, a loss in photosynthesis occurs that leads to induction of senescence (Yoshida, 2003; Lim et al., 2007). This sugar-sensing signal is

possibly mediated by the enzyme hexokinase (HXK). Here, HXK acts as a glucose sensor. Over expression of HXK results in premature senescence (Dai et al., 1999; Xiao et al., 2000).

The physiological functions of a specific hexokinase (HXK1) in the plant glucose-signaling network have been analyzed in the *A. thaliana glucose insensitive2* (*gin2*) mutants (Q432* and G416A substitution in HXK1) (Moore et al., 2003). Analysis of mutants suggests that HXK1 acts as a glucose sensor which correlates nutrient, light, and hormone signaling networks for controlling growth and development in response to environmental changes. HXK1 mutants are found lacking in catalytic activity but they support various signaling functions in gene expression, cell proliferation, root and inflorescence growth, leaf expansion, and senescence (Moore et al., 2003). This demonstrates that glucose signaling and glucose metabolisms have disjointed pathways. The *gin2* mutants are also insensitive to auxin and hypersensitive to cytokinin (Moore et al., 2003; Rolland et al., 2006).

On the whole, the glucose-sensing mechanism appears to be quite complex. It is not just the sugar alone but perhaps sugar concentration, in relation to nitrogen, that plays a role in senescence signaling (Wingler et al., 2006). Sugars may regulate genes during senescence, but with a combination of other developmental signals (Wingler et al., 1998; Rolland et al., 2006).

3. Other Pathways

Other factors namely, ROS, ethylene, jasmonic acid (JA), and salicylic acid (SA) have been reported to be modulators, but not essential factors, for senescence (Buchanan-Wollaston et al., 2003). An age-dependent factor is required along with the above mentioned factors for signaling and controling senescence (Buchanan-Wollaston et al., 2003). ROS is a common factor in developmental or artificially induced leaf senescence. All stress conditions also finally lead to ROS formation and thus modulate senescence. The protective mechanism against ROS involves up-regulation of the members of the glutathione S-transferase (GST) family class phi and quinone reductase during leaf senescence (Hebeler et al., 2008)

Premature senescence is also observed in plants under stress. In most cases, pathways in response to stress appear down-stream of the senescence induction signal. Genomics have revealed that signalling pathways of developmental senescence, stress, starvation or dark induced senescence have overlapping components (Buchanan-Wollaston et al., 2005).

II. We Know What We Do Not Know

In this section we summarize what we do not know about senescence (signaling; associated genes with unknown function; carotenoid metabolism during the process, and biotechnology); as well as about gerontoplasts (their formation and their re-greening).

A. Signaling System for Senescence

It is well known that leaf senescence is initiated upon the decline of photosynthesis with age, but the exact signaling system initiating the senescence still remains unclear. More intriguing are the molecular events. The post genomic era has provided us with a large amount of information regarding genes associated with senescence but a lot more remains to be discovered regarding promoters and repressors of the genes to understand the signaling mechanism (Guo et al., 2004). Though the signaling systems for dark-induced or stress-induced senescence are different from the developmental senescence, there is an interplay between these systems. The area of intersection of these systems remains to be defined (Buchanan-Wollaston et al., 2005).

B. Dismantling of Chloroplasts

Senescence-induced structural changes associated with the dismantling of chloroplasts resulting in the formation of gerontoplasts are well characterized through electron microscopy but the biochemical processes leading to degradation are poorly understood. Several aspects of protein degradation in gerontoplasts during leaf senescence remain speculative. For example, participation of vacuolar proteases in the degradation of plastid proteins is still at the level of hypothesis (see Buchanan-Wollaston et al., 2003; Lin and Wu, 2004).

Degradation of Chl and the enzymes associated with it are understood to a large extent but very little is known about their regulating

processes. The final step of monopyrrole formation from NCC is also poorly understood (see Hörtensteiner, 2006).

Lipid degradation and mobilization through β-oxidation and glyoxylate pathways are known but inductions of the pathways are poorly understood (Lin and Wu, 2004).

Proteomics studies have provided a long list of proteins associated with senescence. Although some of these proteins are functionally characterized, functions of most of the proteins are not known (Guo et al., 2004; Buchanan-Wollaston et al., 2005). The interactions of these proteins and establishing a senescence roadmap with these proteins remains a challenging task for the future.

C. Senescence-Associated Genes with Unknown Function

Genomics yields senescence-associated genes with known functions, predicted functions, and unknown functions. We do not know whether they are ever expressed and if expressed what their roles are. It is a completely dark zone.

D. Re-greening of Gerontoplasts

In a tobacco plant, gerontoplasts revert to chloroplasts during re-greening of a single yellow leaf when the stem above it is removed and it is treated with cytokinin (Zevaleta-Mancera et al., 1999a, b). The ultra-structure clearly shows that gerontoplasts transdifferentiate and become chloroplasts. Increase in the number of plastids does take place. Chls are synthesized, LHCs reappear, thylakoid membrane is regenerated, Rubisco reappears and photosynthesis is restored. However, the molecular pathway of re-greening is not yet known except that the process is not a reversal of the pathway for degradation. What takes place in the re-greening pathway remains unknown.

E. Carotenoid Metabolism During Senescence

Physiology and disposal of carotenoid degradation products remains an exciting field of research for the future. Nothing is known about degradation pathway(s) and the supply of energy involved in carotenoid degradation. We also do not know the end products of carotenoid degradation and their physiological significance if any (Biswal 1995).

F. Biotechnology of Leaf Senescence

Leaf senescence that causes loss in photosynthesis of green leaves is directly linked to plant productivity. Therefore, genetic manipulation leading to the production of *stay-green* mutants has become a fascinating area of research. In the *stay-green* mutants, the process of transformation of chloroplasts to gerontoplasts becomes slower allowing the organelle to carry on photosynthesis and carbon assimilation.

Natural senescence or senescence induced by stress, either biotic or abiotic, may bring about a loss in crop yield. The delay in the process by knocking out the senescence-inducing genes is likely to extend the photosynthetic period and consequently an increase in the crop yield and biomass production. In transgenic plants with delayed senescence, a 50% increase in both seed yield and total biomass production has been observed (Lim and Nam, 2005). Post-harvest biotechnology has become another major area of research in plant biotechnology. Genetic engineering could improve the quality of the post-harvest products especially of the green leaves (Lim and Nam, 2005). Post harvest senescence has also been shown to be significantly delayed in transgenic lettuce and broccoli (Lim and Nam, 2005).

The story of the molecular biology of leaf senescence still remains hazy although many genes associated with the process are cloned and characterized (Biswal, 1999; Buchanan-Wollaston et al., 2003, 2005; Lim et al., 2007). The precise molecular mechanism of the process and the functional analysis of SAGs including senescence-regulatory genes are not known. It will, therefore, not be easy to exploit genetic manipulation of the process even though this has immense potential in the fields of agriculture and food biotechnology. This area needs serious attention by plant molecular biologists.

III. The Unknown: What We Do Not Know

We know much about the SAGs, up- and down-regulated genes associated with senescence but information on upstream sequences that are involved in regulating transcription is not clearly

known (see Buchanan-Wollaston et al., 2003; Guo et al., 2004). We do not know if such a "Senescence-Box" exists and so the characteristics of the Senescence-Box constitute an unknown-unknown domain.

IV. Conclusion

The preceding section has identified the poorly understood areas in leaf senescence, transdifferentiation of gerontoplasts and the fate of this organelle. Research in this area warrants priority. Future work should be devoted to unearth the unknowns.

Before we decide the future course in research on leaf senescence, a time has come to take a pause and define senescence. The debate on this fundamental issue has been raised after the term programmed cell death (PCD) has been applied to this system (Fig. 10.5). PCD refers to systematically programmed biological events leading to death of a cell. Once the cell receives the signal for death, it proceeds to death and there is no point of return (Van Doorn, 2005). Senescence has similar features leading to death hence apparently synonymous with PCD. But two important questions are raised against its similarity with PCD. Firstly, that PCD is a cellular phenomenon whereas senescence is a tissue event and secondly, there is no point of return in PCD but senescence can be reversed (Van Doorn, 2005). Hence the term senescence should be used for the dismantling processes which are still reversible and PCD used after the point of no return is reached (Thomas et al., 2003). The logic put forth in favor of both being synonymous is that senescence is also a cellular phenomenon, which occurs simultaneously in all cells of a tissue. Re-greening can be seen as a part of the reversibility of the program. Re-greening is rare, besides it is artificially induced. Senescence also normally leads to death ultimately. Thus senescence and PCD may be treated as synonymous (Van Doorn and Woltering, 2004).

A roadmap of leaf senescence remains to be drawn for understanding the syntax, the codes, and for the careful compilation of the program of cell death.

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References

Adam Z (1996) Protein stability and degradation in chloroplasts. Plant Mol Biol 32: 773–783

Andersson B and Aro E-M (1997) Proteolytic activities and proteases of plant chloroplasts. Physiol Plant 100: 780–793

Biswal B (1995) Carotenoid catabolism during leaf senescence and its control by light. J Photochem Photobiol B: Biol 30: 3–13

Biswal B (1997) Chloroplast metabolism during leaf greening and degreening. In: Pessarakli M (ed), Handbook of Photosynthesis, pp 71–81. Marcel Dekker Inc, New York

Biswal B (1999) Senescence-associated genes of leaves. J Plant Biol 26: 43–50

Biswal B (2005) Formation and demolition of chloroplast during leaf ontogeny. In: Pessarakli M (ed), Handbook of Photosynthesis, pp 109–122. CRC Press, Boca Raton

Biswal B and Biswal UC (1999) Leaf senescence, physiology and molecular biology. Curr Sci 77: 775–782

Biswal B, Rogers LJ, Smith AJ and Thomas H (1994) Carotenoid composition and its relationship to Chl and D1 protein during leaf development in a normally senescing cultivar and a stay green mutant of *Festuca pratensis*. Phytochemistry 37: 1257–1262

Biswal UC and Biswal B (1984) Photocontrol of leaf senescence. Photochem Photobiol 39: 875–879

Biswal UC and Biswal B (1988) Ultrastructural modifications and biochemical changes during senescence of chloroplasts. Int Rev Cytol 113: 271–321

Biswal UC and Biswal B (1990) Plant senescence and changes in photosynthesis. Biol Edn 7: 56–72

Biswal UC, Biswal B and Raval MK (2003) Chloroplast Biogenesis: From Proplastid to Gerontoplast. Kluwer Academic Publishers, Dordrecht

Biswal UC, Choudhury NK and Biswal B (1983) Degradation of plastid membranes during senescence. In: Sen DN and Kumar S (eds), Indian Review of Life Sciences, Vol. 3, pp. 33–61. Geobios International, Jodhpur

Britton G and Young AJ (1989) Chloroplast carotenoids: function, biosynthesis and effects of stress and senescence. In: Biswal UC and Britton G (eds) Trends in Photosynthesis Research, pp 303–319. Agro Botanical Publishers, Bikaner

Brychkova G, Alikulov Z, Fluhr R and Sagi M (2008) A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the *Atxdh1* Arabidopsis mutant. Plant J 54: 496–509

Buchanan-Wollaston V (1997) The molecular biology of leaf senescence. J Exp Bot 48: 181–199

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T and Pink D (2003) The molecular analysis of leaf senescence: a genomics approach. Plant Biotech J 1: 3–22

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K and Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J 42: 567–585

Callard D, Axelos M and Mazzolini L (1996) Novel molecular markers for late phases of the growth cycle of *Arabidopsis thaliana* cell-suspension cultures are expressed during organ senescence. Plant Physiol 112: 705–15

Cardini F (1983) Carotenoids in ripe green and in autumn senescing leaves of a tree: II-seasonal changes of free carotenoids and xanthophyll esters and relationship between their content and senescing stage. Gion Bot Ital 117: 75–97

Cha K-W, Lee Y-J, Koh H-J, Lee B-M, Nam Y-W and Paek N-C (2002) Isolation, characterization, and mapping of the stay green mutant in rice. Theor Appl Genet 104: 526–532

Chrost B, Daniel A and Krupinska K (2004) Regulation of *a*-galactosidase gene expression in primary foliage leaves of barley (*Hordeum vulgare* L.) during dark-induced senescence. Planta 218: 886–889

Chrost B, Kolukisaoglu U, Schulz B and Krupinska K (2007) An α-galactosidase with an essential function during leaf development. Planta 225: 311–320

Crafts-Brandner SJ, Salvucci ME and Egli DB (1990) Changes in ribulose bisphosphate carboxylase/oxygenase and ribulose-5-phosphate-kinase abundances and photosynthetic capacity during leaf senescence. Photosynth Res 23: 223–230

Dai N, Schaffer A, Petreikov M, Shahak Y, Giller Y, Ratner K, Levine A and Granot D (1999) Over expression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11: 1253–1266

Dangl JL, Dietrich RA and Thomas H (2000) Senescence and programmed cell death. In: Buchanan B, Gruissem W and Jones R (eds) Biochemistry and Molecular Biology of Plants, pp 1044–1100. American Society of Plant Physiologists, Rockville

Drake R, John I, Farrell A, Cooper W, Schuch W and Grierson D (1996) Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. Plant Mol Biol 30: 755–767

Efrati A, Eyal Y and Paran I (2005) Molecular mapping of the chlorophyll retainer (cl) mutation in pepper (*Capsicum spp.*) and screening for candidate genes using tomato ESTs homologous to structural genes of the chlorophyll catabolism pathway. Genome 48: 347–351

Espinozoa C, Medina C, Somerville S and Arce-Johnson P (2007) Senescence-associated genes induced during compatible viral interactions with grapevine and *Arabidopsis*. J Exp Bot 58: 3197–3212

Fang Z, Bouwkamp JC and Solomos T (1998) Chlorophyllase activities and chlorophyll degradation during leaf senescence in nonyellowing mutant and wild type of *Phaseolus vulgaris* L. J Exp Bot 49: 503–510

Fischer A, Brouquisse R and Raymond P (1998) Influence of senescence and of carbohydrate levels on the pattern of leaf proteases in purple nutsedge (*Cyperus rotundus*). Physiol Plant 102: 385–395

Fujiki Y, Nakagauchi Y, Furumoto T, Yoshida S, Biswal B, Ito M, Watanabe A and Nishida I (2005) Response to darkness of late-responsive dark-inducible genes is positively regulated by leaf age and negatively regulated by calmodulin-antagonist-signaling in *Arabidopsis thaliana*. Plant Cell Physiol 46: 1741–1746

Gan S and Amasino RM (1995) Inhibition of leaf senescence by auto-regulated production of cytokinin. Science 270: 1986–1988

Gan S and Amasino RM (1997) Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. Plant Physiol 113: 313–319

Gepstein S (2004) Leaf senescence – not just a 'wear and tear' phenomenon. Genome Biol 5: 212–214

Goldberg AL (1992) The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. Eur J Biochem 203: 9–23

Gottesman S and Maurizi MR (1992) Regulation of proteolysis: energy dependent proteases and their targets. Microbiol Rev 56: 592–621

Graham IA and Eastmond PJ (2002) Pathways of straight and branched chain fatty acid catabolism in higher plants. Prog Lipid Res 41: 156–181

Gresersen PL and Holm PB (2007) Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotech J 5: 192–206

Griffiths CM, Hosken SE, Oliver D, Chojecki J and Thomas H (1997) Sequencing, expression pattern and RFLP mapping of a senescence enhanced cDNA from *Zea mays* with high homology to Oryzain γ and aleurain. Plant Mol Biol 34: 815–821

Guo Y and Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46: 601–612

Guo Y, Cai Z and Gan S (2004) Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ 27: 521–549

He Y and Gan S (2002) A gene encoding an acyl hydrolase is involved in leaf senescence in Arabidopsis. Plant Cell 14: 805–815

Hebeler R, Oeljeklaus S, Reidegeld KA, Eisenacher M, Stephan C, Sitek B, Stuhler K, Meyer HE, Sturre MJ, Dijkwel PP and Warscheid B (2008) Study of early leaf senescence in *Arabidopsis thaliana* by quantitative

proteomics using reciprocal $^{14}N/^{15}N$ labeling and difference gel electrophoresis. Mol Cell Proteomics 7: 108–120

Hensel LL, Grbic V, Baumgarten DA and Bleecker AB (1993) Developmental and age related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. Plant Cell 5: 553–564

Hörtensteiner S (1999) Chlorophyll breakdown in higher plants and algae. Cell Mol Life Sci 56: 330–347

Hörtensteiner S (2006) Chlorophyll degradation during senescence. Annu Rev Plant Biol 57: 55–77

Hortensteiner S and Feller U (2002) Nitrogen metabolism and remobilization during senescence. J Exp Bot 53: 927–937

Ishida H, Makino A and Mae T (1999) Fragmentation of the large subunit of ribulose-1,5-bisphosphatase carboxylase by reactive oxygen species occurs near Gly-329. J Biol Chem 274: 5222–5226

Ishida H, Anzawa D, Kokubun N, Makino A and Mae T (2002) Direct evidence for non-enzymatic fragmentation of chloroplastic glutamine synthetase by reactive oxygen species. Plant Cell Environ 25: 625–631

Jiang H, Li M, Liang N, Yan H, Wei Y, Xu X, Liu J, Xu Z, Chen F and Wu G (2007) Molecular cloning and function analysis of the *stay green* gene in rice. Plant J 52: 197–209

Kawakami N and Watanabe A (1993) Translatable mRNAs for chloroplast targeted proteins in detached radish cotyledons during senescence in darkness. Plant Cell Physiol 34: 697–704

Krupinska K (2006) Fate and activities of plastids during leaf senescence. In: Wise RR and Hoober K (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 433–449. Springer, Dordrecht

Krupinska K and Humbeck K (2004) Photosynthesis and chloroplast break down. In: Nooden LD (ed) Plant Cell Death Processes, pp 169–187. Elsevier Academic Pres, San Diego

Kusuba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M and Tanaka A (2007) Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell 19: 1362–375

Li XP, Gan R, Li PL, Ma YY, Zhang LW, Zhang R, Wang Y and Wang NN (2006) Identification of a leucine-rich repeat receptor-like kinase gene that is involved in regulation of soyabean leaf senescence. Plant Mol Biol 61: 829–844

Lim PO and Nam HG (2005) The molecular and genetic control of leaf senescence and longevity in *Arabidopsis*. Curr Top Dev Biol 67: 49–83

Lim PO, Kim HJ and Nam HG (2007) Leaf senescence. Annu Rev Plant Biol 58: 115–136

Lin J-F and Wu SH (2004) Molecular events in senescing *Arabidopsis* leaves. Plant J 39: 612–628

Lindahl M, Spatea C, Hundal T, Oppenheim AB, Adam Z and Andersson B (2000) The thylakoid Ftsh protease plays a role in the light-induced turnover of the photosystem II D1 protein. Plant Cell 12: 419–431

Liu L, Zhou Y, Zhou G, Ye R, Zhao L, Li X and Lin Y (2008) Identification of early senescence-associated genes in rice flag leaves. Plant Mol Biol 67: 37–55

Liu X-Q and Jagendorf AT (1984) ATP dependent proteolysis in pea chloroplasts. FEBS Lett 166: 248–252

Lohman KN, Gan S, John M C and Amasino RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant 92: 322–328

Makino A, Mae T and Ohira K (1983) Photosynthesis and ribulose 1,5-bisphosphate carboxylase in rice leaves: changes in photosynthesis and enzymes involved in carbon metabolism from leaf development through senescence. Plant Physiol 73: 1002–1007

Malek L, Bogorad L, Ayers AR and Goldberg AL (1984) Newly synthesized proteins are degraded by an ATP stimulated proteolytic process in isolated pea chloroplasts. FEBS Lett 166: 253–257

Martínez DE, Bartoli CG, Grbic V and Guiamet JJ (2007) Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. J Exp Bot 58: 1099–1107

Masferrer A, Arro M, Manzano D, Schaller H, Fernandez-Busquets X, Moncalean P, Fernandez B, Cunillera N, Boronat A and Ferrer A (2002) Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. Plant J 30: 123–132

Matile P (1992) Chloroplast senescence. In Baker N and Thomas H (eds) Crop Photosynthesis: Spatial and Temporal Determinants pp 413–440. Elsevier, Amsterdam

Matile P, Hörtensteiner S and Thomas H (1999) Chlorophyll degradation. Annu Rev Plant Physiol Plant Mol Biol 50: 67–95

Maurizi M (1992) Proteases and protein degradation in *Escherichia coli*. Experientia 48: 178–201

Misra AN and Biswal UC (1982) Changes in the content of plastid macromolecules during aging of attached and detached leaves, and of isolated chloroplasts of wheat seedlings. Photosynthetica 16: 22–26

Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T and Sheen J (2003) Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300: 332–336

Muller B and Sheen J (2007) *Arabidopsis* cytokinin signaling pathway. Sci STKE 407: cm5

Nakabayashi K, Ito M, Kiyosue T, Shinozaki K and Watanabe A (1999) Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. Plant Cell Physiol 40: 504–514

Oh M-H, Kim Y-J and Lee C-H (2000) Leaf senescence in a stay-green mutant of *Arabidopsis thaliana*: disassembly process of photosystem I and II during dark-incubation. J Biochem Mol Biol 33: 256–262

Otegui MS, Yoo-Sun Noh YS, Martínez DE, Vila Petroff MG, Staehelin LA, Amasino RM, and Guiamet JJ (2005) Senescence-associated vacuoles with intense proteolysis activity develop in leaves of Arabidopsis and soybean. Plant J 41: 831–844

Ouelhadj A, Kaminski M, Mittag M and Humbeck K (2007) Receptor-like protein kinase HvLysMR1 of barley (*Hordeum vulgare* L.) is induced during leaf senescence and heavy metal stress. J Exp Bot 58: 1381–1396

Ougham HJ, Morris P and Thomas H (2005) The colors of autumn leaves as symptoms of cellular recycling and defenses against environmental stresses. Cur Top Dev Biol 66: 135–160

Panigrahi PK and Biswal UC (1979) Aging of chloroplasts *in vitro* 1. Quantitative analysis of the degradation of pigments, proteins and nucleic acids. Plant Cell Physiol 20: 775–779

Park SY, Yu J-W, Park J-S, Li J, Yoo S-C, Lee N-Y, Lee S-K, Jeong S-W, Seo HS, Koh H-J, Jeon J-S, Park Y-I and Paek N-C (2007) The senescence-induced stay green protein regulates chlorophyll degradation. Plant Cell 19: 1649–1664

Parrott D, Yang L, Shama L and Fischer AM (2005) Senescence is accelerated, and several proteases are induced by carbon "feast" conditions in barley (*Hordeum vulgare* L.) leaves. Planta 222: 989–1000

Pruzínská A, Anders I, Aubry S, Schenk N, Tapernoux-Lüthi E, Müller T, Kräutler B and Hörtensteiner S (2007) *In vivo* participation of red chlorophyll catabolite reductase in chlorophyll breakdown. Plant Cell 19: 369–38

Reyes-Arribas T, Barrett JE, Huber DJ, Nell TA and Clark DG (2001) Leaf senescence in a non-yellowing cultivar of chrysanthemum (*Dendranthema grandiflora*). Physiol Plant 111: 540–544

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 57: 675–709

Roulin S and Feller U (1998) Dithiothreitol triggers photo oxidative stress and fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in intact pea chloroplasts. Plant Physiol Biochem 36: 849–856

Sato Y, Morita R, Nishimura M, Yamaguchi H and Kusaba M (2007) Mendel's green cotyledon gene encodes a positive regulator of the chlorophyll-degrading pathway. Proc Natl Acad Sci USA 104: 14169–14174

Sitte P (1977) Chromoplasten-bunte objekte der modernen zell biologie. Biol Uns Zeit 7: 65–74

Smart CM (1994) Gene expression during leaf senescence. New Phytol 126: 419–448

Spetea C, Hundal T, Lohmann F and Andersson B (1999) GTP bound to the chloroplast thylakoid membranes is required for light-induced multienzyme degradation of the photosystem II D1 protein. Proc Natl Acad Sci USA 96: 6547–6552

Tevini M and Steinmuller D (1985) Composition and function of plastoglobuli. II. Lipid composition of leaves and plastoglobuli during beech leaf senescence. Planta 163: 91–96

Thayer SS, Choe HT, Tang A and Huffaker RC (1987) Protein turnover during senescence. In: Thompson WW, Nothnagel E and Huffaker RC (eds) Plant Senescence: Its Biochemistry and Physiology pp. 71–80. American Society of Plant Physiologists, Rockville

Thomas H (1986) The role of polyunsaturated fatty acids in senescence. J Plant Physiol 123: 97–105

Thomas H (1994) Aging in the plant and animal kingdoms-the role of cell death. Rev Clinical Gerontol 4: 5–20

Thomas H and Howarth CJ (2000) Five ways to stay green. J Exp Bot 51: 329–337

Thomas H and Stoddart J (1975) Separation of chlorophyll degradation from other senescence processes in leaves of a mutant genotype of meadow fescue (*Festuca pratensis* L.). Plant Physiol 56: 438–441

Thomas H, Ougham H and Hortensteiner S (2001) Recent advances in the cell biology of chlorophyll catabolism. Adv Bot Res 35: 1–52

Thomas H, Ougham HJ, Wagstaff C and Stead AD (2003) Defining senescence and death. J Exp Bot 54: 1127–1132

Thompson J, Taylor C and Wang TW (2000) Altered membrane lipase expression delays leaf senescence. Biochem Soc Trans 28: 775–777

Uauy C, Distelfeld A, Fahima T, Blechl A and Dubcovsky J (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314: 1298–1301

Ülker B, Mukhtar MS and Somssich IE (2007) The WRKY 70 transcription factor of *Arabidopsis* influences both the plant senescence and defense signaling pathways. Planta 226: 125–137

Van Doorn WG (2005) Plant programmed cell death and the point of no return. Trends Plant Sci 10: 478–483

Van Doorn WG and Woltering EJ (2004) Senescence and programmed cell death: substance or semantics? J Expt Bot 55: 2147–2153

Wingler A, von Schaewen A, Leegood RC, Lea PJ and Quick WP (1998) Regulation of leaf senescence by cytokinin, sugars, and light. Effects on NADH-dependent hydroxypyruvate reductase. Plant Physiol 116: 329–335

Wingler A, Purdy S, MacLean JA and Pourtau N (2006) The role of sugars in integrating environmental signals during the regulation of leaf senescence. J Expt Bot 57: 391–399

Woolhouse HW (1987) Regulation of senescence in the chloroplast. In: Thomson WW, Nothnagel EA and Huffaker RC (eds) Plant Senescence: Its Biochemistry and Physiology pp 132–145. The American Society of Plant Physiologists, Rockville

Xiao W, Sheen J and Jang JC (2000) The role of hexokinase n plant sugar signal transduction and growth and development. Plant Mol Biol 44: 451–461

Yoshida S (2003) Molecular regulation of leaf senescence. Curr Opin Plant Biol 6: 79–84

Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H and Scott IM (1999a) Regreening of senescent *Nicotiana* leaves 1. Reappearance of NADPH-protochlorophyllide oxidoreductase and light harvesting chlorophyll *a*/*b* binding protein. J Exp Bot 50: 1677–1682

Zavaleta-Mancera HA, Thomas BJ, Thomas H and Scott IM (1999b) Regreening of senescent *Nicotiana* leaves II. Redifferentiation of plastids. J Exp Bot 50: 1683–1689

Part III

Photosynthetic Responses of Plants to Environmental Stress

Chapter 11

The Role of Membrane Structure in Acclimation to Low-Temperature Stress

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Summary

All photosynthetic protein complexes are embedded in membranes, and lipids surround these complexes. These lipids are indispensable not only in maintaining the functional state/conformation of the photosynthetic reaction centers, but are also needed for protection against environmental stress conditions such as cold and chilling. The redox enzymes, elements of the photosynthetic electron transport chain, which transform the physical energy of the photons to chemical potential, are also in the photosynthetic complexes. During low-temperature stress conditions, the rate of photosynthetic electron transport decreases. This decrease can be enhanced further by exposing the photosynthetic apparatus to high light-intensities. In this chapter, we concentrate on the role of the photosynthetic membrane, especially on the structural aspects of the lipids involved in the protection against low-temperature stress. Genetic manipulations of higher plants and cyanobacteria have suggested that unsaturation of lipids plays an important role in preserving photosynthetic functions at low temperatures. Lipids, in particular phosphatidylglycerol, the only

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phospholipid among the components of the photosynthetic membrane, seem to be active partners of the proteins in the adaptive response to low-temperature stress. In addition to lipids, carotenoids, by exerting local effects, may also participate in membrane dynamics-based protective mechanisms. Fourier transform infrared spectroscopy has proven to be a valuable tool to reveal protein secondary structure/dynamics, and lipid conformation. Since in the infrared spectrum the regions characteristic mostly for proteins or lipids are well separated, infrared spectroscopy is a method of choice to reveal structural and dynamic changes, as well as lipid-protein interactions in the photosynthetic membranes during low-temperature stress conditions.

I. Introduction

Low temperatures represent a frequent and dangerous stress condition in the life of photosynthetic organisms threatening the integrity of photosynthetic membranes. In adaptive responses to low-temperature stress, lipids are the most important membrane factors. Since the physical behavior of fatty acids esterified to the lipid glycerol backbone determines membrane microviscosity, the unsaturation of these fatty acyl chains may increase lipid disorder, thus providing some protection against low-temperature stress. Indeed, cyanobacterial cells modulate the temperature-dependence of the physical state of their lipid molecules by using acyl-lipid-desaturases, which can act on lipids in situ, in the membranes.

Lipid desaturation decreases the extent of low-temperature photoinhibition, a phenomenon also influenced by proteins of the photosynthetic apparatus. Lipids are involved in different interactions with proteins in membrane-related cellular functions but their roles can be very different. For instance, phosphatidylglycerol is the only phospholipid of the photosynthetic membranes participating in photosynthetic electron transport.

Other, hydrophobic, sometimes amphipatic membrane components are carotenoids, which can be embedded in or traverse the membranes. In photosynthetic organisms, carotenoids serve two major functions: they may act as accessory pigments for light harvesting and/or provide protection against photo-oxidative damage. In addition to their direct photosynthetic function, carotenoids also affect the stability of photosynthetic membranes. Carotenoids and other lipids play a major role in determining the dynamics of the photosynthetic membranes.

Membrane stability, which is primarily affected by low-temperature stress, can be sensitively monitored by Fourier transform infrared spectroscopy. This technique by measuring the vibrations of different groups of the molecules provides direct information both about the conformation of membrane proteins and the packing/phase properties of membrane lipids. Since the regions characteristic for proteins and lipids are well separated in the infrared spectrum, these membrane components can be studied not only separately, but also in their interactions.

Here we show how changes in the lipid unsaturation and carotenoid composition affect the physical properties (lipid disorder, dynamics) of the photosynthetic membrane. We also demonstrate how low-temperature stress and the adaptive mechanisms of the cells influence these membrane conditions and what are the consequences of these changes on the functioning of the photosynthetic apparatus.

Abbreviations: ACP – Acyl carrier protein; AGPAT – Glycerol-3-phosphate acyltransferase of chloroplasts from *Arabidopsis thaliana*; D_1 – Core subunit of the Photosystem II reaction center; *DesA+* – *Synechococcus* sp. PCC 7942 cells transformed with the *desA* gene of *Synechocystis* sp. PCC 6803; *DesA−/desD−* – *Synechocystis* sp. PCC 6803 mutant cells with inactivated desaturase genes; ESR – Electron spin resonance; FTIR – Fourier transform infrared; GPAT – Glycerol-3-phosphate acyltransferase; LAHG – Light activated heterotrophic growth; $L\alpha$ – Liquid crystalline lamellar phase of membranes; $L\beta$ – Lamellar gel phase of membranes; PCC – Pasteur Culture Collection; PG – Phosphatidylglycerol; PS I – Photosystem I; PS II – Photosystem II; Rbcs-SQ – Transgenic tobacco plant transformed with the cDNA for GPAT from squash; SGPAT – Glycerol-3-phosphate acyltransferase of chloroplasts from spinach; $\nu_{sym}CH_2$ – Frequency of the symmetric CH_2 stretching mode; X:Y(Z_1,Z_2…) – Fatty acid where X represents the number of carbon atoms, Y represents the number of double bonds and Z_1, Z_2… indicate positions of double bonds, as counted from the carboxyl terminus

II. Low-Temperature Stress: Changes in Membrane Composition in Cyanobacteria and in Higher Plants

A. Cyanobacteria

1. Lipids

The low-temperature sensitivity of cyanobacterial photosynthesis correlates with the extent of the unsaturation of glycerolipids in thylakoid membranes (Murata et al., 1984; Murata, 1989; Wada et al., 1990, 1994). The photosynthetic apparatus is more vulnerable at low temperatures if it is exposed to intensive light as well. This phenomenon is known as low-temperature photoinhibition (Powles et al., 1983; Powles, 1984; Greer et al., 1986; Baker, 1991).

The adaptive responses to low-temperature stress are known to involve lipid and carotenoid biosynthesis. Lipids and carotenoids are regulatory factors necessary for the maintenance of the functioning of photosynthetic reaction centers. To study the role of individual lipids and/or carotenoids in the protection against low-temperature stress, cyanobacteria, which can be readily manipulated genetically, offer a good model system.

Introduction of poly-unsaturated fatty acids into a cyanobacterial (*Synechococcus* sp. PCC 7942) thylakoid membrane, which naturally contains only mono-unsaturated fatty acids, enhanced its cold tolerance (Wada et al., 1990). This experiment was the first direct evidence for the importance of poly-unsaturated glycerolipids in low-temperature adaptation of photosynthetic organisms. By gradually eliminating poly-unsaturated fatty acids in another cyanobacterial model system (*Synechocystis* sp. PCC 6803), we were able to reveal the strong correlation between the susceptibility of photosynthetic cells to low-temperature photoinhibition and the level of unsaturation of glycerolipids in photosynthetic membranes (Gombos et al., 1992, 1994b).

The effect of lipid unsaturation to high-temperature tolerance of the photosynthetic machinery has also been studied (Gombos et al., 1994a; Wada et al., 1994): here, lipid unsaturation was found to be a less decisive factor than in low-temperature tolerance.

Table 11.1. Oxygen-evolving activity of intact cells of wild-type *Synechocystis* sp. PCC 6803, grown at 34°C

Temperature (°C)	O_2-evolving activity, μmol O_2 mg^{-1} Chl h^{-1}
	H_2O to CO_2
18	70
25	130
34	320
	H_2O to BQ
18	130
25	200
34	490

From Gombos et al. (1992)
BQ – 1,4-benzoquinone. Concentrations of BQ and $K_3Fe(CN)_6$ were both 1 mM

a. Effect of Low Temperature on Photosynthetic Oxygen-Evolving Activity

The effect of low-temperature treatment on the oxygen-evolving activity of *Synechocystis* sp. PCC 6803 cells is shown in Table 11.1. Temperature dependent changes were measured either for the whole electron transport from H_2O to CO_2, or for PS II activity alone from H_2O to para-bezoquinone, an artificial electron acceptor (Gombos et al., 1992). Upon exposing the cells to low temperature (18°C), oxygen evolution decreased by about 70%. This decrease could be the result of a low temperature-initiated phase transition in the thylakoid membrane.

b. Elimination of Poly-Unsaturated Lipids

The sequential removal of poly-unsaturated lipids from *Synechocystis* sp. PCC 6803 mutants resulted in a gradually increasing susceptibility of the mutant cells to low-temperature photoinhibition (Gombos et al. 1992, 1994b; Tasaka et al., 1996). The acyl-lipid desaturase genes, *desD* encoding Δ6, and *desA* encoding Δ12 desaturases were inactivated. This mutant contained only mono-unsaturated lipids (Table 11.2) and showed a higher sensitivity to low-temperature photoinhibition than the wild-type cells. The other mutant that had inactivated ω3 and Δ6 desaturases and contained only mono- and di-unsaturated lipids was as resistant to low-temperature photoinhibition as the wild-type cells (Tasaka et al., 1996). These results suggest that minimum di-unsaturated

Table 11.2. Molecular species composition of total glycerolipids from wild type and mutant cells of *Synechocystis* sp. PCC 6803

	Molecular species (mol %)										
	sn-1	16:0	16:1	18:0	18:1	18:1	18:2	18:2	18:3	18:3	18:4
Strain	sn-2	16:0	16:0	16:0	16:0	16:0	16:0	16:0	16:0	16:0	16:0
Wild type		6	8	2	18	tr	tr	20	10	28	8
desA		10	8	2	56	tr	24	0	0	0	0
desB		2	10	4	12	tr	tr	26	0	44	0
desD		4	12	2	16	tr	0	46	22	0	0
desB/*desD*		6	12	2	24	tr	0	58	0	0	0
desA/*desD*		2	14	2	82	tr	0	0	0	0	0

From Tasaka et al. (1996)
Values were calculated on the assumption that the sn-2 position is esterified exclusively with 16:0.
tr trace amount (less than 0.5%)

Table 11.3. Calculated composition of the major molecular species (sn-1/sn-2 fatty acyl groups) of glycerolipids in wild type and *desA*+ cells of *Synechococcus* sp. PCC 7942 grown at 22–34°C

	Growth	Molecular species (mol %)			
Strain	temperature (°C)	16:1/16:0	18:1(9)/16:0	16:2(9,12)/16:0	18:2(9,12)/16:0
Wild type	22	90	2	0	0
desA$^+$	22	38	t	44	2
Wild type	34	72	12	0	0
desA$^+$	34	58	4	10	12

Calculations were made on the basis of the composition of fatty acids of total lipids from cells grown at 22°C and at 34°C (Wada et al., 1990), and on the assumption that in the glycerolipids of *Synechococcus* sp. PCC 7942, the *sn*-2 position of the glycerol backbone is esterified exclusively by 16:0 (Sato et al., 1979)

lipids are needed to protect the photosynthetic apparatus from the deleterious effect of high-light irradiation.

c. Addition of Poly-Unsaturated Lipids

Low-temperature-sensitive *Synechococcus* sp. PCC 7942 cells were transformed by gene *desA* isolated from *Synechocystis* sp. PCC 6803. As a result, the *desA*$^+$ mutant cells contained poly-unsaturated lipids (Table 11.3). The induction of Δ12 desaturase was temperature-dependent. The expression of the gene was temperature- and light-inducible (Kis et al., 1998). This result showed that temperature and light signals are tightly connected with each other. Production of poly-unsaturated lipids is obviously induced by a synergistic effect of light and temperature. It provides protection and regulation whenever a photosynthetic organism faces low-temperature and high-light stress simultaneously. Lipid desaturation may thus be a tool to protect the cells against the deleterious effect of both low temperature and high light.

The *desA*$^+$ mutant cells were more tolerant both to low-temperature stress and to low-temperature photoinhibition than the wild-type cells. The effect of poly-unsaturated lipids on the turnover of D1 protein was localized at the recovery step, where the re-synthesis of D1 takes place (Gombos et al., 1992, 1994b). The processing of preD1 to D1 depended on the saturation level of glycerolipids in the photosynthetic membranes (Gombos et al., 1997).

We note that taking away certain lipids, which are naturally present in a membrane (like poly-unsaturated lipids in *Synechocystis* sp. PCC 6803), is very different from the case when an extra lipid is provided (like di-unsaturated lipids in *Synechococcus* sp. PCC 7942). The former action will necessarily affect the whole membrane structure (vide infra), while in the latter case it is far from evident, how the extra possibility will be manifested in the membranes, either locally, via specific lipid-protein interactions, or

Table 11.4. Photosynthetic electron transport activity of PS I and PS II of thylakoid membranes isolated from cells grown under light activated heterotrophic growth (LAHG) conditions or cells that were transferred to continuous light for 48 h (Light 48 h)

Strain	LAHG		Light 48 h	
	Electron transport activity [μmol O_2 (mg Chl)$^{-1}$ h^{-1}]			
	$H_2O \rightarrow DCBQ$	$DADH_2 \rightarrow MV$	$H_2O \rightarrow DCBQ$	$DADH_2 \rightarrow MV$
Wild type	130 ± 30	−1,290 ± 110	190 ± 30	−380 ± 30
CrtH	−20 ± 20	−1,370 ± 240	220 ± 40	−490 ± 30

From Masamoto et al. (2004)
PS I and PS II activities were measured in terms of electron transport from DAD to MV and from water to DCBQ, respectively. CrtH – mutant with inactivated *cis-trans* isomerase; DAD – 2,3,5,6-tetramethyl-phenylene-1,4-diamine; DCBQ – 2,5-dichloro-1,4-benzoquinone; *MV* methylviologen

Table 11.5. Carotenoid content of cytoplasmic and thylakoid membranes isolated from *Synechococcus* sp. PCC 7942 cells grown at low or high light irradiance

	Carotenoid content [nmol mg^{-1} protein]			
	Cytoplasmic membrane		Thylakoid membrane	
Carotenoids	Low light	High light	Low light	High light
Nostoxanthin	6.3	3.8	2.0	0.7
Caloxanthin	34.7	52.2	9.6	4.3
Zeaxanthin	146.5	401.3	34.6	25.6
β-carotene	6.3	9.6	10.6	7.2

From Masamoto et al. (1999)

globally, by affecting the whole membrane. From ongoing investigations by B. Szalontai (unpublished) it seems that specific local di-unsaturated lipid-protein interactions may enhance the low-temperature tolerance of the *desA*$^+$ *Synechococcus* sp. PCC 7942 mutant cells.

2. Carotenoids

a. Effect of Carotenoids on Photosynthetic Functions

X-ray crystallographic studies revealed that several carotenoid molecules are localized both in PS I and PS II reaction centers (Jordan et al., 2001; Loll et al., 2005). Masamoto et al. (2004) demonstrated, through studies on a carotenoid mutant of *Synechocystis* sp. PCC 6803, that certain carotenoid molecules are integral parts of the photosynthetic apparatus and are involved in the assembly and functioning of photosynthetic reaction centers. PS II simply does not work without carotenoids. The functionality of PS I, however, could be preserved when mutant cells, lacking the *cis-trans* isomerase function, were grown under light activated heterotrophic growth (LAHG) conditions (Table 11.4).

b. Effect of Light Intensity on the Carotenoid Composition of Membranes

In *Synechococcus* sp. PCC 7942 cells, increased light intensity affects the carotenoid content and composition of the cytoplasmic membranes more than that of the thylakoids (Table 11.5) (Masamoto et al., 1999). This observation indicates that these pigment molecules protect not only the photosynthetic membranes but also the outer membranes. The quenching of excess light energy is a well-studied protective effect of carotenoids. Carotenoids can also affect membrane integrity and its phase behavior (Gruszecki and Strzalka, 2005).

c. Effect of Temperature on Carotenoid Composition

The carotenoid content of photosynthetic organisms depends on a whole range of environmental stress phenomena. Shortcomings of energy transfer can be counterbalanced by heat dissipation, which is, in turn, regulated by carotenoid molecules. Photosynthetic processes can also be protected by carotenoids against low-temperature

Table 11.6. Carotenoid and chlorophyll content of thylakoids isolated from *C. raciborskii* cells grown at 25°C or 35°C at 100 μmol photons m^{-2} s^{-1} light intensity

Growth temperature (°C)	Σ carotenoid, nmol mg^{-1} protein	Chlorophyll, nmol mg^{-1} protein	Σcar/Chl (ratio)
35	42.7 ± 5.2	114.2 ± 23.1	0.37
25	36.1 ± 2.8	64.5 ± 9.1	0.56

From Varkonyi et al. (2002)

Table 11.7. Relative amounts of carotenoids in thylakoids isolated from *C. raciborskii* cells grown at 35°C or 25°C at a light intensity of 100 μmol photons m^{-2} s^{-1}

Temperature (°C)	β-Carotene **neutral** (rel. %)	Echinenone (rel. %)	Zeaxanthin (rel. %)	Myxoxanthophyll **polar** (rel. %)	**Polar/neutral** (ratio)
35	32	40	2	26	0.8
25	16	39	2	43	2.7

Values corrected from Varkonyi et al. (2002)

stress. Table 11.6 demonstrates that cyanobacterial cells grown at various temperatures differ in carotenoid composition (Varkonyi et al., 2002).

In *Synecochystis* sp. PCC 6803, lower growth temperature is associated with lower chlorophyll content, and concomitantly, with a higher carotenoid content per cell. Temperature treatment of cyanobacterial cells affects both the overall amount and the individual carotenoid composition (Table 11.7). The amount of the neutral β-carotene was lower in cells grown at 25°C, than in those grown at 35°C. This decrease was accompanied by an increase of the polar myxoxanthophyll. Since polar carotenoids can span the lipid bilayer, and establish strong interaction with water molecules, a shift toward more polar carotenoids may affect the phase transition of a biological membrane. Thus, in cyanobacteria, the observed increase of the myxoxanthophyll content may cause increased membrane rigidity.

B. Higher Plants

1. Phosphatidylglycerol, a Key Lipid in Low-Temperature Stress Resistance

Lyons (1973) and Raison (1973) have suggested that the primary event in chilling injury is an L_α-to-L_β lipid phase (liquid crystalline phase to gel phase) transition in the cellular membranes. According to this proposal, transition from liquid crystalline phase to gel phase would result in alterations in the metabolism of chilled cells and lead to injury and to the death of the chilling-sensitive plants. A similar hypothesis concerning chloroplast membranes was proposed by Norio Murata and coworkers (Murata, 1983; Murata and Yamaya, 1984; Murata and Nishida, 1990). This hypothesis suggested that molecular species of chloroplast phosphatidylglycerol (PG) containing a combination of saturated fatty acid (16:0 and 18:0) or 16:1-*trans* at both the sn-1 and sn-2 positions of the glycerol backbone (high-melting-point PG molecular species) confer chilling sensitivity on plants. Murata and Yamaya (1984) have proposed that the presence of high-melting-point PG molecular species in the chloroplast membranes would induce L_α-to-L_β phase transition at chilling temperatures, and that the phase separation within the membranes would be the direct cause of chilling sensitivity. This hypothesis was supported by the detection of L_α-to-L_β phase transitions in synthetic 16:0/16:0 PG molecular species and in the PG purified from chilling-sensitive but not from chilling-resistant plants (Murata and Yamaya, 1984).

Roughan (1985) has surveyed the fatty acid composition of PG in 74 plant species and found, in broad terms, a correlation between chilling sensitivity and the percentage of saturated trans-mono-unsaturated PG molecular species. From this survey, it appears that the *cis*-unsaturated molecular species of PG in chloroplasts are crucial components in low-temperature tolerance. This finding is even more spectacular if one takes into account the low abundance of PG in

Table 11.8. Fatty acids in phosphatidylglycerol isolated from thylakoid membranes of wild type and of a transgenic tobacco plant, transformed with the cDNA for GPAT from squash (Rbcs-SQ)

	Fatty acid (mol %)								
						18:1	18:1		
Plants	16:0	16:1c	16:1t	16:3	18:0	9-*cis*	11-*cis*	18:2	18:3
Wild type	32	0	32	0	3	7	1	10	15
Rbcs-SQ	46	0	34	0	8	1	1	3	7

From Moon et al. (1995)

chloroplast membranes (≈5–10%). In addition, PG is the only phospholipid of photosynthetic membranes.

Glycerol-3 phosphate acyltransferase (GPAT) is a key enzyme in setting the level of *cis*-unsaturation of PG (Frentzen et al., 1987). This observation offered the possibility of genetic manipulation for the study of the role of PG *cis*-unsaturation in low-temperature tolerance. Accordingly, tobacco (*Nicotina tobacum*) was transformed with the cDNA for GPAT from (i) the chilling tolerant *Arabidopsis thaliana*, and from (ii) the chilling-sensitive *Cucurbita moschata*.

The transgenic plants expressing the *A. thaliana* gene contained less high-melting-point PG and exhibited less chilling damage than the control tobacco plants (Murata et al., 1992a). The tobacco transformed with the squash gene (Rbcs-SQ) contained more saturated lipids than the wild type (Table 11.8). The photosynthetic characterization of the transgenic plants showed that Rbcs-SQ became more sensitive to low-temperature photoinhibition (Murata et al., 1992a; Moon et al., 1995).

A similar conclusion was reached by analyzing the chilling tolerance of transformed rice. The level of *cis*-unsaturated fatty acids in PG from chilling-sensitive rice leaves was genetically altered with cDNAs of chloroplasts GPAT from *A. thaliana* (+AGPAT) and spinach (+SGPAT), which are chilling-resistant plants. In both the wild-type rice and the transformants at 25°C, the O_2 evolution from leaves was similar, and was equally impaired at 5–11°C. However, at around 14–17°C, the +AGPAT and +SGPAT plants showed less impaired O_2 evolution rates than the wild type and –SPGAT plants. These results demonstrate the practical importance of the present technology with GPAT to improve the chilling sensitivity of crops (Ariizumi et al., 2002).

To test the importance of PG in the tolerance of higher plants to low temperature stress, a suitably modified form of the *Escherichia coli* gene (*plsB*), that encodes the bacterial acyl-ACP:glycerol-3-phosphate acyltransferase, was used by Wolter et al.(1992) to transform *A. thaliana* plants. The bacterial enzyme does not discriminate between 16:0-ACP and 18:l-ACP substrates; thus, in the transgenic *A. thaliana* plants, more than 50% of the PG molecules were high-melting-point PGs. These transgenic plants suffered wilting and necrosis after exposure to low temperatures; this again appears to support the hypothesis of PG-dependent chilling sensitivity (Wolter et al., 1992). However, Wu and Browse (1995), by the use of an *A. thaliana* (*fab1*) mutant, could not demonstrate that a high proportion of high-melting-point PG species induced chilling sensitivity, and concluded that the high-melting-point molecular species of PG cannot be the principal determinant of chilling sensitivity in this transgenic plant. This discrepancy has not yet been resolved.

Experiments on the structural and functional role of PG have revealed that this lipid species plays a crucial role in the assembly of both PS I and PS II reaction centers (Sakurai et al. 2003, 2007; Domonkos et al., 2004; Domonkos et al., 2008). PG molecules are localized within the reaction centers as shown by X-ray crystallography (Fromme, 1996; Fromme et al., 2001; Jordan et al., 2001; Loll et al., 2005; Kern and Guskov, 2011; Mizusawa and Wada, 2011). This intimate PG-to-protein interaction may explain the effect of PG on photosynthetic electron transport. Functionally, both electron transport in PS II, around the primary and secondary quinones, and PS I activity, were affected by PG depletion (Gombos et al., 2002; Domonkos et al., 2004). PG depletion of the cells resulted in elevated light-susceptibility, as indicated by the bleaching of cellular pigments (Sakurai et al., 2003).

III. Structural Changes in Thylakoid Membranes

Biological membranes are delicate assemblies of proteins and lipids. The bilayer of amphipathic lipids provides thin (≈6 nm) hydrophobic phases for water-insoluble proteins between the 'vast' water-phases, which are separated by the membrane. For the functioning of the membrane (e.g., barrier properties, signal transduction, energy production and material transport), both the individual properties of the constituent proteins and lipids, and their interactions are decisive. Since the vast majority of biological organisms are not able to regulate their temperature, their membranes have to function properly quite often over a wide temperature range. Proper functioning assumes proper membrane dynamics, which are able to adapt to altering external conditions (e.g., temperature, light/darkness in photosynthetic organisms and stress). As an adaptation mechanism to altering temperatures, homeoviscous adaptation (Sinensky, 1974) has been postulated and was later proven mostly for poikilotherm organisms. The homeoviscous adaptation theory focuses on the role of lipids in maintaining membrane dynamics. Much less is known about the role of protein dynamics, and even less about the role of protein-lipid interactions in maintaining membrane dynamics, and thus, the functionality of a biological membrane, especially around the low- and high-temperature in vivo limits.

Now, we shall discuss the effect of low-temperature stress on the structure of lipids and proteins of photosynthetic membranes, and on the interaction between them.

A. Lipid Dynamics

The structures, which are formed from isolated lipids, vary according to the shape of the particular lipid. The occurrence of a given structure can be explained with the help of the so-called shape-structure concept of lipid polymorphism (Cullis and De Kruijff, 1979). This concept sorts out the lipids according to the relative surface requirement of their head groups versus their fatty acyl chains. If the head group of a lipid molecule has roughly the same cross-sectional area as the acyl chains, the lipid molecule is considered to be cylindrical – these lipid molecules self-assemble into bilayer structures. If the surface cross-sectional area of the head group is larger or smaller than that of the acyl chains, the lipid molecule will have, accordingly, two kinds of conical shape – these lipid molecules assemble preferentially into nonbilayer structures (Van den Brink-Van der Laan et al., 2004).

In biological membranes, however, to our present knowledge, lipids always form bilayers, in spite of the fact that a large fraction of the membrane lipids (sometimes up to 50%) is nonbilayer lipid. The conical lipid molecules facilitate the incorporation of peripheral and integral membrane proteins, and are essential at the curvatures of the membranes (Van den Brink-Van der Laan et al., 2004). In biological membranes, lipids can be in the liquid crystalline lamellar phase (L_α), or in the lamellar gel phase (L_β), which is a highly ordered and tightly packed structure that occurs below the main transition temperature (Casal and Mantsch, 1984). According to general experience, lipids in functional biological membranes are always in the liquid crystalline state, nonbilayer formation may appear only locally and temporarily (e.g., in connection with membrane fusion (Siegel and Epand, 1997; Epand, 1998)). As we shall see below, under low-temperature stress conditions, where the temperature may decrease close to the main phase transition temperature of their membranes, the photosynthetic organisms exert major efforts to maintain the liquid crystalline state of their membranes.

Fourier transform infrared (FTIR) spectroscopy is a well-established, non-invasive technique to measure not only lipid phase properties, but also protein structure and dynamics. In the infrared spectrum, the regions characteristic of lipids and proteins are well separated. Therefore, the technique allows not only the parallel investigation of the membrane components but, by analyzing correlating changes in the lipid- and protein-related spectral features as a function of an external parameter (e.g., temperature), also the study of lipid-protein interactions. Concerning lipid acyl chain disorder, the most frequently utilized spectral parameter is the frequency of the symmetric CH_2 stretching mode ($\nu_{sym}CH_2$), which in gel-phase lipids is near 2,850 cm^{-1}, and it exhibits 2–5 cm^{-1} upshift upon gel-to-liquid crystalline phase-transition. The temperature dependence of this frequency shift is a sensitive measure

of lipid conformation, and of lipid-protein interaction (for a review on $\nu_{sym}CH_2$ in lipids, see Mantsch and McElhaney (1991)). It has also been shown that the frequency upshift of the $\nu_{sym}CH_2$ band can be described as competing contributions coming from ordered, all-trans fatty-acyl chains (gel phase), and from disordered acyl chains containing increasing amounts of "gauche" segments with increasing temperatures (liquid crystalline phase). The $\nu_{sym}CH_2$ band could be decomposed into two bands, the lower frequency one corresponding to the ordered, and the higher frequency one to the disordered acyl chains (Kota et al., 1999). As the temperature is increased, the intensity of the lower frequency component diminishes, and that of the higher frequency one increases (Kota et al., 1999). Since infrared spectroscopy provides a "snapshot" of the investigated system, the $\nu_{sym}CH_2$ frequency reflects the average order/disorder (fluidity) of the fatty acyl chains of the membrane lipids.

As to the physical characterization of membranes, 'fluidity' is a frequently used term. One should be aware, however, that fluidity is a macroscopic parameter. It should be used with caution even in electron spin resonance (ESR) and in fluorescence labeling spectroscopy, when generally only the rotational diffusion coefficients of the labels are determined. In infrared spectroscopy, motions of the segments of the molecules are involved, and their coupling to each other. For instance, this coupling changes during the formation of "gauche" segments in the lipid fatty acyl chains, and this results in more disordered membranes (which would be probably more fluid in viscosity measurements). Since the use of the term 'fluidity' is widespread without deeper consideration of its real meaning, we shall also use it, to conform to the literature, but with the above considerations in mind.

Figure 11.1 shows the C-H stretching region of thylakoid membranes prepared from a cyanobacterium *Synechocystis* sp. PCC 6803 (Szalontai et al., 2000). The frequency of the $\nu_{sym}CH_2$ band obtained from curve fitting was utilized to characterize acyl chain disorder in the membranes. The higher the $\nu_{sym}CH_2$ frequency, the greater the disorder. In Fig. 11.2, the temperature dependences of the $\nu_{sym}CH_2$ frequencies are shown for *Synechocystis* sp. PCC 6803 cells, grown at 25–35°C. The curves represent a clear example of

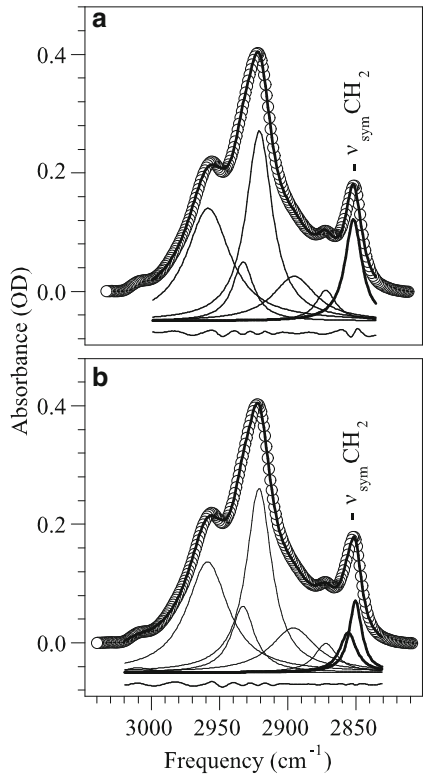

Fig. 11.1. Analysis of the C-H stretching region of the FTIR spectra of thylakoid membranes prepared from wild-type cells of *Synechocystis* sp. PCC 6803 grown at 35°C. *Circles* indicate measured data points. The *continuous line* through the *circles* is the sum of the fitted Lorentzian components, which are displaced for clarity. The $\nu_{sym}CH_2$ components discussed in this chapter are drawn with *thicker lines*. (**a**) Fitting with one component of the $\nu_{sym}CH_2$ band (note the systematic error in the residual curve at the bottom of the panel). (**b**) The $\nu_{sym}CH_2$ band is fitted with two components (observe the disappearance of the systematic fitting error in the residual curve under the components). For details, see the text (Szalontai et al., 2000).

homeoviscous adaptation. In Fig. 11.2a, the level of disorder at the growth temperature (35°C) is indicated with a dashed line. This dashed line, reproduced in Fig. 11.2b, is found to cross the $\nu_{sym}CH_2$ frequency curve around 25°C, at which temperature those cells were grown.

We note that at temperatures lower than the growth temperature, the disorder/fluidity is higher in the membranes of cells grown at 25°C (WT25, WC25) than in those prepared from cells grown at 35°C (Fig. 11.2).

The higher capacity of *Synechocystis* sp. PCC 6803 cells, grown at 25°C, to maintain higher membrane fluidity at lower temperatures is

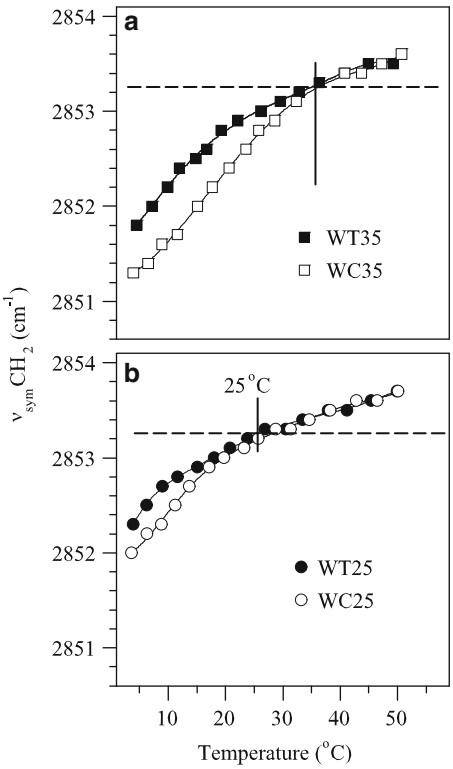

Fig. 11.2. Thermotropic response of the $\nu_{sym}CH_2$ frequencies in thylakoid (*WT*) and in cytoplasmic (*WC*) membranes prepared from wild-type (*W*) cells. (**a**) Cells grown at 35°C; (**b**) Cells grown at 25°C. The *dashed line* indicates the level of lipid disorder found around the corresponding growth temperature. This level is identical in the membranes prepared from cells grown at either 25°C or 35°C (Szalontai et al., 2000).

attributed to an increased amount of poly-unsaturated fatty acyl chains in the membranes of these cells (Murata et al., 1992b). These poly-unsaturated acyl chains are configured in situ in the membranes by desaturase enzymes (Murata et al., 1992b), which can reduce the acyl chains in such a way that they will have *cis*-double bonds between their selected carbon atoms. In wild-type cells, the poly-unsaturated fatty acyl chains are formed by a succession of desaturase enzyme actions initially at positions Δ9 (DesC), Δ12 (DesA), and ω3 (DesB). At any step of this sequence, alternatively, the desaturase D (DesD) enzyme may reduce the fatty acid chains at the Δ6 positions (Murata and Wada, 1995). By insertion methods, the gene of any of these desaturase enzymes can be inactivated. By inactivating the A and D desaturase enzymes one can produce a mutant (*desA*$^-$/*desD*$^-$), which contains only saturated and mono-unsaturated fatty acyl chains in its membranes (Tasaka et al., 1996).

DesA$^-$/*desD*$^-$ mutants provide an experimental system in vivo, in which only the fatty acyl composition of the membranes has been changed; thus, the effect of this parameter on lipid dynamics and – at the organism level – on the physiology of the cells can be studied separately. Of course, this statement would be completely true only if living systems were considered as a linear combination of isolated processes. There are, however, several pieces of experimental evidence showing that changes in other constituents (e.g., proteins and carotenoids) also accompany the alteration of the level of unsaturation of the fatty acyl chains. With confidence, we can only say that in the *desA*$^-$/*desD*$^-$ mutants the fatty acyl composition has been changed. Nevertheless, studies of the different desaturase mutants revealed very interesting structural-functional relations not only between the level of lipid desaturation and membrane dynamics, but also between lipids and proteins (Szalontai, 2009).

The thermotropic response of the $\nu_{sym}CH_2$ frequencies of *desA*$^-$/*desD*$^-$ mutant cells shows that, if grown at 35°C, these cells can maintain a similar lipid disorder/dynamics as the wild-type *Synechocystis* sp. PCC 6803 cells (compare Figs. 11.3a and 11.2a). This is not very surprising, since the amount of poly-unsaturated fatty acyl chains is small from the beginning in the wild-type *Synechocystis* sp. PCC 6803, when it is grown at 35°C (see the fatty acid composition of membrane lipids in Wada and Murata (1989)). On the other hand, when the *desA*$^-$/*desD*$^-$ mutant cells are grown at 25°C, around the low-temperature limit of the wild-type cells, they fail to reach the "ideal" lipid disorder (Fig. 11.3b) of the wild-type cells (indicated by the dashed line, reproduced from Fig. 11.2).

A number of different biochemical studies on cyanocateria have shown that poly-unsaturated lipids are necessary to protect the cells against low-temperature photoinhibition (Gombos et al. 1992, 1994b). In low-temperature photoinhibition, the natural repair process of the light-damaged D1 protein of the photosynthetic reaction center cannot be performed. The pre-D1 proteins are synthesized, inserted into PS II reaction centers but their post-translational processing is not completed (Gombos et al., 1997). Similar

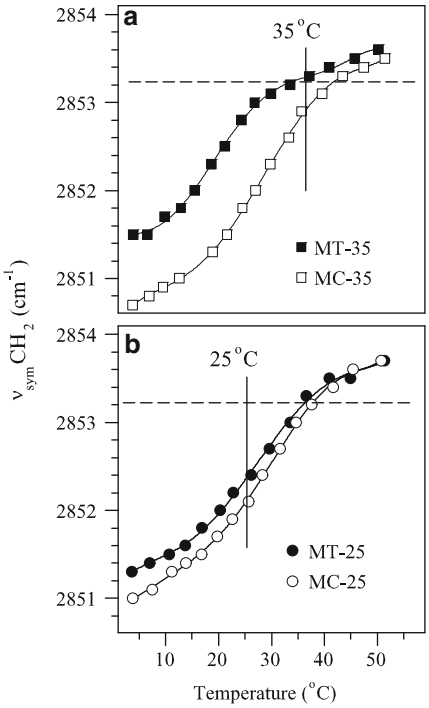

Fig. 11.3. Thermotropic response of $\nu_{sym}CH_2$ frequencies in thylakoid (*MT*) and cytoplasmic (*MC*) membranes prepared from *desA*$^-$/*desD*$^-$ mutant (*M*) cells. (**a**) Mutant cells grown at 35°C; (**b**) Mutant cells grown at 25°C. The *dashed lines* indicate the level of disorder found in membranes prepared from wild-type cells, as shown in Fig. 11.2 (Szalontai et al., 2000).

phenomena have been observed with higher plants as well (Alfonso et al., 2004; Grennan and Ort, 2007). Evidently, the low membrane lipid dynamics do not permit major local membrane reorganizations. The results show that the structure and the dynamics of the thylakoid membrane directly affect the functioning of the photosynthetic apparatus, especially at low temperatures, where the lipid-related physical parameters (e.g., lipid phases, fluidity) change steeply.

The functional entity of the photosynthetic reaction centers and the surrounding membrane lipid matrix at the structural level can be further studied by including light, the main energy source and organizing factor of photosynthesis. A comparison of the photosynthetic activities of *Synechocystis* sp. PCC 6803 cells, grown in the light at 25–35°C, revealed that the temperatures of the maximum photosynthetic activity were at 31–40°C, respectively (Zsiros et al., 2002). This means that the maximum photosynthetic activity correlates with the growth conditions, and consequently with the structure/dynamics of the thylakoid membranes. However, if the same cells were grown at 25–35°C, but under LAHG conditions (Zsiros et al., 2002), the temperatures of the maximum photosynthetic activities were insensitive (39–40°C, respectively) to the growth temperatures. With respect to membrane structure, it turned out that without light there was no lipid desaturation. The level of lipid fatty acyl chain unsaturation in the cells grown under LAHG conditions was very similar to that of cells grown at high temperature (Zsiros et al., 2002).

To reduce the cause of the lack of desaturase activity to the lack of light (the ultimate energy source under phototrophic conditions) would be an oversimplification of the phenomena. Since under LAHG conditions (here light serves only to maintain the photosynthetic apparatus, it is not a primary energy source), the photosynthetic apparatus practically does not work, it seems that it is not affected by either lipid-protein interactions, or membrane dynamics, which would be optimal for its functioning. On the other hand, heterotrophic processes that keep the cells alive in the dark may function optimally with different lipid disorder/dynamics, and therefore they do not force desaturase-mediated lipid disorder optimization at different growth temperatures. (Such comparative studies would be interesting to reveal to what extent the same proteins are used by heterotrophic and autotrophic mechanisms, and how the cell switches from one mode of operation to the other.)

Phosphatidylglycerol seems to play an exceptional role in the chilling resistance of plants. It has been known for some time that the level of unsaturation of the PG fatty acyl chains exhibits a strong correlation with the cold-resistance of the given plant (Murata and Yamaya, 1984). This correlation is very interesting since PG makes up only ≈5–10% of the total plant lipid content. Nevertheless, because of its extremely small head group, this lipid is exceptionally well adapted for specific functions.

By transforming tobacco plants with complementary DNAs for glycerol-3-phosphate acyltransferases (GPATs), the amount of saturated fatty acyl chains in PG molecules could be increased (by the GPAT gene from squash) or decreased (by the GPAT gene from *Arabidopsis*

thaliana) (Murata et al., 1992a). Decomposing the $\nu_{sym}CH_2$ band into two components, as discussed above, revealed that the amount of the rigid/ordered fatty acyl chains was higher in the squash GPAT gene-transformed mutant and lower in the mutant containing the *A. thaliana* GPAT gene than in the wild-type tobacco (Szalontai et al., 2003). It is disturbing, however, that the apparent effect on the structure of the thylakoid membranes in the low-temperature stress region seems to be too large in view of the small amount of PG molecules (Szalontai et al., 2003). The reasons of this enhancement could be any of the following: (i) The inserted GPAT genes had a cauliflower mosaic virus promoter; thus, they were not under the control of the tobacco system. Therefore the alien PG molecules might be more abundant than it is normally needed in the wild-type tobacco. (ii) The method of deconvoluting the $\nu_{sym}CH_2$ band into two bands somewhat enhanced the amount of the rigid/ordered components. The parameters of the lower-frequency (rigid) component had to be fixed at the lowest temperatures so as to make it fit correctly at higher temperatures, where it should gradually diminish. Nevertheless, on the basis of the differences in the lipid disorder/dynamics between the wild type and the mutant cells at low-temperatures, one could predict consequences of the altered PG composition: differences should be exhibited in physiological events, which involve temperatures lower than about 18°C (Szalontai et al., 2003). Indeed, squash GPAT-transformed mutants showed larger damage and slower recovery from low-temperature (1°C) photoinhibition than wild-type tobacco (Moon et al., 1995).

Besides the direct effects of lipid fatty acyl chain disorder, there are other factors that can directly affect membrane dynamics and influence lipid disorder. The change of carotenoid content and composition has been observed to change in practically all the experiments, where the level of lipid fatty acyl chain unsaturation was modified either with genetic methods (M. Kis, personal communication) or by changing the physiological conditions (Varkonyi et al., 2002).

In another cyanobacterium, *Cylindrospermopsis raciborskii*, the relative amounts of poly-unsaturated glycerolipids and myxoxanthophylls in the thylakoid membranes increased markedly when this cyanobacterium was grown at 25°C instead of 35°C. Despite the higher amount of unsaturated lipids in the cells grown at 25°C, the conventional thermotropic responses of the $\nu_{sym}CH_2$ frequencies indicated more rigid/ordered fatty acyl chains in the membranes of the cells grown at 25°C than in those grown at 35°C. This apparent controversy was resolved by two-component analysis of the $\nu_{sym}CH_2$ band, which demonstrated the presence of very rigid, myxoxanthophyll-related lipids in the thylakoid membranes. When this rigid component was excluded from the analysis of the thermotropic responses of the $\nu_{sym}CH_2$ bands, as expected, higher fatty acyl chain disorder was observed in the thylakoids prepared from cells grown at lower temperature. It seems that besides their well-known protective functions, polar carotenoids may have structural effects on the thylakoid membranes as well (Gruszecki and Strzalka, 2005).

Carotenoid effects might be local, by forming protective patches, in-membrane barriers of low dynamics in the more fluid lipid environment, against reactive radicals (Varkonyi et al., 2002).

B. Protein Structure

Of the tens of thousands of proteins, whose structure is known at atomic resolution, only a few dozens are membrane proteins. The reason for this imbalance is that membranes are extremely complex systems, in which proteins and lipids together form functional units. Therefore, investigation of isolated membrane proteins is difficult, and in many cases it is questionable whether the results obtained are relevant in vivo. In addition, when the study of structure and dynamics of biological membranes is the goal, one has to deal with in situ proteins in the membranes, and there are hundreds of them in one membrane.

FTIR spectroscopy, as mentioned above, yields parallel information about membrane proteins and lipids. However, the method is not selective: the FTIR spectrum reflects the sum of all protein contributions in the protein-related spectrum regions, called Amide regions. There are several Amide regions in the infrared spectrum; here we mention only the Amide I (having contribution mostly from the C=O stretch of the peptide bond) between 1,700 and 1,600 cm^{-1}, and the Amide II (here the contribution of the N-H deformation

of the peptide bond is dominant) at around 1,550 cm^{-1}. Decomposition of the Amide I band yields components, which can be assigned to the secondary structure elements [α-helices (1,655–1,648 cm^{-1}), anti-parallel β-sheet (1,690, 1,636–1,630 cm^{-1}), parallel β-sheet (1,645, 1,630 cm^{-1}) and unordered (1,660–1,656 cm^{-1})] of the proteins. From our point of view, the strong, narrow band, which emerges around 1,620–1,615 cm^{-1} in proteins upon heat denaturation, is of particular importance. This band has been assigned to the formation of intermolecular β-structures (Van Stokkum et al., 1995) and can be used as a marker of 'fatal' protein structural changes. For a detailed analysis of the infrared spectra of proteins in general see Arrondo et al. (1993); and for membrane proteins in particular, see Goormaghtigh et al. (1994a, b, c).

In membranes a detailed structural analysis of the Amide I band to determine the relative amounts of the individual secondary structure elements does not make much sense, since the observed spectrum stands for the sum of all proteins present in the membrane. However, differences in the changes of the Amide I region due to changing external conditions can be informative. While the overall structures of the thylakoid membrane proteins, even in different plants, are very similar, slight changes, like altered PG content, as discussed above, may alter the stability of the membrane proteins or a part of them (Fig. 11.4). The progress of protein denaturation can be better visualized if difference spectra in the Amide I region are calculated by subtracting the lowest-temperature spectrum from the higher-temperature ones (Fig. 11.5). If this is done, the protein aggregation appears to take place at the cost of functional α-helical and β-structured conformations (Fig. 11.5). Plotting the intensities of the 1,618 cm^{-1} band as a function of temperature reveals the progress of protein denaturation. Thus the effect of different factors on membrane protein stability/dynamics can be studied. Such curves of membrane protein heat denaturation are shown for three different plants later in the chapter (see Fig. 11.7b). We see no major denaturing of proteins up to the high ends of the physiological temperature range of plants; i.e., no major role of proteins in the low-temperature stress signaling is to be expected. (Since the protein-related parameters change sharply in the high-temperature stress range, one may speculate that some of them might be the trigger of an actual alarm signal there.)

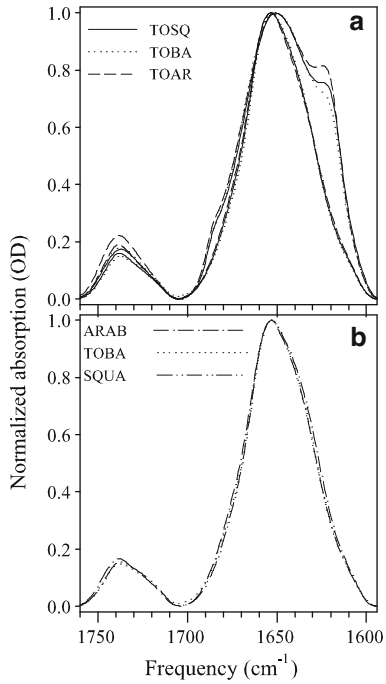

Fig. 11.4. The amide I and ester C=O stretching regions characteristic of higher plant thylakoid membranes. (**a**) Comparison of the spectra of wild-type tobacco (*TOBA*), tobacco transformed with cDNA for squash GPAT (*TOSQ*), and tobacco transformed with cDNA for *Arabidopsis thaliana* GPAT (*TOAR*) at 5°C and at 68°C. (**b**) Comparison of spectra of tobacco (*TOBA*), squash (*SQUA*) and *A. thaliana* (*ARAB*) thylakoids recorded at 5°C. The spectra as shown were normalized after baseline subtraction; the baseline was a Gaussian curve fitted to the regions 1,770–1,767, 1,709–1,706, 1,597–1,594, 1,507–1,505, and 1,365–1,362 cm^{-1}. The major section of the spectrum is not shown (Szalontai et al., 2003).

C. Lipid-Protein Interaction

As mentioned above, there are regions in the infrared spectrum of biological membranes, which can be primarily assigned to lipids (e.g., the C-H stretching region), or to proteins (Amide I and II regions). Very close to each other are a band at around 1,750–1,720 cm^{-1}, which is due to the stretching of the C=O group formed upon the ester bonding of fatty acids to the glycerol backbone of the lipids, and the protein-related Amide I band (1,700–1,600 cm^{-1}). The intensity ratio of these two bands can be used to determine an

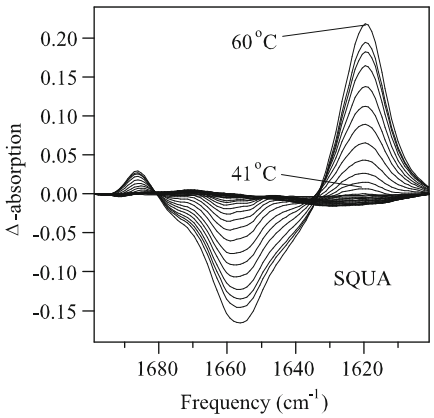

Fig. 11.5. Heat-induced difference spectra in the amide I and ester C=O regions, illustrated on squash thylakoid membranes. The differences were calculated from the spectra as shown in Fig. 11.4a by subtracting the spectrum recorded at 5°C from those registered at increasing temperatures. In the analysis of protein stabilities, the intensity of the difference band at around 1,620 cm^{-1} was taken as a measure of the extent of protein denaturing (Szalontai et al., 2003).

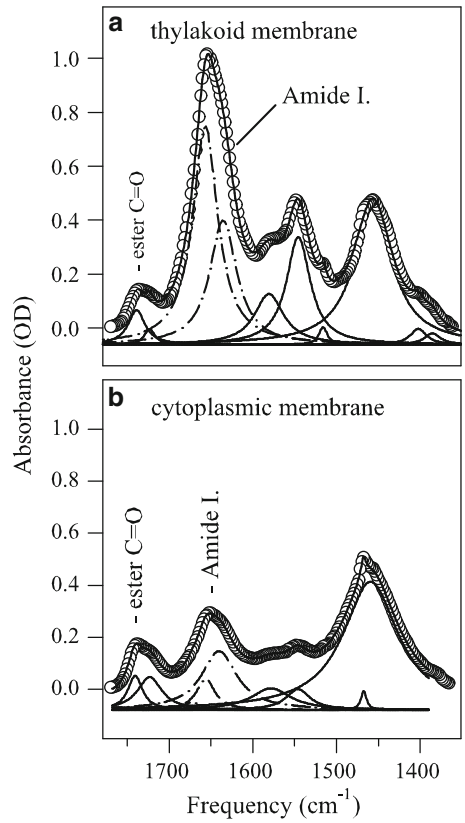

Fig. 11.6. Amide I-II regions of thylakoid (**a**) and cytoplasmic (**b**) membranes prepared from wild-type *Synechocystis* sp. PCC 6803 grown at 35°C. Note the much higher *amide I* to *ester C=O* band intensity ratio for the thylakoid membrane. *Circles* indicate experimental data points (for better visibility, only every second point is displayed). The *continuous curve* through the *circles* is the result of the fit obtained by using the depicted component bands. Component bands are displaced for clarity. *Dashed-dotted lines* represent protein *Amide I* bands, and *thick lines* stand for *ester C=O* vibrations. The ratio of these two types of component bands was used to determine the 'spectroscopic' protein to lipid ratio (Szalontai et al., 2000).

apparent protein-to-lipid ratio in biological membranes (Szalontai et al., 2000). For determining the absolute ratio, a knowledge of the extinction coefficients of the ester C=O bond, of the Amide I bands, and of the underlying amino acid side chains in the Amide I region would be needed, and this is difficult to achieve. For practical reasons, however, the knowledge of the change of the protein-to-lipid ratio from one membrane to another is sufficient. Good examples for largely different protein-to-lipid ratios are the thylakoid and cytoplasmic membranes of *Synechocystis* sp. PCC 6803 (Fig. 11.6).

The much higher protein-to-lipid ratio in the *Synechocystis* sp. PCC 6803 thylakoid membranes affects the structure and dynamics of the lipids as well. In Figs. 11.2 and 11.3, the lipid disorder was lower in the cytoplasmic membranes up to the actual growth temperature than in the thylakoid membranes. According to the 'logic' of the infrared measurements this means that lipids are more disordered in the thylakoid than in the cytoplasmic membranes. Such a conclusion, however, contradicts earlier ESR and fluorescence anisotropy measurements, which indicated a higher probe mobility, and consequently a higher fluidity in cytoplasmic membranes than in thylakoids (Wada et al., 1984). Accordingly, lower phase transition temperatures have been found in cytoplasmic membranes (Ono and Murata, 1981a, b; Wada et al., 1984). It should be remembered, however, that FTIR spectroscopy involves a very different energy range from that of labeling ESR or fluorescence spectroscopy. In the FTIR spectra, elementary movements of segments of all molecules present in the system can be seen, whereas movements of whole labeled molecules are measured with the other two techniques. Accordingly, in consequence of the much higher protein-to-lipid ratios, the rotation of a 'lipid-like' labeled molecule is liable to be more limited in thylakoids (even among more disordered lipids) than

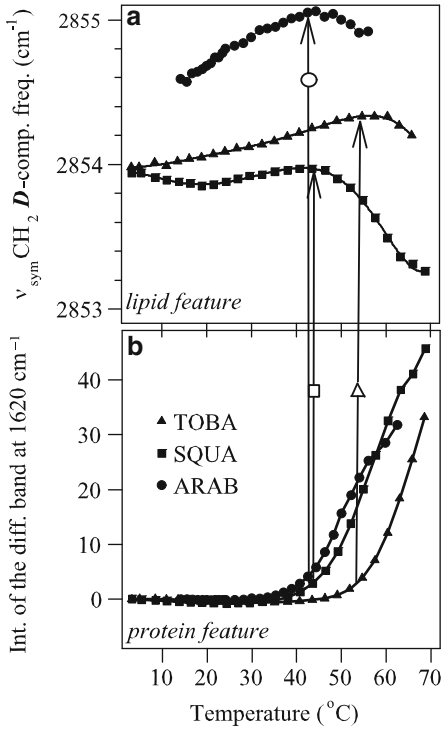

Fig. 11.7. Correlation between lipid and protein dynamics in the thylakoid membranes of tobacco (*TOBA*), squash (*SQUA*) and *Arabidopsis thaliana* (*ARAB*). (**a**) $\nu_{sym}CH_2$ frequencies of the disordered (***D***-comp) population of membrane lipid fatty-acyl chains. Disordered population is the higher frequency component of the two-component analysis of the $\nu_{sym}CH_2$ band discussed above. See also in (Kota et al., 1999; Szalontai et al., 2003). (**b**) Heat-induced denaturing of membrane proteins, as characterized by an increasing intensity of the difference band around 1,620 cm^{-1} (see Fig. 11.5), assigned to intermolecular β-sheets formed upon protein aggregation. *Arrows* connect the corresponding lipid- and protein-related curves (Szalontai et al., 2003).

in the large (and possibly more ordered) lipid matrices of cytoplasmic membranes with low protein content. There is therefore no discrepancy between the infrared spectroscopy-based conclusions and the data obtained by other techniques, when the conditions of the experiments are specified more thoroughly.

It seems that there is a 'structural disorder' in the lipid fatty acyl chains, independently of the actual temperature. This disorder is due to the interaction of the lipids with proteins at the lipid-protein interface. To meet the requirement of the proper membrane physical state and the barrier properties, the lipid fatty acyl chains contain more "gauche" (broken) segments at the interface than in the pure lipid matrix. As the temperature increases, the number of the thermally induced "gauche" segments increases as well. It is noteworthy, however, that the sum of the structural and thermally induced (total) disorder is 'sensed' when *homeoviscous adaptation* takes place (Fig. 11.2).

Since, as discussed above, the structure of the membrane proteins does not exhibit major changes at low temperatures, the lipids of the lipid-protein interface are also expected to be rather insensitive to changes in this temperature range. In contrast, the amount of the thermally induced "gauche" segments, and consequently the structure and the dynamics of the lipid matrices change largely just below the lower limit of the normal physiological temperature range. To prove that if there were changes at the lipid-protein interface they would be visible in the infrared spectra, the high-temperature stress region of the lipid-protein interaction is shown in Fig. 11.7. The frequencies of the disordered component of the $\nu_{sym}CH_2$ band, which should increase continuously with increasing temperatures, exhibit breakdowns (Fig. 11.7a). These breakdowns nicely correlate with the start of heat denaturing of the proteins in the same organism (Fig. 11.7b). An explanation could be that upon protein aggregation, formerly 'structurally disordered' lipids are excluded from the lipid-protein interface and these lipids, now in the lipid matrix, adopt a less disordered conformation, having lower $\nu_{sym}CH_2$ frequencies (Szalontai et al., 2003).

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References

Alfonso M, Collados R, Yruela I and Picorel R (2004) Photoinhibition and recovery in a herbicide-resistant mutant from *Glycine max* (L.) Merr. cell cultures deficient in fatty acid unsaturation. Planta 219: 428–439

Ariizumi T, Kishitani S, Inatsugi R, Nishida I, Murata N and Toriyama K (2002) An increase in unsaturation of fatty acids in phosphatidylglycerol from leaves improves the rates of photosynthesis and growth at low temperatures in transgenic rice seedlings. Plant Cell Physiol 43: 751–758

Arrondo JL, Muga A, Castresana J and Goni FM (1993) Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. Prog Biophys Mol Biol 59: 23–56

Baker NR (1991) A possible role for photosystem-II in environmental perturbations of photosynthesis. Physiol Plant 81: 563–570

Casal HL and Mantsch HH (1984) Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy. Biochim Biophys Acta 779: 381–401

Cullis PR and De Kruijff B (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim Biophys Acta 559: 399–420

Domonkos I, Malec P, Sallai A, Kovacs L, Itoh K, Shen GZ, Ughy B, Bogos B, Sakurai I, Kis M, Strzalka K, Wada H, Itoh S, Farkas T and Gombos Z (2004) Phosphatidylglycerol is essential for oligomerization of photosystem I reaction center. Plant Physiol 134: 1471–1478

Domonkos I, Laczko-Dobos H and Gombos Z (2008) Lipid-assisted protein-protein interactions that support photosynthetic and other cellular activities. Prog Lipid Res 47: 422–435

Epand RM (1998) Lipid polymorphism and protein-lipid interactions. Biochim Biophysi Acta 1376: 353–368

Frentzen M, Nishida I and Murata N (1987) Properties of the plastidial acyl-(acyl-carrier-protein) – glycerol-3-phosphate acyltransferase from the chilling-sensitive plant squash (cucurbita-moschata). Plant Cell Physiol 28: 1195–1201

Fromme P (1996) Structure and function of photosystem I. Curr Opin Struc Bio l6: 473–484

Fromme P, Jordan P and Krauss N (2001) Structure of photosystem I. Biochim Biophys Acta 1507: 5–31

Gombos Z, Wada H and Murata N (1992) Unsaturation of fatty acids in membrane lipids enhances tolerance of the cyanobacterium *Synechocystis* PCC 6803 to low-temperature photoinhibition. Proc Natl Acad Sci USA 89: 9959–9963

Gombos Z, Wada H, Hideg E and Murata N (1994a) The unsaturation of membrane lipids stabilizes photosynthesis against heat stress. Plant Physiol 104: 563–567

Gombos Z, Wada H and Murata N (1994b) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. Proc Natl Acad Sci USA 91: 8787–8791

Gombos Z, Kanervo E, Tsvetkova N, Sakamoto T, Aro EM and Murata N (1997) Genetic enhancement of the ability to tolerate photoinhibition by introduction of unsaturated bonds into membrane glycerolipids. Plant Physiol 115: 551–559

Gombos Z, Varkonyi Z, Hagio M, Iwaki M, Kovacs L, Masamoto K, Itoh S and Wada H (2002) Phosphatidylglycerol requirement for the function of electron acceptor plastoquinone Q_B in the photosystem II reaction center. Biochemistry 41: 3796–3802

Goormaghtigh E, Cabiaux V and Ruysschaert JM (1994a) Determination of soluble and membrane protein structure by fourier transform infrared spectroscopy. I. Assignments and model compounds. Subcell Biochem 23: 329–362

Goormaghtigh E, Cabiaux V and Ruysschaert JM (1994b) Determination of soluble and membrane protein structure by fourier transform infrared spectroscopy. II. Experimental aspects, side chain structure, and H/D exchange. Subcell Biochem 23: 363–403

Goormaghtigh E, Cabiaux V and Ruysschaert JM (1994c) Determination of soluble and membrane protein structure by fourier transform infrared spectroscopy. III. Secondary structures. Subcell Biochem 23: 405–450

Greer DH, Berry JA and Björkman O (1986) Photoinhibition of photosynthesis in intact bean-leaves - role of light and temperature, and requirement for chloroplast-protein synthesis during recovery. Planta 168: 253–260

Grennan AK and Ort DR (2007) Cool temperatures interfere with D1 synthesis in tomato by causing ribosomal pausing. Photosynth Res 94: 375–385

Gruszecki WI and Strzalka K (2005) Carotenoids as modulators of lipid membrane physical properties. Biochim Biophys Acta 1740: 108–115

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W and Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 angstrom resolution. Nature 411: 909–917

Kern J and Guskov A (2011) Lipids in photosystem II: Multifunctional cofactors. J Photochem Photobiol B: Biol 104: 19–34

Kis M, Zsiros O, Farkas T, Wada H, Nagy F and Gombos Z (1998) Light-induced expression of fatty acid desaturase genes. Proc Natl Acad Sci USA 95: 4209–4214

Kota Z, Debreczeny M and Szalontai B (1999) Separable contributions of ordered and disordered lipid fatty acyl chain segments to nu ch2 bands in model and biological membranes: a fourier transform infrared spectroscopic study. Biospectroscopy 5: 169–178

Loll B, Kern J, Saenger W, Zouni A and Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 angstrom resolution structure of photosystem II. Nature 438: 1040–1044

Lyons JM (1973) Chilling injury in plants. Ann Rev Plant Physiol 24: 445–466

Mantsch HH and McElhaney RN (1991) Phospholipid phase transitions in model and biological membranes as studied by infrared spectroscopy. Chem Phys Lipids 57: 213–226

Masamoto K, Zsiros O and Gombos Z (1999) Accumulation of zeaxanthin in cytoplasmic membranes of the cyanobacterium *Synechococcus* sp. strain PCC7942 grown under high light condition. J Plant Physiol 155: 136–138

Masamoto K, Hisatomi S, Sakurai I, Gombos Z and Wada H (2004) Requirement of carotene isomerization for the assembly of photosystem II in *Synechocystis* sp. PCC 6803. Plant Cell Physiol 45: 1325–1329

Mizusawa N and Wada H (2011) The role of lipids in photosystem II. Biochim Biophys Acta doi:10.1016/j.bbabio.2011.04.008

Moon BY, Higashi S, Gombos Z and Murata N (1995) Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants. Proc Natl Acad Sci USA 92: 6219–6223

Murata N (1983) Molecular-species composition of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. Plant Cell Physiol 24: 81–86

Murata N (1989) Low-temperature effects on cyanobacterial membranes. J Bioenerg Biomembr 21: 61–75

Murata N and Nishida I (1990) Lipids in relation to chilling sensitivity of plants. In: Wang CY (ed) Chilling Injury of Horticultural Crops, pp 181–199. CRC Press, Boca Raton, FL

Murata N and Wada H (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. Biochem J 308: 1–8

Murata N and Yamaya J (1984) Temperature-dependent phase-behavior of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. Plant Physiol 74: 1016–1024

Murata N, Wada H and Hirasawa R (1984) Reversible and irreversible inactivation of photosynthesis in relation to the lipid phases of membranes in the blue-green-algae (cyanobacteria) *Anacystis nidulans* and *Anabaena variabilis*. Plant Cell Physiol 25: 1027–1032

Murata N, Ishizaki-Nishizawa O, Higashi S, Hayashi H, Tasaka Y and Nishida I (1992a) Genetically engineered alteration in the chilling sensitivity of plants. Nature 356: 710–713

Murata N, Wada H and Gombos Z (1992b) Modes of fatty-acid desaturation in cyanobacteria. Plant Cell Physiol 33: 933–941

Ono TA and Murata N (1981a) Chilling susceptibility of the blue-green-alga *Anacystis nidulans*. 1. Effect of growth temperature. Plant Physiol 67: 176–181

Ono TA and Murata N (1981b) Chilling susceptibility of the blue-green-alga *Anacystis nidulans*. 2. Stimulation of the passive permeability of cytoplasmic membrane at chilling temperatures. Plant Physiol 67: 182–187

Powles SB (1984) Photoinhibition of photosynthesis induced by visible-light. Ann Rev Plant Physiol Plant Mol Biol 35: 15–44

Powles SB, Berry JA and Björkman O (1983) Interaction between light and chilling temperature on the inhibition of photosynthesis in chilling-sensitive plants. Plant Cell Environ 6: 117–123

Raison JK (1973) The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane associated enzyme systems. Bioenergetics 4: 285–309

Roughan PG (1985) Phosphatidylglycerol and chilling sensitivity in plants. Plant Physiol 77: 740–746

Sakurai I, Hagio M, Gombos Z, Tyystjarvi T, Paakkarinen V, Aro EM and Wada H (2003) Requirement of phosphatidylglycerol for maintenance of photosynthetic machinery. Plant Physiol 133: 1376–1384

Sakurai I, Mizusawa N, Ohashi S, Kobayashi M and Wada H (2007) Effects of the lack of phosphatidylglycerol on the donor side of photosystem II. Plant Physiol 144: 1336–1346

Sato N, Murata N, Miura Y and Ueta N (1979) Effect of growth temperature on lipid and fatty acid compositions in the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. Biochim Biophys Acta 572: 19–28

Siegel DP and Epand RM (1997) The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms. Biophys J 73: 3089–3111

Sinensky M (1974) Homeoviscous adaptation: a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci USA 71: 522–525

Szalontai B (2009) Membrane protein dynamics: Limited lipid control. PMC Biophys 2: 1

Szalontai B, Nishiyama Y, Gombos Z and Murata N (2000) Membrane dynamics as seen by fourier transform infrared spectroscopy in a cyanobacterium, *Synechocystis* PCC 6803 - the effects of lipid unsaturation and the protein-to-lipid ratio. Biochim Biophys Acta 1509: 409–419

Szalontai B, Kota Z, Nonaka H and Murata N (2003) Structural consequences of genetically engineered saturation of the fatty acids of phosphatidylglycerol in tobacco thylakoid membranes. An FTIR study. Biochemistry 42: 4292–4299

Tasaka Y, Gombos Z, Nishiyama Y, Mohanty P, Ohba T, Ohki K and Murata N (1996) Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*: Evidence for the important roles of polyunsaturated membrane lipids in growth, respiration and photosynthesis. EMBO J 15: 6416–6425

Van den Brink-Van der Laan E, Killian JA and de Kruijff B (2004) Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. Biochim Biophys Acta 1666: 275–288

Van Stokkum IH, Linsdell H, Hadden JM, Haris PI, Chapman D and Bloemendal M (1995) Temperature-induced changes in protein structures studied by Fourier transform infrared spectroscopy and global analysis. Biochemistry 34: 10508–10518

Varkonyi Z, Masamoto K, Debreczeny M, Zsiros O, Ughy B, Gombos Z, Domonkos I, Farkas T, Wada H and Szalontai B (2002) Low-temperature-induced accumulation of xanthophylls and its structural consequences

in the photosynthetic membranes of the cyanobacterium *Cylindrospermopsis raciborskii*: an FTIR spectroscopic study. Proc Natl Acad Sci USA 99: 2410–2415

Wada H and Murata N (1989) *Synechocystis* PCC 6803 mutants defective in desaturation of fatty acids. Plant Cell Physiol 30: 971–978

Wada H, Hirasawa R, Omata T and Murata N (1984) The lipid phase of thylakoid and cytoplasmic membranes from the blue-green-algae (cyanobacteria), *Anacystis nidulans* and *Anabaena variabilis*. Plant Cell Physiol 25: 907–911

Wada H, Gombos Z and Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature 347: 200–203

Wada H, Gombos Z and Murata N (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. Proc Natl Acad Sci USA 91: 4273–4277

Wolter FP, Schmidt R and Heinz E (1992) Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane-lipids. EMBO J 11: 4685–4692

Wu JR and Browse J (1995) Elevated levels of high-melting-point phosphatidylglycerols do not induce chilling sensitivity in an *Arabidopsis* mutant. Plant Cell 7: 17–27

Zsiros O, Kis M, Mustardy L, Farkas T, Varkonyi Z, Gombos Z and Szalontai B (2002) Light-driven structural changes in thylakoid and cytoplasmic membranes of a cyanobacterium, *Synechocystis* PCC 6803. J Plant Physiol 159: 403–414

Chapter 12

Heat Stress: Susceptibility, Recovery and Regulation

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Summary

The primary targets of thermal damage in plants are the oxygen-evolving complex and its associated cofactors in photosystem II (PS II), carbon fixation by Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) and the ATP generating system. The enzyme Rubisco activase is extremely sensitive to weak and moderate heat stress. Recent investigations on the combined action of moderate light intensity and heat stress suggest that moderately high temperatures do not cause serious PS II damage but inhibit the repair of PS II. The latter largely involves de novo synthesis of proteins, particularly the D1 protein of the photosynthetic machinery that is damaged due to generation of reactive oxygen species (ROS), resulting in reduced rates of carbon fixation and oxygen evolution, as well as disruption of linear electron flow. The attack of ROS during moderate heat stress principally affects the repair system of PS II, but not directly the PS II reaction center. Heat stress additionally induces cleavage and aggregation of reaction center proteins and pigment-protein complexes, the mechanisms of such processes are as yet unclear. On the other hand, membrane-linked sensors seem to trigger the accumulation of compatible solutes like glycinebetaine in the neighborhood of PS II in thylakoid membranes. They also induce the expression of stress proteins that alleviate the ROS-mediated inhibition of stress damage repair of the photosynthetic machinery and are required for the acclimation process. In this chapter we summarize recent progress in the studies of molecular mechanisms involved during moderate heat stress on the photosynthetic machinery, especially in PS II and the CO_2 assimilation system. We also examine joint effects of high temperature and other stress factors.

I. Introduction

Heat stress due to high ambient temperatures constitutes a serious threat to crop production worldwide (Hall, 2001). Besides, improved knowledge of the photosynthetic responses to high temperatures is essential for the understanding of many phenomena associated with global warming. According to a report of the Intergovernmental Panel on Climatic Change (IPCC), the global mean temperature will rise 0.3°C per decade (Jones et al., 1999) reaching approximately 1°C and 3°C above the present value by years 2025 and 2100, respectively. The origin of the warming is under debate. Gaseous emissions due to human activities, such as CO_2, methane, chlorofluorocarbons, and nitrous oxides, are likely to contribute to the increased temperature. Instrumental observations and reconstructions of global and hemispheric temperature evolution reveal a pronounced warming during the past approximately 150 years. One expression of this warming is the observed increase in the occurrence of heat waves (Schär et al., 2004). Conceptually this increase is understood

Abbreviations: APX – Ascorbate peroxidase; Chl – Chlorophyll; D1/D2/Cyt b_{559} – Isolated Photosystem II complex retaining the D1 D2 and cytochrome b_{559} proteins; DF – Delayed fluorescence; F – (chlorophyll) Fluorescence; F_o – Basal (initial or minimal) (chlorophyll) fluorescence; F_i – (chlorophyll) Fluorescence intensity at 30 ms; F_v – Variable (chlorophyll) fluorescence; F_m – Maximum (chlorophyll) fluorescence; F_p – Maximum measured (chlorophyll) fluorescence intensity; Fp=Fm only when all PS II reaction centers are closed; HSPs – Heat-shock proteins; HSFs – Heat-shock (transcription) factors; LHCII – Light-harvesting complex II; OEC – Oxygen-evolving complex; PS II – Photosystem II; PS I – Photosystem I; QTL – Quantitative trait loci; ROS – Reactive oxygen species; SOD – Superoxide dismutase

as a shift of the statistical distribution towards warmer temperatures, while changes in the width of the distribution are often considered small.

Heat stress is one of the main abiotic stresses that limit plant biomass production, especially in tropical and subtropical countries (Boyer, 1982). The photosynthetic activity of chloroplasts is considered among the most heat sensitive cell functions (Berry and Björkman, 1980; Yordanov et al., 1986). There are, at least, three major stress-sensitive sites in the photosynthetic machinery: the photosystems, mainly photosystem II (PS II) with its oxygen-evolving complex (OEC), photophosphorylation and carbon assimilation (Aro et al., 1993; Bukhov and Mohanty, 1999; Carpentier, 1999; Bukhov and Carpentier, 2000; Nishiyama et al., 2005, 2006; Murata et al., 2007; Mohanty et al., 2007; Allakhverdiev et al., 2008). PS II is the critical site of damage by a variety of stress factors such as drought, salinity, low and high temperatures, high light, and UV radiation (Berry and Björkman, 1980; Al-Khatib and Paulsen, 1989; Allakhverdiev and Murata, 2004, 2008). In vivo, the extent of damage under any type of stress depends on the balance between damage and repair processes during the stress. The above is particularly true for PS II and provides the basis for acclimation and photosynthetic recovery processes (Adir et al., 2003; Mohanty et al., 2007; Murata et al., 2007; Allakhverdiev and Murata, 2004, 2008; Allakhverdiev et al., 2008).

There are two principal modes of stress-induced impairment of photosynthesis: first, a direct damage induced by the stress factor and second, inhibition of de novo protein synthesis by reactive oxygen species (ROS). Further, stresses like CO_2 limitation, drought, cold, or salinity may be referred to as a category of oxidative stresses that inhibit the repair of PS II and/or PS I (Nishiyama et al., 2001; Allakhverdiev and Murata, 2004, 2008; Nishiyama et al., 2005, 2006; Mohanty et al., 2007; Murata et al., 2007). A number of studies demonstrated that ROS-scavenging mechanisms have an important role in protecting plants against high temperature stress or the combination of high light and temperature stresses (Larkindale and Knight, 2002; Suzuki and Mittler, 2006; Allakhverdiev et al., 2008).

There are multiple effects of elevated temperature on the photosynthetic machinery ranging from a reduction in specific activities to a deeper damage of all parts of the photosynthetic machinery. However, heat sensitivity and tolerance of the various functions are different. At the same time, mechanisms such as structural rearrangements of the chloroplast thylakoid membranes or enhanced heat dissipation are involved in the protection of the photosynthetic machinery from damage. The thylakoid membrane integrity, the redox-potential of components of the electron transport chain of the chloroplast and the relationship between antioxidants and oxidants are important factors for resistance and tolerance of the photosynthetic machinery to heat stress.

High temperature tolerance has been genetically engineered in plants mainly by over-expressing heat-shock protein (HSP) genes or indirectly by altering levels of heat-shock transcription factor proteins (Singh and Grover, 2008). Apart from HSPs, thermotolerance has also been altered by elevating the levels of osmolytes, increasing levels of cell detoxification enzymes and through altering membrane fluidity (Miller and Mittler, 2006; Charng et al., 2007; Singh and Grover, 2008). HSPs may be directly implicated in thermotolerance as agents that minimize damage to cell proteins. The approaches leading to thermotolerance in transgenic plants operate in their own specific ways but indirectly might help in the creation of a more reductive and energy-rich cellular environment, thereby minimizing the accumulation of damaged proteins. Hence, intervention in protein metabolism to minimize the accumulation of damaged proteins becomes a major approach for the creation of genetically engineered crops exhibiting resistance to high temperature stress (Singh and Grover, 2008).

In this chapter, we focus on moderate high temperature stress effects in vivo. Here, we have attempted to dissect and separate the mechanisms of stress injuries and impairments, the repair processes and the factors that affect the damage-repair cycle.

II. High Temperature Stress

Threats of global warming and climate changes make heat stress a general concern for all of us, as heat and drought are the two major stresses for tropical agriculture (Hall, 2001). There are several target sites for elevated temperature-induced damage such as the CO_2 fixation system, photophosphorylation, the electron transport chain and the

OEC (Nash et al., 1985; Feller et al., 1998; Bukhov and Mohanty, 1999; Carpentier, 1999; Sharkey, 2005). Thylakoid membrane fluidity might act as a sensor for temperature-induced functional changes as well (Horvath et al., 1998; Los and Murata, 2004). In general, a transient elevation in temperature, usually 10–15°C above ambient, is considered as heat shock or heat stress (Wahid et al., 2007). The severity of heat-induced damage depends not only on the tested systems and the potential for temperature tolerance but also on the temperature gradient and mode of heat application (Bukhov and Mohanty, 1999). Under in vivo conditions, the heat-stress-linked alterations depend on the stage of growth of the photosynthetic tissues. Indeed, the young, developing and the old, senescing tissues exhibit different sensitivities (Kalitulo et al., 2003). Thus, it is important to take into account the sequence of heat injury, plant age, severity of damage and the activity of the post-stress repair processes.

Various parameters of the fast (up to 1 s) chlorophyll (Chl) fluorescence (F) transients such as the ratio of variable fluorescence ($F_v = F_m - F_o$) to maximum fluorescence (F_m), (F_v/F_m), the basal (initial, or minimal) fluorescence (F_o) and fast and slow maxima of Delayed Light (DL; also called Delayed Fluorescence, DF, or Delayed Light Emission, DLE) of Chl are physiological features that have been shown to correlate with heat tolerance. The most convenient test for monitoring heat-induced changes remains the rise in minimum Chl *a* fluorescence or F_o level, as it the critical temperature for PS II inactivation (Havaux, 1993; Yamada et al., 1996; Yamane et al., 2000). Yamane et al. (2000) found that the main causes of F_o increase differ from species to species. In some species (e.g., potato and tobacco) the main cause of high-temperature induced F_o increase is not the inactivation of PS II photochemical reactions but the dark reduction of plastoquinone by stromal donors (see Bukhov et al., 1998; Sazanov et al., 1998; Bukhov and Carpentier, 2000). [For a discussion on Chl fluorescence, see various chapters in Papageorgiou and Govindjee (eds) (2004); for a review on Delayed Light Emission, see Goltsev et al., 2009.]

DF constitutes a useful approach for the assessment of PS II photochemistry and a useful tool to investigate the processes of cell stress-resistance and acclimation. DF can be observed as a long-lived low intensity light emission from photosynthetic organisms after being illuminated and then placed in darkness (Strehler and Arnold, 1951; Lavorel, 1975). DF mainly originates from back reactions in the reaction center of PS II after charge separation. Electrons from the acceptor side of PS II flow back to $P680^+$ resulting in the formation of the excited singlet state of P680, P680*; excitation energy in P680* is then transferred to Chls of the antenna from where light is emitted (Jursinic, 1986). DF intensity represents the integral under the decay curve and is proportional to the number of active PS II centers, the fluorescence yield, and the rate of back reaction (Joliot and Joliot, 1980). The influence of environmental stresses on the overall process of photosynthesis has been measured through DF (see e.g., Yordanov et al., 1987). In addition, induction curves of DF are assumed to be good indicators for the electron transport capacity and the proton gradient across thylakoid membranes, as well as both the energization and the integrity of thylakoid membranes under heat stress (Baker and Rosenqvist, 2004).

On the other hand, the polyphasic Chl *a* fluorescence transient OJIP is also a useful approach; it has been reviewed in a number of publications (see Lazar, 1999, 2006; Govindjee, 2004; Papageorgiou et al., 2007; Stirbet and Govindjee, 2011). These traces illustrate the progressive reduction of the quinones located on the acceptor side of PS II with three main phases; O-J, J-I, and I-P (Strasser et al., 2004). Note that the shape of the OJIP transient is very sensitive to environmental stresses, in particular to high temperature. A positive band appears at around 0.3 ms, which is normally not recognized in the OJIP, but appears as a band in the stressed situation, when subtracted from the normalized transients, i.e., the stressed minus the control samples. This band was defined as the K band and it was first noted from a study with high temperature stress but is also induced by other forms of oxidative stress (Guisse et al., 1995; Srivastava et al., 1997; Strasser, 1997; Wang et al., 2007). A positive K band is indicative of partial inactivation of the OEC (Strasser et al., 2004), and in the case of heat stress this retardation of the O-J rise could be linked to the formation of $P680^+$ during the heat-induced inhibition of oxygen evolution, thus causing fluorescence quenching (Strasser, 1997; Srivastava et al., 1997; Vani et al., 1999). The appearance of the K step paralleled

the appearance of the thermoluminescence-A_T band induced by a short 50°C heat pulse suggesting damage to the charge accumulation at the OEC and this damage was repaired when heat-treated plants were taken back to the growth light condition but was not repaired if stressed plants were kept in the dark. During recovery, again the K band and thermoluminescence A_T band disappeared in a parallel fashion indicating that both the K band of transient fluorescence and the thermoluminescence A_T band could be used as in vivo markers for impairment of the OEC (Toth et al., 2005) (see Chapters 18 and 19).

A. Heat Stress: Early Events

Both low and high temperature stresses cause multi-step injuries to the photosynthetic machinery, although the early steps of impairment seem to be different. The heat-induced changes in the structure of Chl-protein complexes and the inactivation of enzyme activity constitute the early effects of thermal stress. The enzymes of the Calvin-Benson cycle are heat labile. Thus, the carbon assimilation system is sensitive to elevated temperature and gets strongly inhibited at moderate thermal stress (Berry and Björkman, 1980; Weis, 1981, Feller et al., 1998; Sharkey, 2005). The decline in Rubisco activity by moderate thermal stress is correlated with the loss in photosynthesis (Law and Crafts-Brandner, 1999). The Rubisco enzyme of higher plants is heat stable but the loss in activity at elevated temperature is due to Rubisco activase, which is extremely sensitive to elevated temperature (Salvucci and Crafts-Brandner, 2004; Sharkey, 2005). The permeability of the membrane (Toth et al., 2005) and macroscopic structure of the chloroplast gets altered by heat exposure (Vani et al., 2001; Semenova, 2004; Kreslavski et al., 2008); and hence, the integrity of the thylakoid membranes is compromised. A decreased membrane stacking and a general reorganization of the thylakoid membranes are observed with temperatures in the interval of 35–45°C (Gounaris et al., 1983, 1984). These structural transformations are accompanied by ion leakage from heat-stressed leaves (Inaba and Crandall, 1988; Wahid and Shabbir, 2005) and changes in energy distribution between the two photosystems due to high temperature stress in vivo (Sane et al., 1984; Joshi et al., 1995; Mohanty et al., 2002). At moderate heat stress, both photochemical and non-photochemical quenching are altered, but differentially in light state 1 and light state 2. However, at extreme high temperatures exceeding 42°C, the state transition is arrested even though some PS II reaction centers remain active, as revealed from thermoluminescence measurements (Joshi et al., 1995). State transitions are discussed in Chapter 18 (see a historical perspective by Papageorgiou and Govindjee, 2011).

B. The Fragility of Photosystem II (PS II)

Photosystem II is relatively more sensitive to heat stress than PS I and this has been noted from numerous in vitro and in vivo studies. The OEC is fragile and gets impaired even at moderate heat stress (Nash et al., 1985). Cao and Govindjee (1990) analyzed the effect of a 1 min high temperature (55°C) treatment on spinach thylakoids; this caused a faster OI rise in the Chl *a* fluorescence transient, and this heat-induced OI rise was differentially sensitive to different quinone derivatives, such as DCBQ (dichlorobenzoquinone) and DMBQ (dimethylbenzoquinone). The OI rise has been mostly linked to photochemical reduction of Q_A to Q_A^- and the different sensitivity of quinone derivatives was taken to be indicative of conversion of active PS II to inactive PS II centers by heat treatment (Cao and Govindjee, 1990). However, these conclusions have been challenged by Lavergne and Leci (1993) and by Srivastava et al. (1995). In addition, the Q_A^- dark decay components were altered by heat stress and the heat treatment caused slower than normal back recombination reactions at the PS II reaction center reflecting a slowing down of the back reaction between Q_A^- and the S_2^+-state of the OEC by the short temperature treatment (Cao and Govindjee, 1990). It appears that heat stress in vivo enhances heterogeneity of PS II due to conversion of active Q_B-reducing to inactive Q_B-non-reducing PS II reaction centers (Vani et al., 1999; Strasser et al., 2004). Thus the thermal stress may be considered to lead to an increase in the relative fraction of Q_B-non-reducing centers (Havaux et al., 1991; Kreslavski and Khristin, 2003) as well as, loss of thylakoid membrane integrity, especially destacking of the thylakoid membranes (Gounaris et al., 1984; Semenova, 2004) and dysfunction of CO_2 assimilation (Sharkey, 2005). However, among

the various components of the photosynthetic machinery, the OEC of PS II is most sensitive to heat as revealed by in vivo and in vitro studies (Katoh and San Pietro, 1967; Berry and Björkman, 1980; Carpentier, 1999; Mamedov et al., 1993; Allakhverdiev et al., 1994, 1996; Havaux and Tardy, 1996). This appears to be due to a heat-induced loss of two out of four Mn from the Mn-containing cluster of the OEC (Nash et al., 1985; Allakhverdiev et al., 1994; Enami et al., 1994, 1998). However, it has been demonstrated that PS II activity is not inhibited at the minimally elevated temperatures where CO_2 assimilation is already reduced due to a decreased activity of Rubisco activase (Salvucci and Crafts-Brandner, 2004; Tang et al., 2007).

The regulatory mechanisms of energy distribution between the two photosystems are likely to get modified by elevated temperature and this modification may constitute one of the protective mechanisms against heat stress (Joshi et al., 1995; Mohanty et al., 2002; also see Sane et al., 1984). The absorbed energy distribution or partitioning between two interactive photosystems is dynamically balanced and regulated by the process known as the "state transitions" (Bonaventura and Myers, 1969; Murata, 1969; also see recent reviews: Allen and Mullineaux, 2004; Dietzel et al., 2008; Murata, 2009; Tsimilli-Michael et al., 2009; Papageorgiou and Govindjee, 2011; and see Chapter 18). The state transitions are affected by both low and moderately high temperature. The Chl *a* fluorescence long-term slow transient, beyond the OJIP phase, has contributions of non-photochemical quenching including those arising from the state changes, and these are affected by heat as seen in early work with intact cells of *Chlorella* and *Porphyridium* (see reviews in Papageorgiou and Govindjee, 2004). Also early observations of the phycobilisome-sensitized Chl *a* fluorescence rise in DCMU-poisoned *Anacystis nidulans* can now be interpreted in terms of state 2 to state 1 fluorescence yield changes (Papageorgiou and Govindjee, 1967; see Chapter 18). Further, the variable extent of Chl *a* fluorescence quenching by PS I far-red light during the progress of fluorescence induction in *Chlorella pyrenoidosa* reflected state-shift-type transitions (Mohanty et al., 1970). The mechanisms of state transition in plants and in phycobilisome-containing oxygenic photosynthetic organisms are different (Allen and Mullineaux, 2004; Tismilli-Michael et al., 2009) and thus the temperature dependence of the state transitions in these organisms have been shown to be different (Joshi et al., 1995; Vani et al., 1999; Li et al., 2007).

Energy transfer from light harvesting complex II (LHCII) to the core antenna complex of PS II is affected at elevated temperatures. Even at 35°C, the migration of LHCII from the intergranal space towards PS I gets arrested, thus affecting the balance of light absorption between the two photosystems (Pastenes and Horton, 1996). At temperatures above 40–42°C, the loss of photosynthetic activity is partly caused by the inactivation of the acceptor side of PS II and reduction of the rate of electron transport in the chloroplasts.

C. Heat Stress: Oxidant and Antioxidant Balance

Reactive oxygen species (ROS) play a key signaling role in plants and are controlled in cells by a complex network of ROS-metabolizing enzymes found in several different cellular compartments. ROS, especially H_2O_2, can also be generated during moderate heat stress. The generation of ROS was observed both in PS I and PS II (Asada, 1999, 2006; Mubarakshina et al., 2006) as well as in the Calvin-Benson cycle (Kim and Portis, 2004). The pathways of ROS generation are shown in the scheme in Fig. 12.1. In vitro experiments from several laboratories suggested that thermal stress at high enough temperatures produces ROS such as superoxide radicals, hydroxyl radicals and hydrogen peroxide at the PS II reaction center, which are scavenged by antioxidants, including superoxide dismutase (SOD) (Bukhov and Mohanty, 1999). The presence of antioxidant enzymes can protect the organisms by limiting the concentration of singlet oxygen, a very toxic ROS that is also produced under strong light (Krieger-Liszkay, 2005). Like singlet oxygen, hydroxyl radicals are highly toxic for plant cells and their conversion to H_2O_2 reduces their damaging effect (Asada, 2006). Formation of ROS and their scavenging by antioxidants also occurs in vivo (El-Shitinawy et al., 2004; Kreslavski et al., 2007). Major ROS-scavenging mechanisms include the action of the enzymes SOD, catalase and ascorbate peroxidase (APX) (Allen, 1995; Mittler, 2002; Asada, 2006). Peroxides, especially APX, are one of the basic elements participating

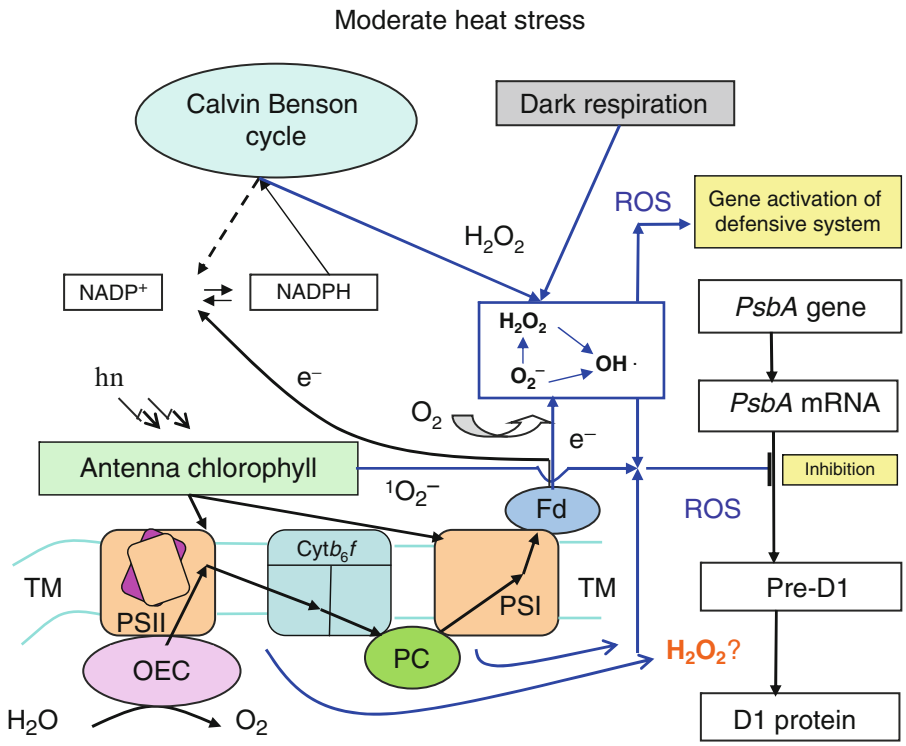

Fig. 12.1. The scheme for ROS production in the photosystems located in the thylakoid membrane and the relationship between ROS formation and the inhibition of the synthesis of the D1 protein. Reduction of O_2 on the acceptor side of PS I during the process of electron transport leads to the formation of O_2^- that can be converted to H_2O_2 by SOD or catalase and finally into OH and other ROS. One of the sources of ROS formation is dark respiration. ROS production is enhanced by strong light and by limitations of the Calvin-Benson cycle, which also results in the production of O_2^- and other ROS, and inhibits the repair of photodamaged PS II via inhibiting the synthesis of pre-D1 at the translation stage (Allakhverdiev et al., 2002, 2004, 2005a, b, 2007, 2008; Nishiyama et al., 2001, 2004, 2005, 2006; Mohanty et al., 2007; Murata et al., 2007; Takahashi and Murata, 2008). Pre-D1 is the precursor of the D1 protein. TM thylakoid membrane, Fd ferredoxin, Pc plastocyanin. ROS are shown in blue.

in the photosynthetic response to heat stress and acclimation of the photosynthetic machinery (Foyer et al., 1997; Dat et al., 1998). To understand how different ROS signals formed in the chloroplast and the cytosol are generated and integrated in cells, Miller et al. (2007) generated a double mutant lacking thylakoid ascorbate peroxidase (*tylapx*) and cytosolic ascorbate peroxidase 1 (*apx*1) in *Arabidopsis thaliana*. The study suggested that two different signals are generated in plants lacking cytosolic APX1 or tylAPX. The lack of a chloroplastic hydrogen peroxide removal enzyme, tylAPX, triggered a specific signal in cells that results in enhanced tolerance to heat stress, whereas the lack of a cytosolic hydrogen peroxide removal enzyme, APX1, triggered a different signal, which resulted in stunted growth and enhanced sensitivity to oxidative stress. Further investigation of the enhanced heat tolerance in plants lacking tylAPX, using mutants deficient in chloroplast-to-nuclei retrograde signaling (see Chapter 8), suggests the existence of a chloroplast-generated stress signal that enhances basal thermotolerance in plants. The activity of cytosolic antioxidant enzymes seems to be important for the protection of the photosynthetic machinery from heat-induced photoinhibition. For instance, the study of Davletova et al. (2005) demonstrated that in the absence of the cytosolic H_2O_2-scavenging enzyme ascorbate peroxidase 1 (APX1), the entire chloroplastic H_2O_2-scavenging system of *Arabidopsis thaliana* collapses, H_2O_2 levels increase, and protein oxidation occurs.

The extent of damage caused to the photosynthetic machinery of wheat seedlings by short-term exposure of wheat seedlings to mild heat

(40–42°C), their capacity to recover from it, and the possible roles of H_2O_2, SOD, catalase and APX on the recovery process, have been investigated by Kreslavski et al. (2008). This allowed the understanding of the link between H_2O_2 production and antioxidant enzyme activities. The damage to the photosynthetic machinery and its recovery depended on temperature and light intensity during seedling recovery**.** High temperature led to an imbalance in which the redox steady state of the cell is altered in the direction of pro-oxidants (H_2O_2) and increased activities of antioxidant enzymes**.** However, the balance was restored during the following light-stimulated recovery. Hence the balance between (pro)oxidant and antioxidant levels during heat stress in the dark and heat-induced photoinhibition may be crucial for the recovery from heat-induced damage.

D. Post-Heat Stress Recovery

The damage to the photosynthetic apparatus due to elevated temperatures in darkness has been studied in higher plants, green algae as well as in cyanobacteria. In each case the thermal inactivation sites were found to be numerous, but it is difficult to describe each step of the damage, as the deterioration can be sequential or simultaneous. However, it is possible to weigh the relative sensitivity of the target sites. Such studies involved monitoring the time and the extent of repair after the thermal stress was removed (Mohanty et al., 1987, 2007). Studies made on wheat plants on the post-dark heat-stress recovery exhibited the complexity of the recovery process. In darkness, the recovery of carbon assimilation and PS II performance depended on mitochondrial phosphorylation, while in the light the recovery was influenced by temperature and light intensity (Kreslavski et al., 2007, 2008). Moreover, moderate heat stress can stimulate dark reduction of plastoquinone and PS I cyclic electron flow in the light (Bukhov et al., 1998; Sharkey, 2005). An assessment of cyclic and non-cyclic photophosphorylation in suspensions of isolated thylakoids from leaves of wheat seedlings damaged by heat stress and recovered in light demonstrated that cyclic electron flow was enhanced in thylakoids from recovered plants (Kreslavski et al., 2008). These observations are in line with suggestions that the cyclic electron flow could provide additional ATP required for protein synthesis and protect PS II from photoinhibition (Havaux et al., 1991; Miyake and Okamura, 2003; Allakhverdiev et al., 2005a; Govindachary et al., 2007). In spite of these complexities, it is clear that post-temperature-stress recovery needs the protein biosynthetic machinery to repair the thermal or the light damage of PS II (Allakhverdiev et al., 2002, 2004, 2005a, b; Nishiyama et al., 2004, 2005, 2006; Mohanty et al., 2007; Murata et al., 2007; Yang et al., 2007). A number of studies have also shown that effective repair of the photosynthetic machinery also requires weak light and a suitable balance of oxidants and antioxidants (Nishiyama et al., 2004; Allakhverdiev et al., 2005a, b; Kreslavski et al., 2007).

III. Role of Cofactors, Extrinsic Proteins of PS II and Heat-Shock Proteins in Thermal Damage

A. The Heat-Shock Proteins and Extrinsic Proteins of the Oxygen-Evolving Complex

At the OEC, the oxidation of two bound water molecules to molecular oxygen is catalyzed by a metal center, which contains four Mn atoms and one calcium as well as one/two chloride ions (Mn_4CaCl_2) as essential cofactors (Dau et al., 2001; Ferreira et al., 2004; Murray et al., 2008; Guskov et al., 2009; for review see McEvoy and Brudvig, 2006). In addition, bicarbonate has been suggested to play a role in the assembly of the OEC (Allakhverdiev et al., 1997), and in the protection of the donor side of PS II against thermal inactivation as well as photoinactivation (Klimov et al., 1997, 2003; Villarejo et al., 2002; Zharmukhamedov et al., 2007; Shutova et al., 2008) (see also Chapter 20). As many as five extrinsic proteins are associated with the OEC in different combinations depending on the type of organism (Roose et al., 2007). The extrinsic proteins are labeled as PsbO, PsbP, PsbQ, PsbR, PsbU, and PsbV. Only the PsbO protein (the 33 kDa or manganese-stabilizing protein) is ubiquitous to all oxygenic photosynthetic organisms. The three most widespread extrinsic proteins of molecular masses of 33, 23, and 17 kDa (PsbO, PsbP, and PsbQ, respectively) are bound at the lumenal side of PS II in many types of photosynthetic organisms (Seidler, 1996; Roose

et al., 2007). The 33 kDa protein is known to stabilize the Mn cluster (Nash et al., 1985; De Las Rivas and Heredia, 1999; Roose et al., 2007), and the 23 kDa protein has been shown to enhance the binding of calcium and chloride in addition to Mn (Bondarava et al., 2005; Roose et al., 2007). The extrinsic proteins and cofactors seem to counteract the negative action of ROS as well; protection of the oxygen-evolving machinery by the extrinsic proteins of PS II has been shown to be essential for the development of cellular thermotolerance in *Synechocystis* sp. PCC 6803 (Kimura et al., 2002).

Thermal stress to PS II particles caused the release of 33, 23 and 17 kDa proteins and loss of cofactors. Loss of the PsbU protein found in cyanobacteria induced the inactivation of PS II, damage of the D1 protein and production of ROS both in light and in dark when thermal stress persists. Balint et al. (2006) suggested that PsbU provides protection from ROS as PsbU mutants have enhanced mechanisms to detoxify exogenous H_2O_2.

Among the intrinsic components of PS II, the D1 protein is the most vulnerable. Moderate heat exposure (40°C for 30 min) of spinach thylakoids could cleave the D1 protein producing both 9 kDa C-terminal and 23 kDa N-terminal fragments. Only the D1 protein was cleaved in the thylakoids (Yoshioka et al., 2006). Degradation of phosphorylated D1 was affected by a FtsH protease located in the stroma which was assumed to diffuse to the granal area as a result of heat-triggered swelling of thylakoids (Yamamoto et al., 1999; Komayama et al., 2007). Yoshioka et al. (2006) have assumed that the degradation of the thermally damaged (heat inhibited) and the high light damaged (photoinhibted) D1 follows the same route. We still do not know if the elevated temperature treatment of intact leaves and mesophyll cells, protoplasts or algal cells could exhibit similar or identical results: no data are available at the moment to support this proposal.

Heat-shock proteins (i.e., HSPs) are important for protecting cells against high temperature and other stresses (Barua et al., 2003). Addition of purified chloroplast-localized HSPs confers heat tolerance to the photosynthetic electron transport chain in isolated chloroplasts. In vivo experiments demonstrated that small HSPs could associate with thylakoids and protect O_2 evolution and OEC proteins of PS II against heat stress. Evidence for the significance of chloroplast-localized HSPs for thermotolerance was obtained in tomato species (Heckathorn et al., 1998, 2002). Neta-Sharir et al. (2005) demonstrated that HSP21, a small heat-shock chloroplast protein, induced by heat treatment in tomato leaves, protects PS II from temperature-dependent oxidative stress. It appears that chloroplast HSPs do not participate in the repair of stress-related damage, but rather they function to prevent damage (Downs et al., 1999; Heckathorn et al., 2002). HSPs can be considered as molecular chaperones that can prevent but not reverse protein denaturation and aggregation (Barua et al., 2003). HSP101 has been shown to be essential for thermotolerance by genetic analysis (Hong and Vierling, 2001). The role and sub-cellular localization of the small (16 kDa) HSP (HspA) was investigated by comparing the cyanobacterium *Synechococcus strain ECT16-1*, with constitutively expressed HspA, with the reference strain (Nitta et al., 2005). The protein possesses the unique property that it can associate with thylakoid membranes during heat stress; further, it supports the stability of thylakoid membranes.

B. Heat-Stress-Inducible Genes in Synechocystis sp. PCC 6803

Acclimation to stress begins with the perception of stress and transduction of the stress signal. A combination of systematic mutation of potential sensors and transducers and DNA microarray analysis has led to significant progress in understanding the mechanisms of the perception of, and reaction to, environmental stress in cyanobacteria. Much progress has been made, in particular, in understanding the *Synechocystis* sp. PCC 6803 system (Suzuki et al., 2005; Los et al., 2008).

Proteomic analysis of the heat-shock response in *Synechocystis* sp. PCC 6803 has led to interesting results (Slabas et al., 2006; Los et al., 2008). The examination of heat-induced proteins in *Synechocystis* sp. PCC 6803 cells grown at 34°C and then exposed to 44°C, for 60 min, indicated that 65 different proteins were up-regulated. The most remarkable changes that occurred upon heat shock corresponded to components involved in the pathways for the folding, breakdown of the aggregates, and the degradation of proteins. The small heat-shock protein HspA showed the

greatest change, with an increase in level of almost 20-fold. Smaller changes in levels were observed for chaperonins GroES, GroEL1, GroEL2, and for HtpG (90 kDa), DnaK (70 kDa), and ClpB. The latter is an ATP-dependent Clp protease. The PS II complex, in particular the oxygen-evolving machinery, is the most heat-sensitive component of the photosynthetic apparatus. After heat shock, there is a two-fold increase in the level of the PsbO protein. Levels of phycobilisome proteins decrease upon heat shock and the level of RbcL, the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), declines to less than half of the control level.

Heat-induced gene expression was studied in *Synechocystis* sp. PCC 6803 at the genome-wide level (Inaba et al., 2003; Suzuki et al., 2005; Singh et al., 2006; Los et al., 2008). Upon heat stress, achieved by exposing cells grown at 34–44°C for 20 min, the levels of expression of 59 genes rose more than three-fold and those of 58 other genes fell more than three-fold (Suzuki et al., 2005). Most of the 59 up-regulated genes were strongly, though transiently, induced within 10–20 min after the upward shift in temperature from 34°C to 44°C. One hour after the onset of heat stress the levels of their transcripts declined significantly.

Table 12.1 lists genes whose expression is rapidly and strongly induced by heat stress. A general scheme showing the responses of cyanobacterial cells to heat stress is illustrated in Fig. 12.2. The transiently induced genes include *hspA*, *groES*, *groEL1*, *groEL2*, *dnaJ*, *htpG*, and *dnaK2*. Heat stress also induces the expression of the *clpB1* and *htrA* genes for proteases, the *sigB* gene for the group 2 sigma factor of RNA polymerase, and the *hik34* gene for the putative heat sensor histidine kinase (Suzuki et al., 2005), as well as the *sodB* gene for superoxide dismutase (i.e., SOD) and the *hliC* gene for a high-light-inducible protein. We note that the expression of none of these genes is specific to heat stress. The expression of these genes is also induced by hyperosmotic stress and salt stress, as well as by oxidative stress, strong visible light and UV-B light (Los et al., 2008). The genes whose expression is specifically induced by heat mainly encode proteins whose functions are, as yet, unknown (*slr0095*, *slr1920*, *slr1232*, among others). The levels of expression of most genes that are involved in energy and lipid metabolism, pigment biosynthesis and photosynthesis fall under heat-stress conditions (Suzuki et al., 2005).

It is also probable that other compounds different from HSPs contribute to heat tolerance

Table 12.1. Heat-inducible genes in *Synechocystis* sp. PCC 6803

ORF	Gene	Encoded protein	Ratio of transcript levels
slr1641	*clpB1*	ClpB protease	92.5 ± 9.1
sll1514	*hspA*	Small heat-shock protein	68.1 ± 2.9
slr2075	*groES*	10-kDa chaperonin	44.7 ± 2.3
sll0416	*groEL2*	60-kDa chaperonin 2	42.1 ± 7.7
slr0093	*dnaJ*	Heat-shock protein 40	36.9 ± 3.6
sll0430	*htpG*	Heat-shock protein 90	34.6 ± 5.6
slr0095		*o*-Methyltransferase	24.4 ± 4.6
sll030	*sigB*	RNA polymerase group 2s-factor	22.9 ± 0.7
slr1963		Water-soluble carotenoid protein	22.8 ± 0.0
sll0170	*dnaK2*	Heat-shock protein 70	22.3 ± 1.5
slr1285	*hik34*	Histidine kinase 34	18.9 ± 2.6
sll1621		AhpC/TSA family protein	11.0 ± 0.6
ssl3044		Ferredoxin-like protein	10.3 ± 0.0
slr2076	*groEL1*	60-kDa chaperonin 1	8.7 ± 1.5
ssl1633	*hliC*	CAB/ELIP/HLIP superfamily	6.9 ± 0.5
slr1204	*htrA*	Serine protease	6.0 ± 0.7
slr1516	*sodB*	Superoxide dismutase	5.6 ± 0.3
sll1483		Salt-inducible periplasmic protein	5.1 ± 1.1
sll1009	*frpC*	Iron-regulated protein	4.9 ± 0.5
slr1675	*hypA1*	Hydrogenase component protein	4.7 ± 0.1
sll1507		Salt-induced peripasmic protein	4.0 ± 0.6

Data adapted from Suzuki et al. (2005)
Cells that had been grown at 34°C were incubated at 44°C for 20 min. Genes with ratios of transcript levels greater than 4 are listed

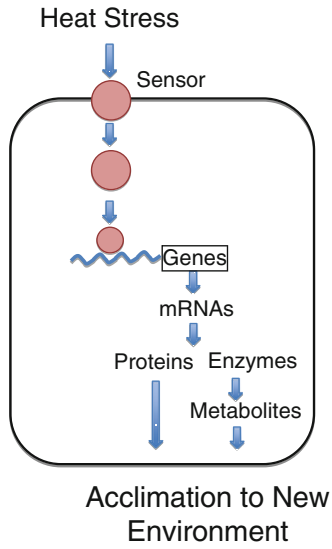

Fig. 12.2. A general scheme showing the responses of cyanobacterial cells to heat stress (adapted from Los et al. (2008) with some modification). Note that the scheme implies the possible existence of both positive and negative regulation by putative sensor(s).

(Nishiyama et al., 1999; Tanaka et al., 2000). Numerous studies have shown that the extrinsically associated proteins are not necessary for oxygen evolution activity in vitro, but they are required to enhance oxygen evolution and play important roles in vivo (reviewed in Seidler, 1996; Roose et al., 2007). In the marine cyanobacterium *Synechococcus* sp. PCC 7002, the oxygen-evolving machinery is stabilized against heat-induced inactivation by PsbU, which is an extrinsic protein of the PS II complex (Nishiyama et al., 1999).

Inactivation of the *psbU* gene lowered the thermal stability of the OEC in the mutant in comparison with the wild type. However, the levels of HSPs, namely the homologs of HSP70, HSP60, and HSP17 remained unaffected by mutation of the *psbU* gene suggesting that HSPs are not involved in cell acclimation to high temperature (Nishiyama et al., 1999). Similarly, Tanaka et al. (2000) reported that thermal acclimation of the OEC of *Chlamydomonas reinhardtii* did not change the levels of well characterized HSPs with molecular masses 70, 60 or 22 kDa. It is possible that the OEC's thermal stability and acclimation depends on the expression level of the *psbO* gene that produces the 33 kDa extrinsic protein (Enami et al., 1998; Pueyo et al., 2002, Ferreira et al., 2004).

IV. Membrane Fluidity and Heat Sensors

A. Membrane Fluidity and its Role in the Perception of Environmental Signals

The extent of molecular disorder and molecular motion within a lipid bilayer is referred to as the fluidity of the membrane (Los and Murata, 2004); it alters the tolerance of photosynthetic machinery to environmental stresses (Allakhverdiev et al., 1999, 2001, 2005b, 2007). A number of studies, focused on the effects of low temperature, demonstrated clearly that the membrane fluidity decreases with a decrease in temperature (Inaba et al., 2003; for reviews, see Murata and Los, 1997; Los and Murata, 2004; Mohanty, 2008; see also Chapter 11). A clear effect of membrane rigidification with cold-induced gene expression was demonstrated by genome-wide analysis, using DNA microarrays of wild-type *Synechocystis* sp. PCC 6803 and *desA*$^{-}$/*desD*$^{-}$ cells, which are deficient in Δ12- (*desA*) and Δ6-(*desD*) desaturases (Tasaka et al., 1996; Inaba et al., 2003). The effects of high temperature on the physical state of membranes have also been studied, albeit less intensively (Carratu et al., 1996; Vigh et al., 1998). High temperatures cause an increase in the fluidity of membranes, which can lead to disintegration of the lipid bilayer. According to the data of Kreslavski et al. (2008), exposure of wheat seedlings to increasing heat stress at 42°C for 20, 30 and 40 min led to a progressive destacking of the thylakoid membranes (see arrows in Fig. 12.3) and reduced grana. Finally, the amount of the thylakoids stacked into grana was diminished to 10% and the thylakoids were expanded compared with the control.

Carratu et al. (1996) and Vigh et al. (1998) have suggested that expression of heat-shock genes depends on membrane fluidity in yeast cells. We may assume that the increase of membrane fluidity inherent to the increased temperatures can activate the expression of HSP genes, some of which code proteins preventing membrane disintegration (Vigh et al., 1998). Evidence to support this possibility in photosynthetic cells was obtained from studies of the response of *Synechocystis* sp. PCC 6803 cells to heat shock (Horvath et al., 1998; Török et al., 2001). At the optimal growth temperature, the membrane fluidizer benzyl alcohol activated the transcription of

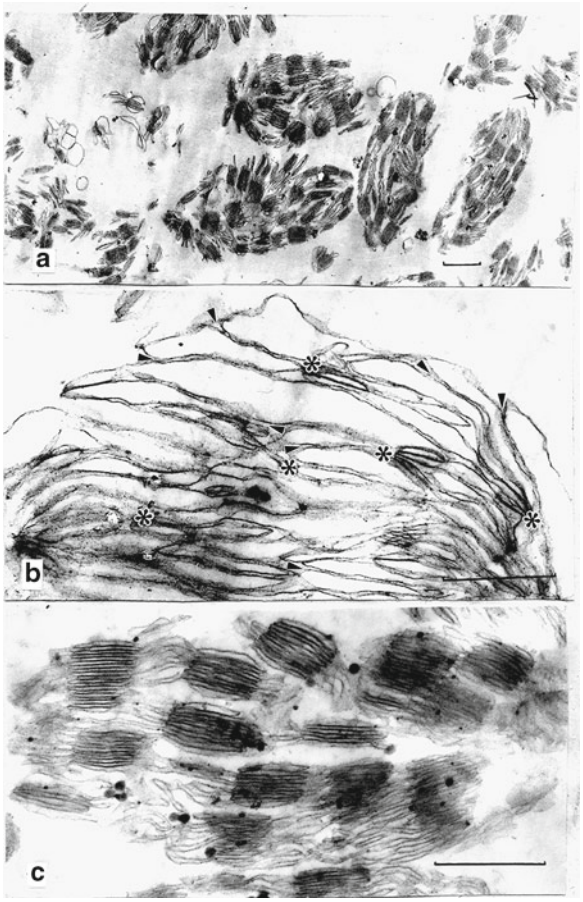

Fig. 12.3. Thin-section electron microscopy showing ultrastructural organization of the chloroplasts isolated from seedlings. Non-treated (**a**) or heat-treated at 42°C for 40 min either used immediately (**b**) or exposed to light (40 μmol photons m^{-2} s^{-1}) at 20°C for 6 h (**c**). *Arrows* denote partial destacking of the thylakoid membranes and *stars* indicate the reduced grana. Bars equal 1 μm (adapted from Kreslavski et al. (2008).

the *hspA* gene as efficiently as heat stress. Horvàth et al. (1998) have suggested that thylakoid membranes act as a cellular thermometer that senses thermal stress. However, other studies have not supported the putative involvement of membrane fluidity in heat-induced gene expression and heat sensing (Inaba et al., 2003). Thus, the detection of heat-stress-induced membrane fluidization by cellular protein sensors still remains a controversial issue (Los and Murata, 2004). Rigidification of thylakoid membranes seems to involve altered expression profiles of heat-shock genes, suggesting that the temperature sensing mechanism may be located in the thylakoid membrane (Horvàth et al., 1998). The idea of the thylakoid membrane acting as a heat sensor is physiologically crucial because it is susceptible to temperature up-shifts owing to its highly unsaturated character, and the presence of photosystems, which are sensitive to temperature changes (Sung et al., 2003).

B. Membrane Lipids

Growth temperature regulates the composition of thylakoid membrane lipids (Barber et al., 1984). Membrane fluidity is thought to be linked to the degree of unsaturation of membrane lipids (Gombos et al., 1994; Vigh et al., 1998; Los and Murata, 2004; Mohanty, 2008). The mechanism of lipid desaturation in the acclimation to high temperature is still unclear and the available data are apparently contradictory. In the green alga *C. reinhardtii*, saturation of membrane lipids was reported to enhance the thermal stability of PS II (Sato et al., 1996), while in cyanobacteria, desaturation of membrane lipids was shown to have no effect on the thermal stability of this photosystem (Gombos et al., 1991, 1994; Wada et al., 1994). Gombos et al. (1994) monitored the changes in heat tolerance after the elimination of dienoic and trienoic fatty acids from the membrane lipids of *Synechocystis* sp. PCC 6803. Heat resistance of photosynthetic oxygen evolution was not associated with fatty acid unsaturation. On the other hand, on the basis of their observations of the differences in the temperature dependence and thermosensitivity of PS II in *Synechocystis* sp. PCC 6803 grown at 25°C and 35°C, Aminaka et al. (2006) suggested that modification in the lipid desaturation levels of plasma and thylakoid membranes is the major factor responsible for the modulation of PS II thermosensitivity.

V. Redox State of Chloroplast Components and Heat Signaling

There is abundant evidence in the literature that the redox state of the electron transfer chain intermediates is involved in the expression of photosynthetic genes in prokaryotes (Oh and Kaplan, 2001; Wilson et al., 2006). In eukaryotes, a consensus is emerging that the expression of chloroplastic genes, as well as retrograde regulation of nuclear genes

encoding photosynthetic and non-photosynthetic proteins, can occur via the modulation of the redox status of the membrane-bound quinone pools associated with photosynthetic and respiratory electron transport (Allen et al., 1995; Pfannschmidt, 2003). The effect of heat stress conditions on the redox state of the plastoquinone pool was examined by Pshybytko et al. (2008). Heat stress (40°C, 3 h, and illumination at 100 μmol photons m^{-2} s^{-1}) clearly influenced the redox state of the plastoquinone pool in thylakoids isolated from heat-treated barley seedlings. N.L. Pshybytko and coworkers (personal communication) have suggested that this thermal induced change and a redistribution of plastoquinone molecules between photoactive and non-photoactive pools could reflect and regulate the response of the photosynthetic apparatus under heat stress conditions. Structural studies have also suggested that redox-dependent changes in the secondary structure of components of the cytochrome b_6f complex may be involved in this signaling process (Allen, 2004). However, strong genetic and biochemical proof for a link between heat signaling and the cytochrome b_6f complex is still lacking. We consider it important to recognize that low-molecular cytosolic and nuclear-encoded heat-shock-stress proteins can be imported into chloroplasts (Lubben and Keegstra, 1986; Vierling et al., 1986).

VI. Genetic Basis of Heat Tolerance

Thermotolerance is a complex cellular feature, which depends on the function of many biochemical pathways, as well as on the current physiological status of the cells and of the whole plant. These aspects of thermotolerance and the set of genes implicated to impart heat tolerance have been studied by several investigators (Maestri et al., 2002; Inaba et al., 2003; Los and Murata, 2004). Besides the genetic stock diversity and analysis of quantitative trait loci (QTL), the following factors are considered important in providing heat tolerance in plant cells: (1) HSPs; (2) antioxidants; (3) membrane lipid unsaturation; (4) gene expression and translation; (5) protein heat stability, and (6) accumulation of compatible solutes. We have discussed earlier how some of these factors participate in acquiring high temperature tolerance to PS II and its OEC.

A. Heat-Shock Transcription Factors and Heat-Stress Sensors

As discussed above, eukaryotic cells respond to elevated temperature or heat shock by inducing the transcription of genes encoding proteins such as molecular chaperones. Many of these proteins are involved in preventing or repairing the damage caused by heat stress and thus confer increased thermotolerance (Vierling, 1991). This phenomenon, known as the heat-shock response, is initiated by the activation of heat-shock transcription factors (HSFs), which are the central regulators of the heat-shock-stress response in all eukaryotic organisms (Baniwal et al., 2004). The heat-shock response was shown to be under the control of HSFs (Volkov et al., 2006). The review of Miller and Mittler (2006) describes several studies, which support the hypothesis that HSFs can function as molecular sensors that are able to directly sense ROS, for instance H_2O_2, and control the expression of oxidative stress response genes during oxidative stress.

Fifty-seven genes for transcriptional factors that comprise 1.7% of genes in the genome of *Synechocystis* sp. PCC 6803 have been found (Vladislav V. Zinchenko, personal communication). Among them, there is the *hrcA* (sll1670) gene, which encodes an ortholog of a negative regulator of the expression of the heat-shock *grpE-dnaK-dnaJ* and *groESL* operons for chaperonins in *Bacillus subtilis* (Hecker et al., 1996). In *Synechocystis* sp. PCC 6803, HrcA negatively regulates the *groESL* operon and the *groEL2* gene (Nakamoto et al., 2003; Kojima and Nakamoto, 2007), suggesting that other heat-shock genes might be regulated by different mechanisms.

Screening of a histidine kinase knock-out library under normal growth conditions using a DNA microarray identified Hik34 as an important contributor to thermotolerance (Suzuki et al., 2005). Mutation of the *hik34* gene enhanced the levels of transcripts of a number of heat-shock genes, including *htpG* and *groESL1*.

Overexpression of the *hik34* gene repressed the expression of these heat-shock genes. In addition, the ΔHik34-mutant cells survived incubation at 48°C for 3 h, while wild-type cells and mutant cells with the other Hiks knocked out failed to do so. However, mutation of the *hik34* gene had only an insignificant effect on the global expression of

genes upon incubation of the mutant cells at 44°C for 20 min. Quantitative two-dimensional gel electrophoresis revealed that levels of GroES and HspA were elevated in ΔHik34 cells after the incubation of cells at 42°C for 60 min. Overexpressed and purified recombinant Hik34 was autophosphorylated in vitro at physiological temperatures, but not at elevated temperatures, such as 44°C. Thus, it seems likely that Hik34 negatively regulates the expression of certain heat-shock genes that might be related to thermotolerance in *Synechocystis* sp. PCC 6803 (Suzuki et al., 2005; Slabas et al., 2006).

B. Effect of Glycinebetaine and Choline

Osmolyte accumulation can enhance high temperature tolerance by plants. We emphasize here the key role of compatible solutes, particularly glycinebetaine (betaine), in protecting photosynthesis from heat stress. Glycinebetaine appears to be an important molecule that imparts specific properties to cellular components that allow them to tolerate a variety of stresses (Chen and Murata, 2002). Application of betaine in order to stabilize PS-II-catalyzed functions has been reviewed by Papageorgiou and Murata (1995). It has been shown that betaine enhances the stability of photochemical performance of the reaction centers in thylakoid membranes (Mamedov et al., 1993), in isolated PS II particles (Allakhverdiev et al., 1996), and in isolated D1/D2/Cyt b_{559} complexes (Allakhverdiev et al., 2003). Effects of betaine on the temperature for 50% inhibition ($T_{1/2}$) of various photoreactions in PS II are shown in Table 12.2 (Allakhverdiev et al., 1996), which demonstrates its significant effect on almost all of the photosynthetic characteristics used in vitro.

Plant genetic engineering may be one of the answers to the global warming phenomenon in terms of producing high-temperature-tolerant crops. Norio Murata's laboratory, in Japan, introduced the bacterial *codA* gene for choline oxidase that converts choline to betaine. Transformed plants such as *Arabidopsis thaliana*, rice, and tomato were endowed with enhanced tolerance to various types of stresses: low-temperature stress, freezing, high light or salt stress (Allakhverdiev et al., 2001; Allakhverdiev and Murata, 2004, 2008; for reviews, see Sakamoto and Murata, 2002; Chen and Murata, 2002; Murata et al., 2007).

Table 12.2. Effect of glycinebetaine[a] on the temperature of 50% inhibition ($T_{1/2}$) of various processes in PS II

	$T_{1/2}$ (°C)	
Parameters	Control	Betaine
O_2 evolution	37	54
$(F_m - F_O)/F_m$	42	54
Mn content	44	55
Energy conservation	46	63
$H_2O \Leftrightarrow$ DCPIP	42	52
H_2O + DPC $\Leftrightarrow$ DCPIP	49	58
A_{685} (Pheo)	41	62

From Allakhverdiev et al. (1996)

[a]1 M betaine in vitro studies was used.

DCPIP -2,6-dichlorophenolindophenol; DPC –diphenylcarbazide; Pheo –pheophytin

The genetically engineered line of tobacco (*Nicotiana tobacum*) synthesizing betaine accumulated osmolytes in chloroplasts, and the transformed seedlings grew at higher temperatures than those of the wild type. Carbon fixation in transgenic plants was significantly more tolerant to elevated temperatures (Yang et al., 2005). Yang et al. (2005, 2007) argued that synthesis of betaine in chloroplasts maintained the activation of Rubisco by sequestering Rubisco activase near thylakoids and preventing its thermal inactivation.

Choline is a precursor of glycinebetaine. Root application or spraying of choline chloride enhanced the activities of antioxidative enzymes such as SOD and APX and provided heat stability for PS II in leaves. These observations suggest that cells with elevated ability for choline synthesis could be more heat-stress resistant than ordinary cells (Kreslavski et al., 2001). However, the cyanobacterium *Synechococcus* sp. PCC 7942 lacks the ability to synthesize choline. *Synechococcus* sp. PCC 7942 cells transformed with the gene *codA* for choline oxidase, named PAMCOD, acquired heat tolerance when these cells were supplemented with exogenous choline chloride but not its non transformant cells (Deshnium et al., 1995; Allakhverdiev et al., 2007). In transformed PAMCOD cells, the heat-induced half inactivation temperature ($T_{1/2}$) of Hill activity for O_2 evolution was shifted from 46°C to 54°C and $T_{1/2}$ for PS II photochemistry was shifted from 51°C to 58°C, exhibiting a significant increase in heat tolerance in transformant PAMCOD cells over the non-transformant cells (Allakhverdiev et al., 2007). Heat stress combined with light stress caused the inactivation of

PS II activity faster than when the two stresses were applied separately as heat stress enhanced the inhibition of PS II repair. The accumulation of betaine in PAMCOD cells alleviated this inhibitory effect of heat stress on the repair of PS II by accelerating the synthesis of new D1 protein. Therefore, betaine accumulation conferred the ability to sustain combined heat and light stresses in transformed cells (Allakhverdiev et al., 2007, 2008).

Analogously, in vivo experiments showed that betaine protects *Synechococcus* sp. PCC 7942 cells against the synergistic actions of salt stress and strong light stress likely by accelerating the repair of PS II (Ohnishi and Murata, 2006). It also appears that betaine protects cyanobacterial cells during heat-induced photoinhibition in a similar manner. Moreover, choline chloride was shown to enhance the repair of PS II from heat-induced photoinhibition on wheat seedlings (V. Kreslavski and S. I. Allakhverdiev, unpublished observations).

One of the explanations for the positive action of betaine on thermotolerance was arrived at on the basis of in vitro experiments with D1/D2/Cyt b_{559} complexes isolated from PS II membranes, which indicated that the presence of betaine enhanced the thermotolerance of such complexes with a shift in temperature for 50% inactivation from 29°C to 35°C (Allakhverdiev et al., 2003). Braun et al. (1990) have suggested that elevated temperatures increase the distance between D1 and D2 proteins. The stabilization and protective effect of betaine against heat inactivation in isolated D1/D2/Cyt b_{559} and PS II complexes can be explained with minimization of the water–protein interfacial area that leads to preferential hydration of the proteins and, hence, to their tighter packaging and stabilization (Allakhverdiev et al., 1996, 2003, 2007).

VII. Combined Effects of High Temperature and Other Stress Factors

A. Light- and Heat-Induced Inhibition

Light and heat stresses usually occur together in nature and their joint action can lead to the weakening or the strengthening of their damaging effects on the photosynthetic machinery. Exposure to low or moderate irradiance during short-term heat treatment is often beneficial to plants diminishing the extent of damage to the photosynthetic machinery (Havaux et al., 1991). Light is often required for switching adaptation mechanisms of the photosynthetic machinery to heat stress, especially the repair of photosystems, which needs weak light for protein photophosphorylation and stimulation of the activity of several enzymes. In contrast, exposure to strong light increases the magnitude of photosynthetic machinery inhibition induced by heat stress. This might be linked to the formation of extra ROS during heat-induced photoinhibition (Al-Khatib and Paulsen, 1989; Kreslavski et al., 2007; Allakhverdiev et al., 2008).

Some studies report that exposure to elevated temperatures higher than growth temperature ensures an advantage for plant acclimation to strong light (Havaux, 1993; Kreslavski et al., 2008). This type of resistance to stress factors is called "cross-tolerance" (Allen, 1995; Pastori and Foyer, 2002) but the mechanism of such cross adaptation is not fully understood. Similar to what happens under conditions of low-temperature stress, pre-exposure of plants to high temperatures affects the photodamage that follows it. In the same way, short-duration heat pre-treatment can increase tolerance of PS II to light and to elevated temperatures and short-duration exposure to strong light can increase the thermostability of PS II in *Lycopersicon esculentum* leaves (Havaux and Tardy, 1996). Short-duration pre-treatment of wheat seedlings at 40°C enhanced their tolerance to high light exposure (Kreslavski et al., 2008) that can be explained by the accumulation of HSPs, protecting photosynthetic machinery from photodamage. Other reasons of the enhancement might be the higher rate of CO_2 assimilation indicated in hardening plants and, likely, higher rate of cyclic photophosphorylation indicated in chloroplasts isolated from heat-pre-treated seedlings. Accumulation of HSPs seems to contribute to the protection of the photosynthetic apparatus from photoinhibition as well (Schroda et al., 1999).

High temperature inactivates Rubisco activase (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000), which is essential for the activity of Rubisco. Under such conditions, inhibition of the repair of photodamaged PS II as a response to limitations in fixation of CO_2 might accelerate

photoinhibition. According to this hypothesis, heat accelerates photoinhibition by inhibiting the repair of photodamaged PS II (Allakhverdiev et al., 2007; Yang et al., 2007). Although these various stresses might inhibit the repair of photodamaged PS II directly, their inhibitory effects might be attributable, in part, to the limitation of the fixation of CO_2 in the Calvin-Benson cycle (Allakhverdiev et al., 2007; Murata et al., 2007; Takahashi and Murata, 2008).

B. Salt- and Heat-Induced Inhibition

There is cross-tolerance between high salinity and heat stress. Salinity treatment showed no effects on PS II photochemistry, but increased the resistance of PS II to heat stress in the halophytes *Suaeda salsa* (Lu et al., 2003) and *Artemisia anethifolia* (Wen et al., 2005). High salinity (100–400 mM NaCl) had no effects on PS II photochemistry in dark- or light-adapted states; however, heat tolerance of PS II was increased after salt treatment. The ratio $(F_i\text{-}F_o)/(F_p\text{-}F_o)$, which is an expression of the proportion of the Q_B-non-reducing PS II centers, and the K band of the polyphasic fluorescence transients used as a specific indicator of partial damage to the OEC, were applied for the evaluation of the state of the reaction center and OEC (Strasser et al., 2004). The results suggested that the increased thermostability of PS II was associated with the increased resistance of the OEC and the reaction center to high temperature (Lu et al., 2003; Wen et al., 2005). In addition to this, in leaves of salt-adapted plants the CO_2 assimilation system was more thermo-resistant than in the control (Lu et al., 2003).

C. Drought- and Heat-Induced Inhibition

The most severe stress combination for crop productivity, which frequently occurs in the field, is drought and heat stress. The proteomic and metabolic analysis of plants subjected to joint action of drought and elevated temperature indicated 45 different proteins that accumulated in response to this stress combination (Koussevitzky et al., 2008). Cytosolic ascorbate peroxidase 1 (APX1) protein and mRNA accumulated under stress combination. The data obtained with various *Arabidopsis thaliana* mutants deficient in APX1, as well as thylakoid or stromal/mitochondrial peroxidases, demonstrated that the APX-deficient mutant (*apx1*), in contrast to other mutants, was significantly more sensitive to the stress combination than the wild type. Koussevitzky et al. (2008) suggested that APX1 plays a key role in the response to the combination of heat stress and drought.

VIII. Conclusions and Future Perspectives

Recent advances in the study of the effect of heat stress on photosynthesis have led to the following conclusion: the primary target sites of thermal impairment or inactivation are the OEC with associated cofactors and carbon fixation by Rubisco due to, most likely, inactivation of Rubisco activase. These two sites of impairment occur together under moderately elevated temperatures for short duration. The reduction in carbon fixation and oxygen evolution activities is followed by disruption of linear electron flow, which induces the generation of ROS. ROS inhibits the repair of damaged PS II, which largely depends on de novo synthesis of proteins of the photosynthetic machinery (Fig. 12.1). The repair system for stress-induced damage is complex and involves multi-step mechanisms, whose nature is not completely understood.

The most recent investigations on the heat-stress responses were focused on the role of HSFs, chaperones and heat-induced proteins important for plant cell heat acclimation. However, there is little information about chloroplastic (photosynthetic) heat tolerance. HSFs could be sensitive sensors for heat stress; however, the underlying mechanisms will need further examination. Several important areas remain in focus for future research. Some examples are: (1) the relationship between the generation of ROS during heat stress and the regulation of the activity of genes responsible for the synthesis of stress proteins important for protection and acclimation of the photosynthetic machinery; (2) the role of thylakoid membrane changes and the redox-potential of components of the electron transfer chain, notably the plastoquinone pool, in the regulation of photosynthetic tolerance during heat stress, and (3) the molecular nature of stress-induced inhibition of PS II repair in relation to suppression of protein synthesis by ROS.

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References

Adir N, Zer H, Shochat S and Ohad I (2003) Photoinhibition: a historical perspective. Photosynth Res 76: 343–370

Al-Khatib K and Paulsen GM (1989) Enhancement of thermal injury to photosynthesis in wheat plants and thylakoids by high light intensity. Plant Physiol 90: 1041–1048

Allakhverdiev SI and Murata N (2004) Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage - repair cycle of photosystem II in *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 1657: 23–32

Allakhverdiev SI and Murata N (2008) Salt stress inhibits photosystems II and I in cyanobacteria. Photosynth Res 98: 529–539

Allakhverdiev SI, Klimov VV and Carpentier R (1994) Variable thermal emission and chlorophyll fluorescence in photosystem II particles. Proc Natl Acad Sci USA 91: 281–285

Allakhverdiev SI, Feyziev YM, Ahmed A, Hayashi H, Aliev JA, Klimov VV, Murata N and Carpentier R (1996) Stabilization of oxygen evolution and primary electron transport reactions in photosystem II against heat stress with glycinebetaine and sucrose. J Photochem Photobiol 34: 149–157

Allakhverdiev SI, Yruela Y, Picorel R and Klimov VV (1997) Bicarbonate is an essential constituent of the water-oxidizing complex of photosystem II. Proc Natl Acad Sci USA 94: 5050–5054

Allakhverdiev SI, Nishiyama Y, Suzuki I, Tasaka Y and Murata N (1999) Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress. Proc Natl Acad Sci USA 96: 5862–5867

Allakhverdiev SI, Kinoshita M, Inaba M, Suzuki I and Murata N (2001) Unsaturated fatty acids in membrane lipids protect the photosynthetic machinery against salt-induced damage in *Synechococcus*. Plant Physiol 125:1842–1853

Allakhverdiev SI, Nishiyama Y, Miyairi S, Yamamoto H, Inagaki N, Kanesaki Y and Murata N (2002) Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of psbA genes in *Synechocystis*. Plant Physiol 130: 1443–1453

Allakhverdiev SI, Hayashi H, Nishiyama Y, Ivanov AG, Aliev Ja.A, Klimov VV, Murata N and Carpentier R (2003) Glycine betaine protects the D1/D2/Cytb_{559} complex of photosystem II against photo-induced and heat-induced inactivation. J Plant Physiol 160: 41–49

Allakhverdiev SI, Mohanty P and Murata N (2004) Dissection of photodamage at low temperature and repair in darkness suggests the existence of an intermediate form of photo-damaged photosystem II. Biochemistry 42: 14277–14283

Allakhverdiev SI, Nishiyama Y, Takahashi S, Miyairi S, Suzuki I and Murata N (2005a) Systematic analysis of the relation of electron transport and ATP synthesis to the photodamage and repair of photosystem II in *Synechocystis*. Plant Physiol 137: 263–273

Allakhverdiev SI, Tsvetkova N, Mohanty P, Szalontai B, Moon BY, Debreczeny M and Murata N (2005b) Irreversible photoinhibition of photosystem II is caused by exposure of *Synechocystis* cells to strong light for a prolonged period. Biochim Biophys Acta 1708: 342–351

Allakhverdiev SI, Los DA, Mohanty P, Nishiyama Y and Murata N (2007) Glycinebetaine alleviates the inhibitory effect of moderate heat stress on the repair of photosystem II during photoinhibition. Biochim Biophys Acta 1767:1363–1371

Allakhverdiev SI, Kreslavski VD, Klimov VV, Los DA, Carpentier R and Mohanty P (2008) Heat stress: an overview of molecular responses in photosynthesis. Photosynth Res 98: 541–550

Allen JF (2004) Cytochrome b_6f: structure for signalling and vectorial metabolism. Trends Plant Sci. 9: 130–137

Allen JF, Alexciev K and Hakansson G (1995) Regulation of redox signalling. Curr Biol 5: 869–872

Allen JF and Mullineaux CW (2004) Probing the mechanisms of state transitions in oxygenic photosynthesis by chlorophyll fluorescence spectroscopy: Kinetics and Imaging. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluroscence: a signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 447–461. Springer, Dodrecht

Allen R (1995) Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol 107: 1049–1054

Aminaka R, Taira Y, Kashino Y, Koike H and Satoh K (2006) Acclimation to the growth temperature and thermosensitivity of photosystem II in a mesophilic cyanobacterium, *Synechocystis* sp. PCC6803. Plant Cell Physiol 47:1612–1621

Aro E-M, Virgin I and Andersson B (1993) Photoinhibition of photosystem II: inactivation, protein damage and turnover. Biochim Biophys Acta 1143:113–134

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50: 601–639

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141: 391–396

Baker NR and Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J Exp Bot 55: 1607–1621

Balint I, Bhattacharya J, Perelman A, Schatz D, Moskovitz Y, Keren N and Schwarz R (2006) Inactivation of the extrinsic subunit of photosystem II, PsbU, in *Synechococcus* PCC 7942 results in elevated resistance to oxidative stress. FEBS Lett 580: 2117–2122

Baniwal SK, Bharti K, Chan KY, Fauth M, Ganguli A, Kotak S, Mishra SK, Nover L, Port M, Scharf KD, et al. (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J Biosci 29: 471–487

Barber J, Ford RC, Mitchell RAC and Millner PA (1984) Chloroplast thylakoid membrane fluidity and its sensitivity to temperature. Planta 161: 375–380

Barua D, Downs CA and Hechthorn SA (2003) Variation in chloroplast small heat-shock protein function is a major determinant of variation in thermotolerance of photosynthetic electron transport among ecotypes of *Chenopodium album*. Funct Plant Biol 30: 1071–1079

Berry JA and Björkman O (1980) Photosynthetic response and adaptation to temperature in higher plants. Annu Rev Plant Physiol 31: 491–543

Bonaventura C and Myers J (1969) Fluoresence and oxygen evolution from *Chlorella pyrenoidosa*. Biochim Biophys Acta 189: 366–383

Bondarava N, Beyer P and Krieger-Liszkay A (2005) Function of the 23 kDa extrinsic protein of photosystem II as a manganese binding protein and its role in photoactivation. Biochim Biophys Acta 1708: 63–70

Boyer JS (1982) Plant productivity and environment. Science 218: 443–448

Braun P, Greenberg BM and Scherz A (1990) D1-D2-Cytochrome b_{559} complex from the aquatic plant *Spirodela oligorchiza*: Correlation between complex integrity, spectroscopic properties, photochemical activity and pigment composition. Biochemistry 29: 10376–10387

Bukhov NG and Mohanty P (1999) Elevated temperature stress effects on photosystems: Characterization and evaluation of the nature of heat induced impairments. In: Singhal GS, Renger G, Sopory SK, Irrgang K-D and Govindjee (eds) Concepts in Photobiology: Photosynthesis and Photomorphogenesis, pp 617–648. Narosa Publishing House, New Delhi

Bukhov NG and Carpentier R (2000) Heterogeneity of photosystem II reaction centers as influenced by heat treatment of barley leaves. Physiol Plant 110: 279–285

Bukhov NG, Boucher N and Carpentier R (1998) Loss of the precise control of photosynthesis and increased yield of nonradiative dissipation of excitation energy after mild heat treatment of barley leaves. Physiol Plant 104: 563–570

Cao J and Govindjee (1990) Chlorophyll *a* fluorescence transient as an indicator of active and inactive photosystem II in thylakoid membranes. Biochim Biophys Acta 1015: 180–189

Carpentier R (1999) Effect of high-temperature stress on the photosynthetic apparatus. In: Pessarakli M (ed) Handbook of Plant and Crop Stress, pp 337–348. Marcel Dekker Inc, New York

Carratu L, Franceschelli S, Pardini CL, Kobayashi GS, Horvath I, Vigh L and Maresca B (1996) Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. Proc Natl Acad Sci USA 93: 3870–3875

Charng YY, Liu HC, Liu NY, Chi WT, Wang CN, Chang SH and Wang TT (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. Plant Physiol 143: 251–262

Chen THH and Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaine and other compatible solutes. Curr Opin Plant Biol 5: 250–257

Crafts-Brandner SJ and Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc Natl Acad Sci USA 97:13430–13435

Dat JF, Lopez-Delgado H, Foyer CH and Scott IM (1998) Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol 116: 1351–1357

Dau H, Iuzzolino L and Dittmer J (2001) The tetramanganese complex of photosystem II during its redox cycle. X-ray absorption results and mechanistic implications. Biochim Biophys Acta 1503: 24–39

Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Shulaev V, Schlauch K and Mittler R (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell, 17: 268–281

De Las Rivas J and Heredia P (1999) Structural predictions on the 33 kDa extrinsic protein associated with the oxygen-evolving complex of photosynthetic organisms. Photosynth Res 61: 11–21

Deshnium P, Los DA, Hayashi H, Mustardy L and Murata N (1995) Transformation of *Synechococcus* with a gene for

choline oxidase enhances tolerance to salt stress. Plant Mol Biol : 897–907

Dietzel L, Bräutigam K and Pfannschmidt T (2008) Photosynthetic acclimation: state transition adjustment of photosystem stoichiometry – functional relationship between short-term and long-term acclimation. FEBS J 275: 1080—1088

Downs CA, Coleman JS and Heckathorn SA (1999) The chloroplast 22-kDa heat-shock protein: a lumenal protein that associates with the oxygen-evolving complex and protects photosystem II during heat stress. J Plant Physiol 155: 477–487

El-Shitinawy F, Ebrahim MKH, Sewelam N and El-Shourbagy MN (2004) Activity of photosystem 2, lipid peroxidation, and the enzymatic antioxidant protective system in heat shocked barley seedlings. Photosynthetica 42: 15–21

Enami I, Kitamura M, Tomo T, Isokawa Y, Ohta H and Katoh S (1994) Is the primary cause of thermal inactivation of oxygen evolution in spinach PS II membranes release of the extrinsic 33 kDa protein or of Mn? Biochim Biophys Acta 1186: 52–58

Enami I, Kamo M, Ohta H, Takahashi S, Miura Taro, Kusayanagi M, Tanabe S, Kamei A, Motoki A, Hirano M, Tomo T and Satoh K (1998) Intramolecular cross-linking of the extrinsic 33-kDa protein leads to loss of oxygen evolution but not its ability of binding to photosystem II and stabilization of the manganese cluster. J Biol Chem 273: 4629–4634

Feller U, Crafts-Brandner SJ and Salvucci ME (1998) Moderately high temperatures inhibit ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. Plant Physiol 116: 539–546

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Foyer CH, Lopez-Delgado H, Dat JF and Scott IM (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol Plant 100: 241–254

Goltsev V, Zaharieva I, Chernov P and Strasser RJ (2009) Delayed fluorescence in photosynthesis. Photosynth Res 101: 217–232

Gombos Z, Wada H and Murata N (1991) Direct evaluation of effects of fatty-acid unsaturation on the thermal properties of photosynthetic activities, as studied by mutation and transformation of *Synechocystis* PCC6803. Plant Cell Physiol 32: 205–211

Gombos Z, Wada H, Hideg E and Murata N (1994) The unsaturation of membrane lipids stabilizes photosynthesis against heat stress. Plant Physiol 104: 563–567

Gounaris K, Brain ARR, Quinn PJ and Williams WP (1983) Structural and functional changes associated with heat-induced phase separation of non-bilayer lipids in chloroplast thylakoid membranes. FEBS Lett 153: 47–53

Gounaris K, Brain ARR, Quinn PJ and Williams WP (1984) Structural reorganization of chloroplast thylakoid membranes in response to heat stress. Biochim Biophys Acta 766: 198–208

Govindachary S, Bigras C, Harnois J, Joly D and Carpentier R (2007) Changes in the mode of electron flow to photosystem I following chilling-induced photoinhibition in a C3 plant, *Cucumus sativus* L. Photosynth Res 94: 333–345

Govindjee (2004) Chlorophyll *a* fluorescence: a bit of basics and history. In Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 1–42. Springer, Dordrecht

Guisse B, Srivastava A and Strasser RJ (1995) The polyphasic rise of chlorophyll *a* fluorescence (OKIJP) in heat stressed leaves. Arch Sci Geneve 48: 147–160

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A and Saenger W (2009) Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nature Struc Mol Biol 16: 334–342

Hall AE (2001) Crop Responses to Environment. CRS press LLC, Boca Raton

Havaux M (1993) Rapid photosynthetic adaptation to heat stress triggered in potato leaves by moderately elevated temperatures. Plant Cell Environ 16: 461–467

Havaux M and Tardy F (1996) Temperature-dependent adjustment of the thermal stability of photosystem II in vivo: possible involvement of xanthophyll-cycle pigments. Planta 198: 324–333

Havaux M, Greppin H and Strasser RJ (1991) Functioning of photosystems I and II in pea leaves exposed to heat stress in the presence or absence of light. Planta 186: 88–98

Heckathorn S, Downs SA, Sharkey TD and Soleman JS (1998) The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. Plant Physiol 116: 439–444

Heckathorn SA, Ryan SL, Baylis JA, Wang D, Hamilton EW, Cundiff L and Luthe DS (2002) *In vivo* evidence from an *Agrostis stolonifera* selection genotype that chloroplast small heat-shock proteins can protect photosystem II during heat stress. Funct Plant Biol 29: 933–944

Hecker M, Schumann W and Volker U (1996) Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol 19: 417–428

Hong SW and Vierling E (2001) Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J 27: 25–35

Horvath I, Glatz A, Varvasovszki V, Torok Z, Pali T, Balogh G, Kovacs E, Nadasdi L, Benko S, Joo F and Vigh L (1998) Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of *hsp17* as a fluidity gene. Proc Natl Acad Sci USA 95: 3513–3518

Joshi MK, Deasai TS and Mohanty P (1995) Temperature dependent alterations in the pattern of photochemical and nonphotochemical quenching and associated changes in the photosystem II conditions of the leaves. Plant Cell Physiol 36: 1221–1227

Inaba M and Crandall P (1988) Electrolyte leakage as an indicator of high-temperature injury to harvested mature green tomatoes. J Amer Soc Hortic Sci 113: 96–99

Inaba M, Suzuki I, Szalontai B, Kanesaki Y, Los DA, Hayashi H and Murata N (2003) Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. J Biol Chem 278: 12191–12198

Joliot P and Joliot A (1980) Dependence of delayed luminescence upon adenosine triphosphatase activity in *Chlorella*. Plant Physiol 65: 691–696

Jones PD, New M, Parker DE, Mortin S and Rigor IG (1999) Surface area temperature and its change over the past 150 years. Rev Geophys 37: 173–199

Jursinic P (1986) Delayed fluorescence: Current concepts and status. In: Govindjee, Amesz J, Fork DJ (eds) Light emission by plants and bacteria. Academic Press, Orlando, pp 291–328

Kalitulo LN, Pshybutko NL, Kabashnikova LF and Jahns P (2003) Photosynthetic apparatus and high temperature: role of light. Bulg J Plant Physiol 32: 281–289

Katoh S and San Pietro A (1967) Photooxidation and reduction of cytochrome-552 and NADP photoreduction by *Euglena* chloroplast. Arch Biochem Biophys 121: 211–219

Kim K and Portis AR (Jr) (2004) Oxygen-dependent H_2O_2 production by rubisco. FEBS Lett 571: 124–128

Kimura A, Eaton-Rye JJ, Morita EH, Nishiyama Y and Hayashi H (2002) Protection of the oxygen-evolving machinery by the extrinsic proteins of photosystem II is also essential for development of cellular thermotolerance in *Synechocystis* sp. PCC 6803. Plant Cell Physiol 43: 932–938

Klimov VV, Baranov SV and Allakhverdiev SI (1997) Bicarbonate protects the donor side of photosystem II against photoinhibition and thermoinactivation. FEBS Lett 418: 243–246

Klimov VV, Allakhverdiev SI, Nishiyama Y, Khorobrykh AA and Murata N (2003) Stabilization of the oxygen-evolving complex of photosystem II by bicarbonate and glycinbetaine in thylakoid and subthylakoid preparations. Fun Plant Biol 30: 797–803

Kojima K and Nakamoto H (2007) A novel light- and heat-responsive regulation of the *groE* transcription in the absence of HrcA or CIRCE in cyanobacteria. FEBS Lett 581: 1871–1880

Komayama K, Khatoon M, Takenaka D, Horie J, Yamashita A, Yoshioka M, Nakayama Y, Yoshida M, Ohira S, Morita N, Velitchkova M, Enami I and Yamamoto Y (2007) Quality control of Photosystem II: cleavage and aggregation of heat-damaged D1 protein in spinach thylakoids. Biochim Biophys Acta 1767: 838–846

Koussevitzky S, Suzuki **N**, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V and Mittler R (2008) Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination J Biol Chem 283: 34197–34203

Kreslavski VD and Khristin MS (2003) After effect of heat shock on fluorescence induction and low-temperature fluorescence spectra of wheat leaves. Rus J Biophys 48: 865–872

Kreslavski VD, Balakhnina TI, Khristin MS and Bukhov NG (2001) Pretreatment of bean seedlings by choline compounds increases the resistance of photosynthetic apparatus to UV radiation and elevated temperatures. Photosynthetica 39:353–358

Kreslavski VD, Carpentier R, Klimov VV, Murata N and Allakhverdiev SI (2007) Molecular mechanisms of stress resistance of the photosynthetic apparatus. Membrane and Cell Biol 1: 185–205

Kreslavski V, Tatarinzev N, Shabnova N, Semenova G and Kosobrukhov A (2008) Characterization of the nature of photosynthetic recovery of wheat seedlings from short-time dark heat exposures and analysis of the mode of acclimation to different light intensities. J Plant Physiol 165: 1592–1600

Krieger-Liszkay A (2005) Singlet oxygen production in photosynthesis. J Exp Bot 56: 337–346

Larkindale J and Knight MR (2002) Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol 128: 682–695

Lavergne J and Leci E (1993) Properties of inactive photosystem II centers. Photosynth Res 35: 323–343

Lavorel J (1975) Luminescence. In: Govindjee (ed) Bioenergetics of Photosynthesis. Academic Press, New York, pp 223–317

Law R and Crafts-Brandner SJ (1999) Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 120: 173–182

Lazar D (1999) Chorophyll *a* fluorescence induction. Biochim Biophys Acta 1412: 1–26

Lazar D (2006) The polyphasic chlorophyll *a* fluorescence rise measured under high intensity of exciting light. Functional Plant Biol 33: 9–30

Li H, Yang S, Xie J and Zhau J (2007) Probing the connection of PBSs to photosystems in *Spirulina platensis by* artificially induced fluorescence fluctuations. J Lumin 122/123: 294–296

Los DA and Murata N (2004) Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta 1666:142–157

Los DA, Suzuki I, Zinchenko VV and Murata N (2008) Stress responses in *Synechocystis*: regulated genes and regulatory systems. In: Herrero A and Flores E (eds) The Cyanobacteria: Molecular Biology, Genomics and Evolution, pp 117–157. Caister Academic Press, London

Lu C, Qiu N, Wang B and Zhang J (2003) Salinity treatment shows no effects on photosystem II photochemistry, but increases the resistance of photosystem II to heat stress in halophyte *Suaeda salsa*. J Exp Bot 54: 851–860

Lubben TH and Keegstra K (1986) Efficient *in vitro* import of a cytosolic heat shock protein into pea chloroplasts. *Proc Natl Acad Sci USA 83*: 5502–5506

Maestri E, Klueva N, Perrotta C, Gulli1 M, Nguyen HT, Marmiroli N (2002) Molecular genetics of heat tolerance and heat shock proteins in cereals. Plant Mol Biol 48: 667–681

Mamedov MD, Hayashi H and Murata N (1993) Effects of glycinebetaine and unsaturation of membrane lipids on heat stability of photosynthetic electron transport and phosphorilation reactions in *Synechocystis* PCC 6803. Biochim Biophys Acta 1142: 1–5

McEvoy JP and Brudvig GW (2006) Water-splitting chemistry of photosystem II. Chem Rev 106: 4455–4483

Miller G and Mittler R (2006) Could heat shock transcription factors function as hydrogen peroxide sensors in plants? Ann Bot 98: 279–288

Miller G, Suzuki N, Rizhsky L, Hegie A, Koussevitsky S and Mittler R (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. Plant Physiol 144: 1777–1785

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci.7: 405–410

Miyake C and Okamura M (2003) Cyclic electron flow within PSII protects PSII from its photoinhibition in thylakoid membranes from spinach chloroplasts. Plant Cell Physiol 44: 457–462

Mohanty N, Murthy SD and Mohanty P (1987) Riversal of heat induced alteration in photochemical activities in wheat primary leaves. Photosyn Res 14: 259–267

Mohanty P (2008) Perspective of membrane perception of temperature. Physiol Mol Biol Plants 14: 273–275

Mohanty P, Munday JC and Govindjee (1970) Time dependent quenching of chlorophyll *a* fluorescence from (pigment) system II by (pigment) system I of photosynthesis in *Chlorella*. Biochim Biophys Acta 223: 198–200

Mohanty P, Vani B and Prakash S (2002) Elevated temperature treatment induced alteration in thylakoid membrane organization and energy distribution between the two photosystems in *Pisum sativum*. Z Naturforsch C 57: 836–842

Mohanty P, Allakhverdiev SI and Murata N (2007) Application of low temperature during photoinhibition allows characterization of individual steps in photodamage and repair of photosystem II. Photosynth Res 94: 217–234

Mubarakshina M, Khorobrykh S and Ivanov B (2006) Oxygen reduction in chloroplast thylakoids results in production of hydrogen peroxide inside the membrane. Biochim Biophys Acta 1757: 1496–1503

Murata N (1969) Control of excitation transfer in photosynthesis: Light induced chlorophyll a fluorescence in *Prophyridium cruentum*. Biochim.Biophys Acta 172: 242–251

Murata N (2009) The discovery of state transitions in photosynthesis 40 years ago. Photosynth Res. 99: 155–160

Murata N and Los DA (1997) Membrane fluidity and temperature perception. Plant Physiol 115: 875–879

Murata N, Takahashi S, Nishiyama Y and Allakhverdiev SI (2007) Photoinhibition of photosystem II under environmental stress. Biochim Biophys Acta 1767: 414–421

Murray JW, Maghlaoui K, Kargul J, Ishida N, Lai TL, Rutherford AW, Sugiura M, Boussac A and Barber J (2008) X-ray crystallography identifies two chloride binding sites in the oxygen evolving centre of photosystem II. Energy Environ Sci 1: 161–166

Nakamoto H, Suzuki M and Kojima K (2003) Targeted inactivation of the *hrcA* repressor gene in cyanobacteria. FEBS Lett 549: 57–62

Nash D, Miyao M and Murata N (1985) Heat inactivation of oxygen evolution in photosystem II particles and its acceleration by chloride depletion and exogenous manganese. Biochim Biophys Acta 807: 127–133

Neta-Sharir I, Isaacson T, Lurie S and Weiss D (2005) Dual role for tomato heat shock protein 21: Protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. Plant Cell 17: 1829–1838

Nishiyama Y, Los DA and Murata N (1999) PsbU, a protein associated with photosystem II, is required for the acquisition of cellular thermotolerance in *Synechococcus* species PCC 7002. Plant Physiol 120: 301–308

Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A and Murata N (2001) Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. EMBO J 20: 5587–5594

Nishiyama Y, Allakhverdiev SI, Yamamoto H, Hayashi H and Murata N. (2004) Singlet oxygen inhibits the repair of photosystem II by suppressing the translation elongation of the D1 protein in *Synechocystis* sp. PCC 6803. Biochemistry 43: 11321–11330

Nishiyama Y, Allakhverdiev SI and Murata N (2005) Inhibition of the repair of photosystem II by oxidative stress in cyanobacteria. Photosynth Res 84: 1–7

Nishiyama Y, Allakhverdiev SI and Murata N (2006) A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. Biochim Biophys Acta 1757: 742–749

Nitta K, Suzuki N, Honma D, Kaneko Y and Nakamoto H (2005) Ultrastructural stability under high temperature or intensive light stress conferred by a small heat shock protein in cyanobacteria. FEBS Lett 579: 1235–1242

Oh JI and Kaplan S (2001) Generalized approach to the regulation and integration of gene expression. Mol Microbial 39: 1116–1123

Ohnishi N and Murata N (2006) Glycinebetaine counteracts the inhibitory effects of salt stress on the degradation and synthesis of D1 protein during photoinhibition in *Synechococcus* sp. PCC 7942. Plant Physiol 141: 758–765

Papageorgiou GC and Govindjee (1967) Changes in intensity and spectral distribution of fluorescence effect of light treatment on normal and DCMU poisoned *Anasystis nidulans*. Boophysical J 7: 375–389

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration Series, Vol 19, Springer, Dordrecht

Papageorgiou GC and Govindjee (2011) Photosystem II fluorescence: Slow changes -Scaling from the past. J Photochem Photobiol B:Biol104: 258–270

Papageorgiou GC and Murata N (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosynth Res 44: 243–252

Papageorgiou GC, Tsimilli-Michael M and Stamatakis K (2007) The fast and slow kinetics of chlorophyll *a* fluorescence induction in plants, algae and cyanobacteria: a viewpoint Photosynth Res 94: 275–290

Pastenes C and Horton R (1996) Effect of high temperature on photosynthesis in beans. Plant Physiol 112: 1245–1251

Pastori GM and Foyer CH (2002) Common components, networks and pathways of cross-tolerance to stress. The central role of "redox" and abscisic-acid-mediated controls. Plant Physiol 129: 460–468

Pfannschmidt T (2003) Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33–41

Pshybytko NL, Jerzy Kruk J, Kabashnikova LF and Strzalka K (2008) Function of plastoquinone in heat stress reactions of plants. Biochim Biophys Acta. 1777: 1393–1470

Pueyo JJ, Alfonso M, Andres C and Picorel R (2002) Increased tolerance to thermal inactivation of oxygen evolution in spinach photosystem II membranes by substitution of the extrinsic 33-kDa protein by its homologue from a thermophilic cyanobacterium. Biochim Biophys Acta 1554: 22–35

Roose JL, Wegener KM and Pakrasi HB (2007) The extrinsic proteins of Photosystem II. Photosynth Res 92: 369–387

Sakamoto A and Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ 25: 163–171

Salvucci ME and Crafts-Brandner SJ (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments. Plant Physiol 134: 1460–1470

Sane PV, Desai TS, Tatake VG and Govindjee (1984) Heat-induced reversible increase in photosystem I emission in algae, leaves and chloroplasts: spectra, activities, and relation to state changes. Photosynthetica 18: 439–444

Sato N, Sonoike K, Kawaguchi A and Tsuzuki M (1996) Contribution of lowered unsaturation levels of chloroplast lipids to high temperature tolerance of photosynthesis in *Chlamydomonas reinhardtii*. J Photochem Photobiol 36: 333–337

Sazanov LA, Borrows PA and Nixon PJ (1998) The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. FEBS Lett 429: 115–118

Schär C, Vidale PL, Lüthi D, Frei C, Häberli C and Liniger MA (2004) The role of increasing temperature variability in European summer heatwaves. Nature 427: 332–336

Schroda M, Vallon O, Wollman F-A and Beck CF (1999) A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. Plant Cell 11: 1165–178

Seidler A (1996) The extrinsic polypeptides of Photosystem II. Biochim Biophys Acta 1277: 35–60

Semenova GA (2004) Structural reorganization of thylakoid systems in response to heat treatment. Photosynthetica 42: 521–527

Sharkey TD (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant Cell Env 28: 269–277

Shutova T, Kenneweg H, Buchta J, Nikitina J, Terentyev V, Chernyshov S, Andersson B, Allakhverdiev SI, Klimov VV, Dau H, Junge W and Samuelsson G (2008) The photosystem II-associated Cah3 in *Chlamydomonas* enhances the O_2 evolution rate by proton removal. EMBO J 27: 782–791

Singh A and Grover A (2008) Genetic engineering for heat tolerance in plants. Physiol Mol Biol Plant 14: 155–166

Singh AK, Summerfield TC, Li H and Sherman LA (2006) The heat shock response in the cyanobacterium *Synechocystis* sp. strain PCC 6803 and regulation of gene expression by HrcA and SigB. Arch Microbiol 186: 273–286

Slabas AR, Suzuki I, Murata N, Simon WJ and Hall JJ (2006) Proteomic analysis of the heat shock response in *Synechocystis* PCC 6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6: 845–864

Srivastava A, Guisse B, Greppin H and Strasser RJ (1997) Regulation of antenna structure and electron transport in photosystem II of *Pisum sativum* under elevated temperature as probed bythe fast polyphasic chlorophyll *a* fluorescence transient OKJIP. Biochim Biophys Acta 1320: 95–106

Srivastava A, Strasser RJ and Govindjee (1995) Differential effects of dimethylquinone and dichlorobenzoquinone on chlorophyll fluorescence transient in spinach thylakoids. J Photochem Photobiol 31: 163–169

Stirbet A and Govindjee (2011) On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. J Photochem Photobiol B:Biol 104: 236–257

Strasser BJ (1997) Donor side capacity of PSII probed by chlorophyll *a* fluorescence transients. Photosynth Res 52: 147–155

Strasser RJ, Tsimilli–Michael M and Srivastava A (2004) Analysis of chlorophyll *a* fluorescence transient In: Papageorgiou GG and Govindjee (eds) Chlrophyll *a* Fluroscence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 322–358. Springer, Dodrecht

Strehler BL and Arnold W (1951) Light production by green plants. J Gen Physiol 34: 809–820

Sung DY, Kaplan F, Lee KJ and Guy CL (2003) Acquired tolerance to temperature extremes. Trends Plant Sci 8: 179–187

Suzuki I, Kanesaki Y, Hayashi H, Hall JJ, Simon WJ, Slabas AR and Murata N (2005) The histidine kinase Hik34 is involved in thermotolerance by regulating the expression of heat shock genes in *Synechocystis*. Plant Physiol 138: 1409–1421

Suzuki N and Mittler R (2006) Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiol Plant 126: 45–51

Takahashi S and Murata N (2008) How do environmental stresses accelerate photoinhibition? Trends Plant Sci 13: 178–182

Tanaka Y, Nishiyama Y and Murata N (2000) Acclimation of the photosynthetic machinery to high temperature in *Chlamydomonas reinhardtii* requires synthesis de novo of proteins encoded by the nuclear and chloroplast genomes. Plant Physiol 124: 441–450

Tang Y, Wen X, Lu O, Yang Z, Cheng Z and Lu C (2007) Heat stress induces an aggregation of the light-harvesting complex of photosystem II in spinach plants. Plant Physiol 143: 629–638

Tsimilli-Michael M, Stamatakis K and Papageorgiou GC (2009) Dark to light transition in *Synechoccus* sp. PCC 7942 cells studied by fluorescence kinetics asseses plastoquinone redox poise and photosytem II coimponents and dynamics during state 2 to state 1 transition. Photosynth Res 90: 243–255

Tasaka Y, Gombos Z, Nishiyama Y, Mohanty P, Ohba T, Ohki K and Murata N (1996) Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*: evidence for the important roles of polyunsaturated membrane lipids in growth, respiration and photosynthesis. EMBO J 15: 6416–6425

Török Z, Goloubinoff P, Horvath I, Tsvetkova NM, Glatz A, Balogh G, Varvasovszki V, Los DA, Vierling E, Crowe JH and Vigh L (2001) *Synechocystis* HSP17 is an amphitropic protein that stabilizes heat stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. Proc Natl Acad Sci USA 98: 3098–3103

Toth SZ, Schsanker G, Kissiam J, Garab G and Strasser RJ (2005) Biophysical studies of photosystem II related recovery processes after a heat pulse. J Pant Physiol 162: 181–194

Vani B, Mohanty P, Eggenberg P, Strasser B and Strasser RJ (1999) Heat stress on rice leaves probed by chlorophyll *a* fluorescence transient OKJIP. In: Garab G (ed) Photosynthesis: Effects and Mechanisms, pp 3837–3840. Kluwer Academic Publishers, Dordrecht

Vani B, Saradhi PP and Mohanty P (2001) Characterization of high temperature induced stress impairments in thylakoids of rice seedlings. Indian J Biochem Biophys 38: 220–229

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42: 579–620

Vierling E, Mishkind ML, Schmidt GW and Key JL (1986) Specific heat shock proteins are transported into chloroplasts. Proc Natl Acad Sci USA 83: 361–365

Vigh L, Maresca B and Harwood JL (1998) Does the membrane's physical state control the expression of heat shock and other genes? Trends Biochem Sci 23: 369–374

Villarejo A, Shutova T, Moskvin O, Forssen M, Klimov VV and Samuelsson G (2002) A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. EMBO J 21: 1930–1938

Volkov R, Panchuk I, Mullineaux P and Schoffl F (2006) Heat stress-induced H_2O_2 is required for effective expression of heat shock genes in *Arabidopsis*. Plant Mol Biol 61: 733–746

Wada H, Gombos Z and Murata N (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. Proc Natl Acad Sci USA 91: 4273–4277

Wahid A and Shabbir A (2005) Induction of heat stress tolerance in barley seedlings by pre-sowing seed treatment with glycinebetaine. Plant Growth Reg 46: 133–141

Wahid A, Gelani S, Ashraf M and Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61: 199–223

Wang J, Xing D, Zhang L and Jia L (2007) A new principle photosynthesis capacity biosensor based on quantitative measurement of fluorescence *in vivo*. Biosen Bioelect 22: 2861–2868

Weis E (1981) The temperature-sensitivity of dark inactivation and light activation of the ribulose-1,5-bisphosphate carboxylase in spinach chloroplasts. FEBS Lett 129: 197–200

Wen X, Qiu N, Lu Q and Lu C (2005) Enhanced thermotolerance of photosystem II in salt-adapted plants of the halophyte *Artemisia anethifolia*. Plants 220: 486–497

Wilson KE, Ivanov AG, Öquist G, Grodzinski B, Sarhan F and Huner NPA (2006) Energy balance, organellar redox status, and acclimation to environmental stress. Can J Bot 84: 1355–1370

Yamada M, Hidaka T and Fukamachi H (1996) Heat tolerance in leaves of tropical fruit crops as measured by chlorophyll fluorescence. Scientia Hortic 67: 39–48

Yamamoto H, Miyake C, Dietz K-J, Tomizawa K, Murata N and Yokota A (1999) Thioredoxin peroxidase in the cyanobacterium *Synechocystis* sp. PCC 6803. FEBS Lett 447: 269–273

Yamane Y, Shikanai T, Koike H and Satoh K (2000) Reduction of Q_A in the dark: another cause of fluorescence F_o increases by high temperatures in higher plants. Photosynth Res 63: 23–34

Yang X, Liang Z and Lu C (2005) Genetic engineering of the biosynthesis of glycinebetaine enhances photosynthesis

against high temperature stress in transgenic tobacco plants. Plant Physiol 138: 2299–2309

Yang X, Wen X, Gong H, Lu Q, Yang Z, Tang Y, Liang Z and Lu C (2007) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225: 719–733

Yordanov IS, Dilova R, Petkova T, Pangelova V, Goltsev V and Süss K-H (1986) Mechanisms of the temperature damage and acclimation of the photosynthetic apparatus. Photobiochem Photobiophys 12: 147–155

Yordanov I, Goltsev V, Stoyanova T and Venediktov P (1987) High-temperature damage and acclimation of the photosynthetic apparatus. I. Temperature sensitivity of some photosynthetic parameters of chloroplasts isolated from acclimated and non-acclimated bean leaves. Planta 170: 471–477

Yoshioka M, Uchida S, Mori H, Komayama K, Ohira S, Morita N, Nakanishi T and Yamamoto Y (2006) Quality control of photosystem II. Cleavage of reaction center D1 protein in spinach thylakoids by FtsH protease under moderate heat stress. J Biol Chem 281: 21660–21669

Zharmukhamedov SK, Shirshikova GN, Maevskaya ZV, Antropova TM and Klimov VV (2007) Bicarbonate protects the water-oxidizing complex of photosystem II against thermoinactivation in intact *Chlamydomonas reinhardtii* cells. Russian J Plant Physiol 54: 302–308

Chapter 13

Photosynthetic Responses of Plants to Excess Light: Mechanisms and Conditions for Photoinhibition, Excess Energy Dissipation and Repair

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Summary

Plants as sessile organisms must be capable of rapidly coping with changes in environmental conditions. In nature light is the most variable environmental parameter. During the day, plants must deal with changes of several orders of magnitude in the light quantity but also changes in light quality take place. Light is an absolute prerequisite for photosynthesis as an energy source; however, excess light can also be harmful and lead to a destruction of the photosynthetic apparatus. Photoinhibition of photosynthesis has been defined as a light-dependent decline in photosynthetic efficiency as a result of absorption of light. However, a strong consensus is still missing concerning the term photoinhibition and whether it describes a decrease in photosynthetic efficiency due to photodamage and thereby a reduction in the population of functional photosystems or regulatory adjustments, like reduced energy transfer from the antenna to reaction centers or both of these processes. Diurnal photoinhibition is a common phenomenon in most plants exposed to direct sunlight. Depending on the season and also on the diurnal cycle, plants have developed various adaptation systems to cope with highly, as well as frequently, changing light intensity and quality. Although a number of mechanisms have evolved to dissipate excess absorbed light energy by harmless pathways, the photosynthetic apparatus still remains a fragile system and vulnerable to damage by light. This chapter describes briefly the mechanisms of photoinhibition and plant response to light stress. In this chapter, we have used the term photoinhibition to describe the process that finally leads to a photodamage and repair of the reaction centers, while the dissipative regulatory processes are regarded as sole photoprotective processes.

I. Introduction

In photosynthesis, photoautotrophic organisms convert solar energy into chemical energy. Changes in the quality and quantity of solar radiation in turn can modulate the function and structure of the photosynthetic apparatus. Photosynthetic electron transport can be down regulated or inhibited if the absorbed light energy becomes excessive. Light conditions experienced by a plant can be classified into two types: (i) *optimal light*; in such optimal steady-state environmental conditions the absorbed light quanta are utilized via well established biochemical reactions of photosynthesis, and the photodamage and repair are in balance, and (ii) *excess light*, here, absorbed light quanta exceeds their utilization, thus causing photooxidative damage to the photosynthetic apparatus i.e., photoinhibition occurs (Fig. 13.1). One should however note that photodamage to Photosystem II (PS II) occurs at all light intensities, but the net loss of photosynthetic activity occurs only when the rate of photodamage exceeds the rate of repair (Fig. 13.1). Indeed, in addition to light, photoinhibition also depends on other environmental conditions and can occur even at low light. On the other hand, UV radiation has been shown to have a high impact on photosynthesis and thinning of the ozone layer enhances UV-induced photoinhibition of photosynthetic organisms.

The mechanisms of photoinhibition have been under intense discussions and investigations for half a century (Adir et al., 2003; Osmond and Förster, 2005). At early stages of photoinhibition research, it was shown that the action spectrum of photoinhibition follows the action spectrum of photosynthesis. Already in 1956, Bessel Kok proposed that the primary target of photoinhibition is the photosynthetic reaction center (Kok, 1956). Subsequently a decrease in the maximum quantum yield for CO_2 fixation and O_2 evolution has been correlated with a decrease in the variable fluorescence of chlorophyll *a* in PS II in vivo, which implies decreased photochemical efficiency of PS II (for a review see Long et al., 1994). This led to an idea that photoinhibition results from excess energy absorbed by the photosynthetic pig-

Abbreviations: Cyt – Cytochrome; ELIP – Early light-induced protein; LHCI – Light-harvesting complex of PS I; LHCII – Light-harvesting complex of PS II; NPQ – Non-photochemical quenching; PAR – Photosynthetically active radiation; PS I – Photosystem I; PS II – Photosystem II; ROS – Reactive oxygen species; SOD – Superoxide dismutase; Viol – violaxanthin; Zea – zeaxanthin

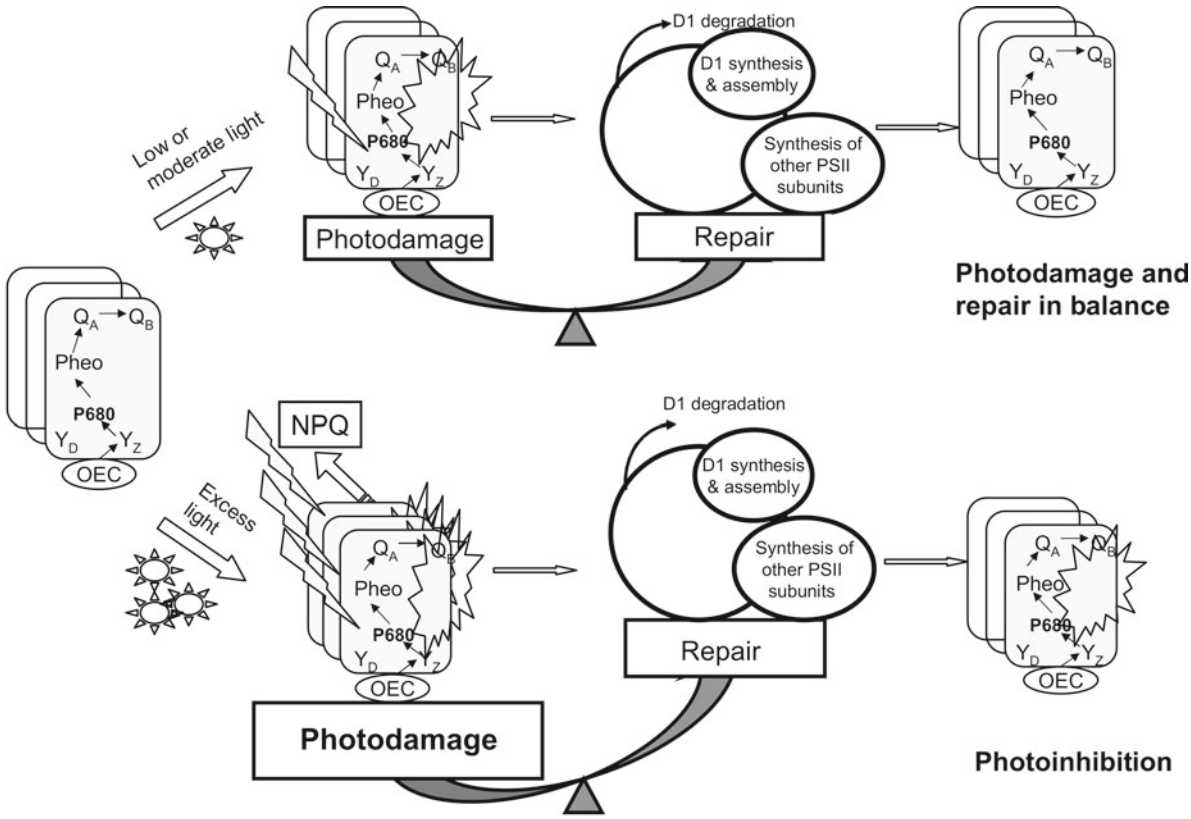

Fig. 13.1. Schematic model of the photoinhibition mechanism. In vivo photoinhibition of the PS II complex is the net difference between the rate of photodamage and the rate of repair. Under low and moderate light the rate of photodamage to PS II and the rate of repair are in balance. Under excess light, photodamage to PS II exceeds the rate of its repair and as a consequence net photoinhibition occurs.

ments and channeled to the PS II complex. Light induced damage is targeted mainly to PS II and leads to inactivation of electron transport and subsequent oxidative damage to the reaction center, in particular to the D1 protein. Excessive damage of PS II may, in turn, be regulated by the repair of the D1 protein and the entire PS II complex and/or by increasing the thermal dissipation of excess energy for example by a high capacity of the xanthophyll cycle. For a long time, the PS II complex captured all attention of investigators as a major site of photoinhibition. However, a series of reports also have been published demonstrating the contribution of also the PS I complex to photoinhibition of photosynthesis, yet the PS I complex shows a relatively high resistance to photodamage.

In general, the conditions where photosynthetic carbon metabolism is suppressed result in a gap between photosynthetic light absorption and its utilization, leading to over-excitation of the photosynthetic electron transfer chain and eventually to photoinhibition of the photosynthetic apparatus. It is also well known that low as well as high temperatures (Powles, 1984; Long et al., 1994; Bongi and Long, 1987), drought, or distortions in nitrogen metabolism (Herrig and Falkowski, 1989) can intensify photoinhibition by inhibiting reactions related to photosynthetic carbon metabolism.

Despite the extensive research in the field, the specific site(s) and mechanisms by which the light inactivates the photosynthetic reaction centers are still under discussion. Here we outline only the major photoinhibition mechanisms and their repair processes. A closely related issue, photoprotection against excess absorbed light energy as an essential and universal attribute of oxygenic photosynthetic organisms, is also discussed in this chapter.

II. Mechanisms and Targets of Photoinhibition

A. Mechanisms of Photoinhibition of the Photosystem II Complex

PS II is a multisubunit pigment protein complex embedded in the thylakoid membrane and catalyses the light-induced reduction of plastoquinone by splitting water (see for review Diner and Rappaport, 2002; Barber, 2006; Nelson and Yocum, 2006; Renger and Renger, 2008; Renger, 2011). Light energy absorbed by the pigment molecules of the light-harvesting antenna system is transferred to the PS II reaction center and causes charge separation between the primary electron donor, P_{680}, and Pheo, the primary electron acceptor. At the acceptor side, the charge separation is stabilized upon electron transfer to Q_A, the primary plastoquinone molecule, and then to Q_B, the secondary plastoquinone acceptor and the plastoquinone pool. Q_A is a tightly bound electron acceptor, whereas Q_B acts as a two-electron and two-proton acceptor. A semiquinone (Q_B^-) intermediate state is stable and tightly bound, while Q_BH_2 state can easily exchange with the plastoquinone pool. At the donor side of the PS II complex, the photooxidized P_{680}^+ is reduced by a redox active Y_Z, which in turn oxidizes the oxygen-evolving complex.

Photoinhibition of PS II in its simplest mode demonstrates that the excess photosynthetically active radiation (PAR) first leads to photoinactivation of PS II electron transport, which is followed by photodamage to the D1 reaction center protein and sometimes also to other proteins of PS II (for reviews see Barber and Andersson, 1992; Aro et al., 1993; Andersson and Aro, 2001).

1. Acceptor Side Photoinhibition

High light stress is known to induce reversible conformational changes at the acceptor side of the PS II complex thereby slowing down Q_A^- to Q_B electron transfer and leading to irreversible D1 protein photodamage (Ohad et al., 1990). During PS II turnover, the primary plastoquinone acceptor (Q_A) is reduced to its semiquinone form (Q_A^-) and then the latter is reoxidized by Q_B. However, under high light conditions, when the plastoquinone pool is highly reduced, the Q_B binding pocket in many PS II centers remains unoccupied due to limitation of oxidized plastoquinone molecules. This in turn leads to a stabilization of Q_A^-. Indeed, in vitro measurements have demonstrated that when the plastoquinone pool is highly reduced and electron transfer from Q_A^- to Q_B is inhibited, Q_A^- can be double reduced and protonated to Q_AH_2 (Vass et al., 1992; Styring and Jegerschöld, 1994) and finally released from its binding site (Koivuniemi et al., 1993). If Q_A is reduced or absent, $P_{680}^+Pheo^-$ cannot relax to form $P_{680}^+Q_A^-$ and there is an increased probability for a spin dephasing on the radical pair resulting in triplet spin configuration $^3(P_{680}^+Pheo^-)$. Recombination of the triplet state of the radical pair gives rise to a $^3P_{680}$ – reaction center triplet state in the nanosecond (ns) time scale (Danielius et al., 1987). $^3P_{680}$ can easily react with molecular oxygen, which is in triplet configuration in its ground state, to produce highly reactive singlet oxygen $^1O_2$ (Durrant et al., 1990), which, in turn, can easily destroy surrounding molecules (also see Vass (2011)).

Taking into account the fact that the inactivation of PS II electron transport due to double protonation of Q_A occurs only under high light under anaerobic conditions, the singlet oxygen that is observed in aerobic photoinhibition conditions must be generated via $^3P_{680}$ formed in PS II centers containing stabilized singly reduced Q_A^- (Vass and Aro, 2007). In such PS II centers the photodamage is the consequence of singlet-oxygen-mediated destruction of the PS II reaction center, rather than blockage of electron transport at Q_A.

Studies on site-directed D2-A249S mutant of *Thermosynechococcus elongatus* (Fufezan et al., 2007) give support to the hypothesis that the redox potential of Q_A determines the charge recombination and hence the ^{3}P680 mediated singlet oxygen formation and consequent amount of PS II photodamage (Krieger-Liszkay and Rutherford, 1998; Rutherford and Krieger-Liszkay, 2001). However, taking into account the significant difference in the measured E_m values of Q_A in *Thermosynechococcus* cells (+84 mV) and in spinach (−80 mV), the application of this model to higher plants needs further investigation.

Hideg et al. (2000) compared the PS II photoinhibition mechanisms when photodamage was induced by excess visible light, by UV-B irradiation in combination with visible light, by low temperature stress in light and by paraquat treatment in light. Although the experimental steps were

arranged such that they resulted in approximately the same extent of PS II photodamage, clearly different molecular mechanisms could be dissected among these treatments. Singlet oxygen production in inactivated PS II reaction centers was shown to be a unique characteristic of photoinhibition by excess visible light. Neither the accumulation of inactive PS II reaction centers (as observed in UV-B/visible light or low temperature/light stress), nor the photooxidative damage of PS II (as observed in paraquat treatment) resulted in production of singlet oxygen, which is characteristic of acceptor-side-induced photoinhibition in excess visible light (Hideg et al., 2000).

2. Donor Side Photoinhibition

Donor side photoinhibition occurs in low and high light under conditions when the electron donation from the oxygen-evolving complex to the P_{680} radical cation, P_{680}^{+}, is disrupted. The P_{680}^{+} has a very high redox potential, estimated to be approximately 1.3 V (Rappaport et al., 2002). This high-oxidation state is required to drive water-splitting reactions on the oxidizing side of the PS II complex by removing electrons from the oxygen-evolving Mn_4Ca-cluster. If there is no natural donor to re-reduce P_{680}^{+} upon illumination, the generation of long-lived P_{680}^{+} and Y_Z radicals takes place. These dangerous radicals, especially P_{680}^{+}, may then oxidize components in their environment leading to destruction of PS II reaction center proteins (Jegerschold et al., 1990). A model proposed by Jan Anderson et al. (1998) suggests that only one photon is required for the photodamage to PS II and that the primary cause of the damage to the D1 protein is P_{680}^{+}, rather than singlet oxygen, which supports mostly the donor-side photoinhibition mechanism under in vivo conditions.

Donor side photoinhibition occurs directly upon illumination of samples in vitro if the activity of the oxygen-evolving complex is inhibited a priori by depletion of manganese with Tris or NH_2OH treatment or by removal of Cl^- and Ca^{2+} ions from the oxygen-evolving complex (Jegerschold et al., 1990; Chen et al., 1992; Jegerschold and Styring, 1996; Krieger-Liszkay and Rutherford, 1998). High sensitivity of PS II to light can also be observed in vivo under conditions in which the activity of the oxygen-evolving complex is inhibited by e.g., site directed mutagenesis with single amino acid changes at the C-terminus of the D1 protein in cyanobacteria (Debus, 2001; Diner and Rappaport, 2002; Allahverdiyeva et al., 2004).

A different 'donor-side photoinhibition' model was presented, based mainly on experiments suggesting that there exists a significant electron-transfer-independent photoinhibitory pathway (Hakala et al., 2005). Moreover, it was reported that the release of a manganese ion to the thylakoid lumen is the earliest detectable step of both the UV- and visible-light induced photoinhibition and that the absorption of light by the oxygen-evolving manganese cluster actually triggers the photoinhibition of the PS II complex (Hakala et al., 2005). This model was extended to a two-step model of photodamage to the PS II complex (Ohnishi et al., 2005), where the light-induced inactivation of the oxygen-evolving complex is postulated to be the first step followed by damage to the photochemical reaction center. There are still ongoing discussions concerning the validity of both the 'acceptor and donor-side photoinhibition' models in explaining the photoinhibition mechanisms in vivo (Tyystjärvi, 2008).

3. Photodamage at Low Light

It is well known that the D1 protein turnover also occurs in vivo at low light intensities, which are far below the intensities that induce PS II photoinhibition. At low light intensities the probability of charge recombination between Q_B^- and the $S_{2/3}$ states of the oxygen-evolving complex is higher than the probability for production of Q_B^{2-} and Q_BH_2 (Keren et al., 1995) and consequently the generation of ^{3}Chl may occur in a process of recombination of long lived Q_B^- with the $S_{2,3}$ states, during the formation of the $P_{680}^{+}Pheo^-$ radical pair. Interaction of ^{3}Chl with molecular oxygen then generates a damaging factor, like singlet oxygen, triggering D1 protein degradation, as has been proposed by experiments with flashes of light or continuous low light illumination in vivo (Keren et al., 1995).

B. Alleviation of Photosystem II Photoinhibition by Rapid Repair

The extent of photoinhibition in vivo can be found as a dynamic imbalance between photodamage to the PS II complex and its repair (Ohad et al., 1984;

Aro et al., 1993, 2005). In other words, in vivo photoinhibition of the PS II complex is the net difference between the rate of photodamage and the rate of repair. As a consequence net photoinhibition occurs when photodamage exceeds the rate of its repair (Fig. 13.1).

Light-induced photodamage of photosystems in many environmental conditions is targeted mainly to the PS II complex. Therefore, the ability of the PS II complex, particularly the D1 protein, to undergo repair during and after the plants have been exposed to light stress is of crucial importance for maintaining the photosynthetic capacity also under stress conditions.

1. D1 Protein and its Degradation

The D1 protein has five transmembrane α-helices (named A to E) that are connected by stromal and lumenal loops. Based on the crystal structure of the PS II complex from a higher plant, the D1 protein together with the homologous D2 protein, forms the reaction center complex, which is the core part of PS II consisting of more than 25 proteins (Hankamer et al., 2001). The D1 and D2 proteins provide the binding sites for all the redox-active cofactors involved in the primary and secondary electron transfer in PS II. Despite the binding of cofactors to both D1 and D2, the unique 'suicide' D1 protein is subjected to irreversible oxidative photodamage by Reactive Oxygen Species (ROS) generated mainly within PS II during exposure to light (Andersson and Barber, 1996; Andersson and Aro, 2001; Yamamoto, 2001). Occasionally, the D2, PsbH, and Cyt b_{559} proteins also become damaged and, thus, require replacement during the PS II repair cycle (Ortega et al., 1999; Rokka et al., 2005).

A partial disassembly of the PS II complex and the presence of a membrane-associated proteolytic enzyme system are prerequisites for degradation of the photodamaged D1 protein in all oxygenic photosynthetic organisms. The mechanisms of D1 protein degradation have been extensively studied in vitro, especially using isolated PS II particles (Aro et al., 1993; Koivuniemi et al., 1995; Adir et al., 2003; Nixon et al., 2010).

The repair process of PS II starts with the monomerization of the PS II complex, which occurs right after the detachment of the Light Harvesting Complex II (LHCII) antenna complex from the PS II dimer. Oxygen-evolving complex proteins dissociate from PS II and a partial disassembly of the PS II core proteins takes place. Such PS II monomers migrate from the grana to the stroma-exposed membranes, where the degradation and synthesis of the new D1 protein take place.

Thus, degradation of the light-damaged D1 protein is a proteolytic process occurring in the stroma-exposed thylakoids and leaves the other PS II proteins most often intact.

2. Involvement of Proteases in the Degradation and Removal of Damaged Photosystem II Subunits

The D1 protein degradation process has been under characterization for the past two decades but only recently have the proteases been identified and characterized. Several different pathways for the removal of the damaged D1 protein and likewise different degradation patterns have been conceived during the past years. These pathways include: (i) D1 protein cleavage on the stromal side by unknown proteases (Andersson and Aro, 2001; Adir et al., 2003); (ii) cleavage in the lumenal side by unknown proteases (Andersson and Aro, 2001; Adir et al., 2003); (iii) degradation by the FtsH protease complex (Bailey et al., 2002; Sakamoto et al., 2002); (iv) degradation of D1 protein aggregates under heat/light stress by unknown proteases (Ohira et al., 2005); (v) a two-step degradation model (Kapri-Pardes et al., 2007); and finally (vi) a model considering the simultaneous cleavage and cross-linking reactions crucial for the complete degradation of the D1-protein in vivo (Mizusawa et al., 2003).

So far, two key chloroplast protease families, the DegP proteases and the FtsH proteases, have been shown to participate in D1 protein degradation and have been studied extensively. Below we shall discuss those proteases more.

a. Deg-family Proteases

The Deg family proteases are present in many organisms from bacteria to higher plants and even in *Homo sapiens*. They are ATP-independent serine endopeptidases. In the *Arabidopsis thaliana* genome 16 *deg* protease genes (originally named

deg1-16) have been identified. Out of 16 Deg proteases, four were found in the thylakoid membrane; Deg1, Deg5, Deg8 were shown to reside in the thylakoid lumen (Itzhaki et al., 1998; Peltier et al., 2002; Schubert et al., 2002), whereas Deg2 was shown to be associated with the stromal side of the thylakoid membrane (Haussuhl et al., 2001). Deg6, Deg9 and Deg16 have only been predicted to be targeted to the chloroplast.

In high light exposed thylakoids, the Deg2 protease was shown to perform the primary cleavage of photodamaged D1 protein in vitro (Haussuhl et al., 2001). Later on, in vivo experiments, however, demonstrated that the rate of the D1 protein degradation under high light stress conditions in the *Arabidopsis thaliana deg2* knock-out mutant was similar to that in the wild type, indicating that the loss of Deg2 might be compensated by other proteases (Huesgen et al., 2006). Thus it has been proposed that several D1 protein degradation pathways might exist in vivo.

Kapri-Pardes et al. (2007) have demonstrated that another Deg protease, Deg1, is involved in degradation of the D1 protein in vivo during the process of PS II repair from photoinhibition. Transgenic plants containing reduced amounts of Deg1 were shown to be more sensitive to photoinhibition than wild-type plants. These transgenic plants also contain higher levels of full-length D1 protein but less of its degradation products and, interestingly, one of the C-terminal degradation products, which has been observed in vivo, can be generated in vitro by recombinant Deg1 (Kapri-Pardes et al., 2007).

In the D1 protein degradation model, Deg1 cooperates with stroma-exposed thylakoid proteases FtsH and Deg2 during the repair from photoinhibition by cleaving the lumen-exposed regions of the protein. Degradation of the D1 protein should therefore be viewed as a two-step process: First a single cleavage at hydrophilic regions of the protein takes place on both sides of the thylakoid membrane by Deg1, Deg2 or other proteases producing a number of fragments and second, the remaining proteolytical degradation is completed by the ATP-dependent FtsH protease (Kapri-Pardes et al., 2007). It is likely that these two steps occur simultaneously.

On the other hand, there are also reports indicating that the FtsH proteases are mainly responsible for D1 protein degradation both in higher plants and in cyanobacteria (Bailey et al., 2001a; Silva et al., 2003; Nixon et al., 2005; also see below).

b. FtsH Family Proteases

The *Arabidopsis thaliana* genome has 12 predicted *FtsH* protease genes (*FtsH1-12*) and the products of nine of them are located in the thylakoid membrane, of which FtsH1, 2, 5–9, 11 and 12 have their protease domains facing the stroma (Sakamoto et al., 2003; Sakamoto, 2006). Proteomic analysis of *Arabidopsis thaliana* thylakoid membranes demonstrated that only four isozymes, FtsH1, 2, 5 and 8, accumulate in *Arabidopsis thaliana* grown under optimal conditions. It is possible that the other isozymes are expressed only at very low levels at specific developmental stages, or under specific environmental conditions. Attempts to clarify the distribution of FtsH proteases in the thylakoid membrane revealed that FtsH2 and/or FtsH8 are abundant mainly in the stroma thylakoids (Lindahl et al., 1996; Komayama et al., 2007). Despite this, detection of FtsH2/FtsH8 proteases also in the PS II-enriched membrane particles confirmed a close association of these FtsH proteases mainly with the PS II complex (Komayama et al., 2007).

The importance of the FtsH protease in the degradation of the D1 protein (Bailey et al., 2002; Nixon et al., 2005) has been confirmed by both in vitro and in vivo studies (Lindahl et al., 2000; Bailey et al., 2002; Sakamoto et al., 2002; Yoshioka and Yamamoto, 2011).

Type A FtsH proteins are encoded by duplicated genes *ftsh1* and *ftsh5*, and Type B proteins by duplicated genes *ftsh2* and *ftsh8*. The two types of subunits, however, are not redundant, and at least one member of each type is essential for the accumulation of the active FtsH protease complex (Zaltsman et al., 2005b). It has been suggested that the loss of one FtsH protein does not cause lethality because of the functional interchangeability among the FtsH homologs and that the variegation specifically detected as a result of the loss of either FtsH2 or FtsH5 shows that their expression could be coordinately regulated (Sakamoto, 2006).

Mutation of *FtsH5 (var1)* and *FtsH2 (var2)* gives rise to variegated plants with green and white-sectored leaves (Chen et al., 2000; Sakamoto

et al., 2002). Variegated mutants are sensitive to light stress, and they accumulate PS II centers with photodamaged D1 protein. Interestingly, cells in green sectors of the leaf contain morphologically normal chloroplasts, whereas cells in white sectors are blocked in chloroplast biogenesis. Detailed characterization of the *Arabidopsis thaliana FtsH* double mutants showed that the appearance of white tissues are more intense in *ftsh1/ftsh5* and *ftsh2/ftsh8* double mutants than in *ftsh2/ftsh5* double mutants (Zaltsman et al., 2005a). Expression studies on FtsH isomers at the transcript level indicate that some chloroplastic FtsHs, such as FtsH8, are highly inducible by high light (Zaltsman et al., 2005b). High-light dependent degradation of D1 protein did not occur in *var2-2* leaves, confirming that FtsH is a critical factor in the PS II repair cycle.

3. De novo Synthesis and Assembly of the D1 Protein

A constant repair of photodamaged PS II complexes by D1 protein turnover is essential for the maintenance of a sufficient level of functional PS II complexes. Thus the degradation of photodamaged D1 protein is rapidly followed by insertion of the de novo synthesized D1 protein into the PS II complex.

In the synthesis of the new D1 protein, the nascent D1 protein is co-translationally inserted into the thylakoid membrane where the Cyt b_{559} and D2 act as first assembly partners. It was demonstrated that not only the insertion into the membrane but also the assembly of the D1 protein into PS II complex, composed of Cyt b_{559}, D2, and possibly also of CP47 and several low-molecular- weight subunits, occur co-translationally during the repair process (Zhang et al., 2000; Rokka et al., 2005). Re-synthesis of the D1 partner subunits is not needed, since they are already present in the existing PS II centers that undergo repair. After processing of the D1 protein at its C-terminus by a protease on the lumenal side of the thylakoid membrane, the reassembly of the internal core antenna protein CP43 occurs. Before reassembly of oxygen-evolving-complex proteins, most of the low-molecular-weight subunits have to be assembled to the PS II complex. The reassembled PS II monomer migrates back to the grana thylakoids, where dimerization, and association with the LHCII antenna proteins take place.

One should bear in mind that light- and redox-controlled protein phosphorylation is an important issue in the modulation of photosynthesis under fluctuating environmental conditions. The stage from photodamage to degradation of the D1 protein is regulated by phosphorylation-dephosphorylation events of the core proteins (Koivuniemi et al., 1995; Rintamäki et al., 1996).

C. Photoinhibition of Photosystem I

PS I is a membrane-bound pigment-protein complex, which drives the photosynthetic electron transfer from plastocyanin to ferredoxin, thus also mediating electron transfer from water to $NADP^+$. The 3.4 Å resolution crystal structure of the PS I supercomplex from a higher plant (*Pisum sativum*) has been determined (Amunts et al., 2007). The plant PS I complex is composed of a reaction center complex (PsaA/PsaB) with up to 15 other protein subunits and 4 different types of abundantly expressed membrane-associated light-harvesting antenna complex proteins (Lhca1 – Lhca4) (Nelson and Yocum, 2006).

1. Mechanisms of Photosystem I Photoinhibition

During early stages of the studies of high light effects on the photosynthetic apparatus, the inhibition of both the PS II and PS I complexes was reported (Satoh, 1970; Satoh and Fork, 1982). However, for a long time the term photoinhibition has mainly been restricted to photoinhibition of PS II, and the D1 protein has been considered as the primary target of photoinhibition (Andersson and Styring, 1991; Barber and Andersson, 1992; Aro et al., 1993). This is because the PS I complex is more resistant to high light than the PS II complex particularly under conditions where high light is the sole stress factor (Powles, 1984).

We now know that in chilling sensitive plants, at chilling and low light conditions, the activity of the PS I complex selectively decreases while that of the PS II complex remains practically unchanged (Havaux and Davaud, 1994; Sonoike and Terashima, 1994; Terashima et al., 1994). Chilling temperature and concomitant oxidative stress are considered as major requirements for

PS I photoinhibition in vivo (Sonoike, 1996; Scheller and Haldrup, 2005). It is apparent that photoinhibition of PS I at chilling temperatures is also an important phenomenon for cold-tolerant plant species (Ivanov et al., 1998; Tjus et al., 1998, 1999). Thus photoinhibition of the PS I complex is a universal phenomenon and is not limited to cold-sensitive plants. It has been suggested that the photodamage to PS I, occurring in vivo during low light illumination at chilling temperatures, results from insufficient defense against ROS by reactive-oxygen-scavenging enzymes at these specific conditions. However, strong illumination simultaneously inhibits both the PS I and the PS II complexes.

PS I generates the most negative redox potential in photosynthesis. The mechanism of damage to the PS I complex is mainly associated with the reduction of molecular oxygen at the reducing side of the PS I complex and thus oxidative destruction of the iron-sulfur clusters, F_A and F_B, which are bound to the PsaC subunit at the stromal side of the PS I complex (Sonoike et al., 1995). Earlier electron acceptors, like the F_X iron-sulfur center, as well as A_0/A_1, the primary and secondary electron acceptors of PS I, may also undergo oxidative destruction under low light and chilling temperatures. Photoinhibition under moderate light and chilling temperature accelerates the rate of P_{700}^+ dark re-reduction, via an increased rate of electron donation from stromal reductants to PS I through the plastoquinone pool (Bukhov et al., 2004). However, P_{700}, the primary electron donor of the PS I complex, is known as a relatively stable cofactor of the electron transport chain under photoinhibitory conditions.

We have known for some years that the mechanism of PS I photoinhibition involves several steps; ultimately, there is a complete destruction of the PS I complex. Inactivation of the Fe-S clusters at the acceptor side of PS I, which is believed to be the first step of PS I photoinhibition, is followed by the destruction of P_{700}. This is followed by the destruction of the other PS I reaction center chlorophylls, and the degradation of the PS I core proteins. Repair of PS I centers starts only when the plants are shifted to normal growth temperatures.

Photoinhibition of PS I is highly dependent on the presence of oxygen, suggesting that the production of ROS is directly involved in PS I photoinhibition. Interestingly, the ROS, produced in PS I, may exert damage not only to PS I, but also to the PS II complex (Tjus et al., 2001). PS I photodamage is suggested to be caused by superoxide produced in the complex. Indeed, contrary to earlier suggestions (Chung and Jung, 1995), singlet oxygen is not produced in PS I under photoinhibitory conditions (Hideg and Vass, 1995). It is well known that chloroplastic Cu/Zn superoxide dismutase (Cu/ZnSOD) and ascorbate peroxidase are effective scavengers of ROS generated on the reducing (electron acceptor) side of the PS I complex during photoinhibition. However, Choi et al. (2002) have demonstrated that the light-chilling stress specially inhibits the activity of Cu/Zn-SOD, without affecting other antioxidant enzymes such as ascorbate peroxidase. Furthermore, in vivo photoinhibition of PS I is accelerated by an inhibitor of SOD given during the chilling-light treatment (Hwang et al., 2004). Thus, the insufficiency of the defense against ROS by active scavenging systems, particularly SOD, under these specific conditions makes the PS I complex sensitive to photodamage at chilling temperatures. This is also in line with the finding that photoinhibition of PS I in vitro in isolated thylakoids occurs irrespective of the temperature during the light treatment.

2. Degradation of the Subunits and Repair of Photosystem I

Similar to PS II, photoinhibition of the PS I complex causes degradation of the reaction center heterodimer subunits– PsaA and PsaB as a consequence of photosynthetic electron transfer inactivation at chilling-light stress (Tjus et al., 1999; Kudoh and Sonoike, 2002; Zhang and Scheller, 2004). Degradation products of PsaB protein can be easily detected. In chilling-tolerant barley plants, a degradation product from the nuclear encoded PsaD subunit was revealed as a result of photoinhibition of the PS I complex (Tjus et al., 1999). In contrast to the results obtained with barley, the extent of PS I reaction center degradation in chilling-sensitive cucumber leaves is very small, whereas the activity of the PS I complex decreased to a larger extent than in barley. According to Tjus et al. (1999), this may reflect species-specific differences with respect to protease availability and acclimation mechanisms (Tjus et al., 1999). Another interesting explanation given to this phenomenon is that the cold-tolerant barley may be

'optimized' to degrade the photodamaged proteins more efficiently thereby allowing their faster replacement with de novo synthesized subunits. The pronounced protein degradation in barley may constitute a part of the repair cycle, whereas the limited degradation in cold-sensitive cucumber may indicate irreversible damage.

Although the PS I protein degradation is induced by ROS, it is rather a proteolytic process, than a passive photo-destructive process (Kudoh and Sonoike, 2002). In the past, it had been assumed that the protein degradation of damaged PS I subunits is not the primary effect of photodamage to PS I, rather, it follows the initial damage in photosynthetic electron transfer cofactors under light-chilling stress in barley and cucumber plants (Tjus et al., 1999; Kudoh and Sonoike, 2002). Nevertheless, in *Arabidopsis thaliana,* despite observed severe damage to PS I, no degradation of PS I proteins was detected during chilling-light stress (Zhang and Scheller, 2004).

Since the first observation of PS I photoinhibition, it took a long time before the repair cycle of the PS I complex was considered. Teicher et al. (2000), Kudoh and Sonoike (2002) and Zhang and Scheller (2004) have indicated that the repair of the PS I complex, upon transfer of plants to room temperature after chilling-light damage, is a very slow process taking many days and even after 1 week it is still incomplete. In *Arabidopsis thaliana* the recovery from chilling-light photoinhibition, and the re-synthesis of PS I, is a very slow process, but more complete than in cucumber (Kudoh and Sonoike, 2002; Zhang and Scheller, 2004).

The repair of the PS I complex is not similar to the PS II repair cycle, where the D1 protein undergoes a very fast turnover and the rest of the subunits are mostly reused. During recovery at 20°C after the chilling-light stress, all the PS I core subunits were completely degraded, indicating that no photodamaged PS I remained in the thylakoid membrane in the initial phase of recovery. According to Zhang and Scheller (2004), the degradation of damaged PS I is not a primary result of photodamage, but it can be considered as a step in its recovery. The LHCI proteins degraded much more slowly and to a smaller extent than the core subunits (Zhang and Scheller, 2004).

There is still a fundamental lack of information both about the turnover of PS I subunits after photodamage and about de novo synthesis and assembly of the PS I complex. Moreover, the knowledge of the proteases involved in the repair cycle of PS I is completely missing.

III. Susceptibility of Plants to Photoinhibition in the Presence of Other Environmental Stress Factors

In nature, excess light often prevails in combination with other environmental stress conditions, which collectively intensify the photoinhibition effects to different extents.

A. Photoinhibition Under Limitation of Electron Transfer to CO_2 and Other Terminal Electron Acceptors

The products of photosynthetic light reactions, which convert absorbed light energy into chemical energy (NADPH and ATP), are primarily used for CO_2 fixation to form carbohydrates. Another main consumer of photosynthetically generated electrons is the production of 2-phosphoglycolate in the photorespiratory pathway. Both competing reactions are catalyzed by Rubisco, the most abundant enzyme of the planet.

The photorespiratory pathway has been listed to be one of the mechanisms responsible for protecting PS II against photoinhibition (Osmond, 1981; Kozaki and Takeba, 1996; Osmond et al., 1997; Wingler et al., 2000). It has been argued that the utilization of molecular oxygen as a terminal electron acceptor, both in the Rubisco oxygenase photorespiration pathway and in the water-water cycle, protects the PS II complex against photoinhibtion in vivo (Park et al., 1996). This idea was based on the fact that the consumption of photochemical energy through the photorespiratory pathway plays an important role in protecting PS II against photooxidative damage by alleviating the generation of $^1O_2$. Contrary to these reports, data obtained by *Arabidopsis thaliana* mutants of the photorespiratory pathway suggest that the impairment of the photorespiratory pathway accelerates photoinhibition by suppression of the repair of photodamaged PS II, not by accelerating the photodamage to PS II (Takahashi et al., 2007).

It is well known that CO_2 fixation is sensitive to various environmental stresses, such as low temperature or drought, which decrease the

Calvin-Benson cycle activity. This in turn results in inhibition of the photosynthetic electron transport activity and also to a limitation of other metabolic processes like photorespiration, and nitrogen metabolism. Moderately elevated temperatures also can inhibit the activation of Rubisco via a direct effect on Rubisco activase (Crafts-Brandner et al., 1997; Feller et al., 1998) and thus enhance photoinhibition. The primary target of photoinhibition in these cases is supposed to be mainly the PS II complex. Inhibition of CO_2 fixation might limit the synthesis of the D1 protein during the repair of photodamaged PS II complexes (Takahashi and Murata, 2006).

It has been reported that severe CO_2 limitation by lack of functional Rubisco enzyme leads to photoinhibition of PS I (Allahverdiyeva et al., 2005). These authors have also investigated the response of the thylakoid components to conditions of severe deficiency of terminal electron acceptors of the photosynthetic light reactions by using a transplastomic Δ*rbcL* tobacco line lacking the large subunit of Rubisco, the key enzyme of CO_2 fixation. (Allahverdiyeva et al., 2005). Illumination of these mutant plants even at low light induced a strong pressure on PS I, which in turn resulted in donation of electrons to molecular oxygen with concomitant production of highly dangerous ROS. The Δ*rbcL* plants had, however, adopted several mechanisms to avoid PS I-mediated ROS production. Allahverdiyeva et al. (2005) showed significant down-regulation of PS I contents, thus, revealing an acclimation strategy of the photosynthetic apparatus known to occur at high light. We also observed free LHCI proteins in Δ*rbcL* plants suggesting an impaired interaction between LHCI and the PS I core, thereby reducing PS I excitation.

B. Combination of Light and Chilling Temperature

Exposure of leaves to chilling temperatures in the light can result in severe damage to the photosynthetic apparatus (see for a review Huner et al., 1998). At chilling temperatures, the target of photodamage in the photosynthetic apparatus differs depending on plant species and environmental conditions. The damage to the function of the photosynthetic apparatus can be induced by chilling temperatures even in darkness; however, it is always more marked under light.

There are several and sometimes controversial opinions about the main targets and causes of chilling-light stress. These include: (i) PS I; (ii) PS II repair cycle; (iii) downstream components of photosynthetic electron transfer; (iv) other regulatory processes, like the xanthophyll cycle, saturation level of thylakoid membrane lipids and others.

As discussed above, PS I is particularly sensitive to photoinhibition at chilling temperatures. One should also notice that the damage to photosynthesis due to light-chilling stress has an irreversible characteristic. Upon low light-chilling stress, along with PS I photoinhibition, an uncoupling of ATPase (Terashima et al., 1991) and an inactivation of Calvin-Benson cycle enzymes were also observed. However, the uncoupling of ATPase and the activity of the Calvin-Benson cycle enzymes are rapidly restored after the damaged leaves are returned to normal growth conditions. Nevertheless, the recovery of the PS I complex from chilling-light stress under normal growth conditions is clearly slower than the recovery of PS II and it is incomplete (Kudoh and Sonoike, 2002; Zhang and Scheller, 2004; Scheller and Haldrup, 2005). Thus the irreversible nature of light-chilling damage can be explained partially by the chilling induced photoinhibition of PS I.

Besides the sensitivity of PS I to photoinhibition at chilling temperatures, PS II also shows enhanced photoinhibition upon lowering of the temperature. Higher susceptibility of plants to photoinhibition at low temperatures seems to be related to the temperature dependence of the recovery of photodamaged PS II. Indeed, the rate of recovery of the PS II complex is very slow at chilling temperatures (Aro et al., 1990; Salonen et al., 1998) suggesting that low temperature accelerates photoinhibition through suppression of the repair of PS II. Likewise, the studies on the relationship between the saturation level of thylakoid membrane lipids and the susceptibility of higher plants to chilling-light stress showed that the extent of unsaturation of phosphatidylglycerol in the thylakoid membrane do not affect the process of photoinhibition per se, but higher unsaturation enhanced the recovery process of the photosynthetic apparatus (Moon et al., 1995).

Even when the repair of the PS II complex is inhibited, the cold-tolerant plants are more

resistant to photoinhibition than the cold-sensitive plants. This difference between the cold-tolerant and cold-sensitive species was shown to result from more severe inhibition of photosynthesis in cold-sensitive plants rather than from the differences in low-temperature inhibition of the PS II repair cycle (Hurry and Huner, 1992; Öquist et al., 1993). It was further demonstrated that given a certain excitation pressure on the PS II complex, the susceptibility of photosynthesis to photoinhibition occurs independently of the temperature in the range from 0°C to 25°C (Öquist et al., 1993). Yet several other factors are involved in chilling-induced photoinhibition. Enhanced PS II and PS I photoinhibition in vivo at chilling temperatures may be triggered by reduced functional activity of the xanthophyll cycle or the antioxidative scavenging systems. Thus, the resistance of chilling tolerant spinach to photoinhibition of PS II and PS I was explained with faster de-epoxidation kinetics of violaxanthin as compared to three chilling-sensitive species (Barth and Krause, 1999; Barth et al., 2001).

It is also likely that the photoinhibition of PS II and PS I might be only a secondary response to the light-chilling stress while the primary targets are possibly found in the downstream steps of the photosynthetic electron transfer chain, in carbon metabolism and in stomatal conductance (Allen and Ort, 2001). Indeed, there are reports showing chilling damage of Rubisco itself in darkness (Kingston-Smith et al., 1997). Exposure of cucumber leaf discs to light-chilling stress also produced the fragmentation of the large subunit of Rubisco which, however, could be completely inhibited by ROS scavengers (Nakano et al., 2006). In their experiments, the fragmentation of Rubisco occurred a few hours after PsaB degradation. It is therefore conceivable that the limitation of CO_2 fixation at low temperature resulted in accumulation of reducing power on the acceptor side of PS I thus damaging the Fe-S centers via PS I photoinhibition, which subsequently caused the fragmentation of Rubisco (Nakano et al., 2006).

Indeed, the combination of light and chilling temperatures modulate the function of the photosynthetic apparatus in a number of different ways and the primary targets of damage are likely to be dependent on plant species and specific environmental conditions to which the plants are exposed.

C. Combination of High Light and High Temperature

Often, both high temperature and bright light occur together in many places in nature, particularly in deserts but also during noon hours in many other habitats.

In darkness, heat stress is known to induce a release of several extrinsic PS II oxygen–evolving complex proteins, PsbO, PsbP and PsbQ; this, in turn, significantly modifies the degradation and aggregation of the D1-protein upon subsequent exposure to light (Komayama et al., 2007). Thus, modifications of the PS II donor side under heat stress conditions leads to donor side photoinhibition of the PS II complex (Yamamoto et al., 1998; Yamamoto, 2001).

In addition to the detrimental effect on the PS II oxygen-evolving complex, moderately high temperatures also inhibit light-dependent activation of Rubisco, and this inhibition has been shown to be closely correlated with inhibition of photosynthetic CO_2 fixation (Berry and Björkman, 1980; Weis, 1981; Feller et al., 1998; and Law and Crafts-Brandner, 1999). It still remains unclear whether the inhibition of Rubisco activase is a key regulatory process affected by high-temperature stress or whether the primary target of high-temperature inactivation is the photosynthetic thylakoid membrane, particularly the PS II complex (Havaux and Tardy, 1996).

D. Combination of Light and Drought Stress

Plants in nature are frequently exposed to drought, which is likely to promote the photoinhibitory stress of plants (Björkman and Powles, 1984; Medrano et al., 2002). In general, the inhibition of photosynthesis under mild drought stress is mostly due to stomatal restriction of CO_2 availability (Cornic, 2000; Chaves, 1991). Drought stress in plants, however, clearly has two components: stomatal and nonstomatal (Flexas and Medrano, 2002). During the onset of drought, stomatal conductance normally decreases (stomatal limitation), which prevents the plants from extensive water loss, but at the same time limits CO_2 uptake. In addition, a nonstomatal limitation of net photosynthesis, directly related with metabolism in chloroplasts, has been observed in a wide range of experiments.

In fact, drought stress of plants closely resembles the situation of impaired CO_2 fixation, where the interplay between the production of photosynthetic reducing power and its consumption is disturbed. From this point, it is understandable that plants grown under drought stress conditions show saturation of photosynthesis at lower light intensities compared to those grown under well-hydrated conditions.

Despite drought-induced limitation of CO_2 fixation, the photosynthetic electron-transport chain appears relatively resistant to drought (Cornic and Briantais, 1991; Tourneux and Peltier, 1995). This was interpreted to result from stimulation of O_2 reduction in the absence of available CO_2 (Cornic and Briantais, 1991). Nevertheless, more recent studies have demonstrated a decrease in PS II quantum yield under dehydration (Loggini et al., 1999; Golding and Johnson, 2003) but this phenomenon was interpreted as a down-regulation of the PS II complex by increased non-photochemical quenching (NPQ) of excited state chlorophyll (Chl*), rather than as a photodamage to the PS II complex. High NPQ in turn resulted from an increased cyclic electron flow around the PS I complex that maintains the pH gradient across the thylakoid membrane and eventually protects the PS II complex under drought conditions (Golding and Johnson, 2003). Other protection mechanisms against drought include a decreased conductance of the electron-transport chain between PS II and PS I, thus decreasing the electron flux to PS I and eventually to O_2. This is a specific feature of regulation of the photosynthetic apparatus under drought conditions (Ott et al., 1999; Golding and Johnson, 2003).

IV. Mechanisms to Avoid Photoinhibition

Plants employ multiple mechanisms to adapt to the seasonal and daily fluctuations in light and temperature regimes associated with their habitats (Takahashi and Badger, 2011). Photosynthetic organisms have developed a number of protective mechanisms in order to avoid damage to the photosynthetic apparatus under excess light conditions. These include a regulation strategy at the level of the leaf and/or the chloroplasts. Strategy at the leaf level for long-term acclimation to light intensity includes changes in the number of chloroplasts per unit leaf area, which was found to be associated with the photosynthetic capacity (Murchie and Horton, 1997) and also an alteration in leaf thickness and orientation. The potential for acclimation of the photosynthetic apparatus at the leaf level can be quantitatively related to the habitat preference of a given species (Murchie and Horton, 1997).

Chloroplast-level regulation strategies can be divided into short-term and long-term responses (Bailey et al., 2001b). Short-term regulation of light harvesting occurs on a timescale of minutes by various distinct mechanisms: the state-transitions (Allen and Forsberg, 2001; Allen, 2003), xanthophyll and/or PsbS dependent NPQ (Kulheim et al., 2002; Demmig-Adams and Adams, 2003; Niyogi et al., 2005), reversible phosphorylation of thylakoid membrane proteins and the ratio of distribution of electrons between the linear and cyclic electron transfer routes (Kanervo et al., 2005). Long-term acclimation takes days and weeks and involves changes in the amounts of antenna proteins associated with PS II and PS I, as well as in the stochiometry of the reaction center complexes (Bailey et al., 2001b) and the amount of Rubisco (Stitt, 1986; Seemann et al., 1987).

A. Regulation of the Antenna Systems

1. Dynamic Composition of Light-Harvesting Antenna

Photosynthesis is driven by the absorption of a photon by a chlorophyll or carotenoid pigment embedded in the light-harvesting antenna complex. The light-harvesting antenna complexes of PS I and PS II show significant structural and functional flexibility, which makes a photosynthetic organism capable of adapting to continuously changing environmental conditions. Growth conditions also modify the composition and dynamics of the antenna proteins in order to adapt to the changes in the quality and quantity of the light. Small PS II antenna size is typical of high-light-acclimated plants and transfer of plants to low light results in an increase in the antenna size. Interestingly, different antenna polypeptides show different behavior. The Lhcb1, Lhcb2, Lhcb3 and Lhcb6 proteins undergo major levels of regulation by the light intensity, clearly decreasing under high light conditions. In contrast, Lhcb4 and Lhcb5 do

not show any significant difference at different growth light intensity, and they maintain a strict stoichiometry with respect to the PS II reaction center (Ballottari et al., 2007). However, comparing results from different laboratories is difficult due to differences in light intensities and regimes used in experiments.

PS I light harvesting is also regulated through different mechanisms, like, e.g., the control of the PS I/PS II ratio and regulation of the association of Lhcb polypeptides with PS I (Tikkanen et al., 2006). Dissociation of LHCI from the PS I core is yet another structural mechanism for protection of the PS I complex and serves to limit the excitation of the reaction center (Hwang et al., 2004).

Adjusting the antenna capacity to increasing light intensities involves the degradation of Lhcb polypeptides and other related proteins (Lindahl et al., 1995; Forsberg et al., 2005). Strong light induces monomerization of trimeric Lhcb complexes (Garab et al., 2002) prior to their degradation (Lindahl et al., 1995). This might be related to the fact that the trimeric Lhcb complex is protected from degradation due to a close interaction between the N-terminal parts of the Lhcb proteins, which hide the protease recognition side (Yang et al., 2000).

Another high-light-induced property of the antenna system is the induction of Early Light Inducible Proteins (ELIP). Like Lhcb proteins, the ELIP family members in higher plants are nuclear-encoded proteins located in the thylakoid membrane. Synthesis of ELIP proteins is triggered by various abiotic stress conditions, when the expression of Lhcb proteins is down-regulated (Montane and Kloppstech, 2000; Adamska et al., 2001; Heddad et al., 2006). ELIPs show very high sequence homology with the Lhcb proteins but differ from those proteins by their smaller size and transient appearance (Adamska, 1997) (see Chapter 14). ELIP proteins have a photoprotective role under light stress conditions either by transient binding of released Chls, thus preventing the formation of ROS, and/or by participating in energy dissipation. In *Arabidopsis thaliana*, ELIP1 and ELIP2 proteins are differently expressed in response to light stress and their expression is controlled at transcriptional and/or translational/posttranslational levels (Heddad et al., 2006). The accumulation of ELIP1 was shown to increase with increasing light intensity and was correlated with the photodamage of the PS II centers. Interestingly, a large accumulation of ELIP2 was observed only when approximately 40% of PS II was damaged and had lost their D1 protein. Thus, it was suggested that most probably ELIP1 and ELIP2 function at different phases of photoinhibition and occur in different LHCII subpopulations (Heddad et al., 2006).

Among photosynthetic pigments of the major antenna systems, the carotenoids are crucially important molecules in photoprotection, since they are able to quench singlet oxygen and also they can react directly with triplet chlorophyll, thus avoiding production of $^1O_2$ (Krinsky, 1979; Edge and Truscott, 1999). Hence an increase in the carotenoid content is clearly a protective mechanism, and the increase of this pigment has been observed in various plant species upon long term acclimation to excess light (Adams et al., 1995) and chilling-high light conditions (Havaux and Kloppstech, 2001).

2. Non-photochemical Quenching and State Transitions

Plants protect themselves against the destructive effect of absorbed excess light energy by quickly and reversibly switching on non-radiative and harmless pathways for dissipation of energy as heat. This process is known as NPQ and observed as a quenching of chlorophyll fluorescence by increased dissipation of energy as heat. NPQ has three components and each of them has characteristic induction and relaxation kinetics: rapidly reversible, ΔpH dependent component (q_E); state transition related component (q_T); and slowly reversible, photoinhibition related component (q_I).

The major component of NPQ, $\mathbf{q_E}$, which is also called feedback de-excitation, can quench up to 80% of singlet chlorophyll molecules $^1Chl^*$ (Demmig-Adams and Adams, 1996; Li et al., 2000). q_E is mainly regulated by acidification of the lumenal compartment of the thylakoids (Muller et al., 2001), but how exactly the process is triggered is not yet precisely known. Plants grown under high light conditions demonstrate a faster induction of energy dissipation (q_E). This very often correlates with higher accumulation of a specific PS II subunit, PsbS. The essential role of PsbS in the development of q_E and its importance in plant fitness has been demonstrated

(Kulheim et al., 2002). Nevertheless, the exact functional mechanism of the PsbS protein is still unclear. Also the results on the location of PsbS are contradictory; in some cases, it has been suggested to be closely associated with the LHC antenna, but in another case with the PS II core (Holt et al., 2004), thus PsbS also may have mobility in the thylakoid membrane (Teardo et al., 2007).

Another key component responsible for q_E establishment is the conversion of violaxanthin (Viol) to zeaxanthin (Zea) via the xanthophyll cycle. This process occurs under strong light upon acidification of thylakoid lumen. Zea is directly involved in quenching of chlorophyll excitation. The *Arabidopsis thaliana npq1* mutant, which lacks functional violaxanthin de-epoxidase and consequently is not able to convert Viol to Zea in high light, showed reduced qE. Interestingly, mutant plants could compensate inhibited NPQ by a strong reduction of the PS II antenna size, in order to avoid excessive excitation pressure on PS II and subsequent photoinhibition (Havaux and Kloppstech, 2001). As a consequence, *npq1* chloroplasts are intrinsically acclimated to high light conditions, whereas wild-type chloroplasts are more flexible, keeping large chlorophyll antenna for optimal light harvesting in limiting light conditions and exhibiting high NPQ capacities for dissipation of excess energy in high light (Havaux and Kloppstech, 2001).

In addition to direct quenching, Zea has been proposed to have a role also in indirect quenching by organizing the light-harvesting antenna complexes and resulting in Chl-Chl quenching. In the *Arabidopsis thaliana* double mutant *lut2npq2*, all xanthophylls are replaced constitutively by Zea. While the PS II photoinhibition in the wild type and *lut2npq2* double mutant is similar when exposed to chilling-high light stress, the double mutant is much more resistant to photooxidative stress and lipid peroxidation than the wild type (Havaux et al., 2004). Thus, most likely Zea plays a role in long-term photoacclimation.

q_T – another component of NPQ – is related to the state-transition, which is one of the important mechanisms in short-term adaptation of the photosynthetic apparatus to changes in light quality and quantity (Allen and Forsberg, 2001; Haldrup et al., 2001; Wollman, 2001; Allen, 2003). State-transition balances the energy between PS I and PS II, and is based on the association of LHCII with PS II (State I) or PS I (State II). (State I is attained when plants are exposed to light absorbed in PS I, and State II when plants are exposed to light absorbed in PS II). When PS II excitation is favored, occurring generally also under low light conditions, the mobile LHCII is phosphorylated and attaches to the PS I complex. Consequently, due to reduction in the PS II antenna size, PS II fluorescence yield diminishes and this is considered as a q_T component of NPQ. Under strong light conditions and when the PS I excitation is favored, LHCII is dephosphorylated and attaches to the PS II complex, increasing PS II antenna size, and, thus, PS II fluorescence yield.

In *Arabidopsis thaliana,* state transitions are catalyzed by the thylakoid associated STN7 kinase (Bellafiore et al., 2005); besides the phosphorylation of the Lhcb1 and Lhcb2, the phosphorylation of Lhcb4.2 (CP29) is also under the control of the STN7 kinase (Tikkanen et al., 2006). STN7 kinase and state transitions were shown to function as a buffering system upon short high irradiance peaks by shifting the thylakoids from State II to State I and thereby down regulating the induction of stress-responsive genes, a likely result from transient over-reduction of PS I acceptors (Tikkanen et al., 2006).

q_I – the third component of NPQ is fluorescence quenching related to photoinhibited PS II reaction centers. The q_I component is a very slowly reversible (or irreversible) process and also considered as a photoprotective process. When a substantial number of nonfunctional PS II centers have accumulated in thylakoids, the photoinhibited PS II centers become strong quenchers by dissipating excitation energy harmlessly in vivo (Matsubara and Chow, 2004; Sun et al., 2006). Thus photoinhibited PS II centers can avoid further damage to them and protect their undamaged neighbors by acting as strong energy sinks. The q_I – state is also associated with Zea accumulation in LHCII.

3. Non-photochemical Quenching of Photosystem I

A nonphotochemical type of fluorescence quenching in PS I in vitro was proposed by Rajagopal et al. (2003). Nonphotochemical energy dissipation in PS I can be promoted by $NADP^+$ which

triggers protein conformational changes and thereby reduces the efficiency of energy transfer from the antenna Chls to the reaction center of PS I, P_{700}, consequently decreasing the rate of P_{700} photooxidation. Importantly, this process is fully reversed if NADPH is added. It has been suggested that this energy dissipation mechanism could play a role in proper redox poising of the intersystem electron carriers (Rajagopal et al., 2003).

B. Regulation of Photosynthetic Electron Transfer Pathways

Preacclimation of plants to high light or to chilling temperature increases the resistance of PS I to photoinhibition (Ivanov et al., 1998). It is also known that the damage to PS I under chilling-light stress is progressive until a certain point and thereafter it levels off (Zhang and Scheller, 2004). The reason for these observations probably involves a combination of adaptation mechanisms triggered by a particular stress condition.

In a simple model, the photosynthetic electron transfer rate within PS I is mainly controlled (i) by the flow of electrons donated by PS II through the Cyt b_6f complex and (ii) by the activity of CO_2 fixation, which utilizes NADPH formed at the acceptor side of PS I. Several protection mechanisms of the PS I complex against photoinhibition have been proposed. These include the water-water cycle, cyclic electron transport around PS I, and the down-regulation of the PS II complex, which may occur by means of the xanthophyll cycle, dissociation of LHCII from the PS II complex or by D1 protein turnover.

1. Water-Water Cycle

In the Mehler reaction, a reduction of molecular oxygen to superoxide radical takes place on the reducing side of the PS I complex. Under different stress conditions leading to unbalance between the production of reducing power and its consumption, the electron flow to molecular oxygen is increased and thus also the generation of ROS. Highly toxic ROS are capable of damaging various cellular components, especially those of the photosynthetic apparatus. However, the chloroplasts, the major source of ROS in plants, are very well equipped with multiple defense systems for detoxifying such oxidative species.

In the so-called water-water cycle (Asada, 1999), the electrons originated from water via PS II are donated to molecular oxygen on the acceptor side of PS I, thus generating the superoxide anion radical (O_2^-), which is, in turn, disproportionated to H_2O_2 catalyzed with SOD and consequently H_2O_2 is reduced to water by ascorbate catalyzed with ascorbate-specific peroxidase located in the thylakoid membranes and/or in the stroma. Thus this pathway creates the pseudocycle where the electrons flow from water to oxygen forming water, with no net changes in oxygen and electrons. Unlike the water-water cycle, the Mehler reaction does not include the reducing steps of oxidized ascorbate for regeneration of ascorbate. The dual photoprotective function of the water-water cycle in leaves includes a rapid and immediate scavenging of O_2^- and H_2O_2 at the site of their generation to avoid interaction with the target proteins and thereby safely dissipating excess photon energy by cycling (Asada, 1999). This pathway functions in (i) controlling the over-reduction of PS I and (ii) down-regulating the electron flow from PS II to PS I by generating a pH gradient across the thylakoid membrane.

The PS I complex, a potentially dangerous ROS generator, may exert damage not only to PS I, but also to the PS II complex (Tjus et al., 2001). In higher plants the PS I complex is situated in the stroma-exposed lamellae well separated from PS II, which is preferentially located in the appressed granal region of thylakoid membranes. Thus the structural heterogeneity of the thylakoid membrane allows keeping PS II apart from the dangerous PS I complex (Tjus et al., 1999).

2. Cyclic Electron Transport Around Photosystem I and Photosystem II

Cyclic electron transfer around PS I has a capacity to regulate the relative production of ATP and NADPH in photosynthetic light reactions. Moreover, cyclic electron transport has an important function in the protection of the photosynthetic apparatus against photooxidative damage (Endo et al., 1999; Munekage et al., 2002). Joliot and Joliot (2006) have proposed that an efficient cyclic electron transport around PS I occurs during the first minutes of dark-light transition, which leads to an increase in ATP concentrations.

There exist at least two different cyclic electron transfer routes around PS I. The route from the reduced Fd or NADPH leads directly to the plastoquinone pool (Clark et al., 1984; Zhang et al., 2001; Iwai et al., 2010), whereas the other route goes via the NDH-1 complex to the plastoquinone pool and Cyt b_6f complex (Burrows et al., 1998; Shikanai et al., 1998; Peng and Shikanai, 2011). Although the physiological roles and the exact functions of these two routes of cyclic electron transfer still remain unclear, yet it has been shown that cyclic electron transfer is essential for photosynthesis (Munekage et al., 2002). Cyclic electron transfer around PS I, which is induced under light-chilling stress (Teicher et al., 2000; Kudoh and Sonoike, 2002; Bukhov et al., 2004) and drought stress (Golding and Johnson, 2003), by the formation of a high pH gradient across the thylakoid membrane, supports extra ATP production and regulates the activity of PS II via development of NPQ.

Cyclic electron transfer has been proposed to occur also around PS II, particularly when (i) the PS II acceptor side is reduced and (ii) the oxygen evolving complex is inefficient in donating electrons. Under these conditions, electrons are suggested to cycle around PS II through Cyt b_{559}. Cyt b_{559} can be photoreduced by plastoquinol and photooxidized by $P680^+$. The role of such cyclic electron flow around PS II was suggested to be similar to that of the water-water cycle in dissipating excitation energy under high light intensities (Miyake et al., 2002).

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References

Adams WW III, Hoehn A and Demmig-Adams B (1995) Chilling temperatures and the xanthophyll cycle. A comparison of warm-grown and overwintering spinach. Aust J Plant Physiol 22: 75–85

Adamska I (1997) ELIPs - light-induced stress proteins. Physiol Plant 100: 794–805

Adamska I, Kruse E and Kloppstech K (2001) Stable insertion of the early light-induced proteins into etioplast membranes requires chlorophyll-*a*. J Biol Chem 276: 8582–8587

Adir N, Zer H, Shochat S and Ohad I (2003) Photoinhibition – a historical perspective. Photosynth Res 76: 343–370

Allahverdiyeva Y, Deak Z, Szilard A, Diner B, Nixon P and Vass I (2004) The function of D1-H332 in photosystem II electron transport studied by thermoluminescence and chlorophyll fluorescence in site-directed mutants of *Synechocystis* 6803. Eur J Biochem 271: 3523–3532

Allahverdiyeva Y, Mamedov F, Maenpaa P, Vass I and Aro EM (2005) Modulation of photosynthetic electron transport in the absence of terminal electron acceptors: characterization of the *rbcL* deletion mutant of tobacco. Biochim Biophys Acta 1709: 69–83

Allen DJ and Ort DR (2001) Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci 6: 36–42

Allen JF (2003) State transitions - a question of balance. Science 299: 1530–1532

Allen JF and Forsberg J (2001) Molecular recognition in thylakoid structure and function. Trends Plant Sci 6: 317–326

Amunts A, Drory O and Nelson N (2007) The structure of a plant photosystem I supercomplex at 3.4 Å resolution. Nature 447: 58–63

Anderson J, Park YI and Chow WS (1998) Unifying model for the photoinactivation of photosystem II in vivo and steady-state photosynthesis. Photosynth Res 56: 1–13

Andersson B and Aro EM (2001) Photodamage and D1 protein turnover in photosystem II. In: Aro EM and Andersson B (eds) Regulation of photosynthesis, Advances in Photosynthesis and Respiration, Vol 11, pp 377–393. Kluwer Academic Publishers, Dordrecht

Andersson B and Barber J (1996) Mechanisms of photodamage and protein degradation during photoinhibition of photosystem II. In: Baker NR (ed) Photosynthesis and the Environment, Advances in Photosynthesis, Vol 5, pp 101–121. Kluwer Academic Publishers, Dordrecht

Andersson B and Styring S (1991) Photosystem II: molecular organization, function, and acclimation. Curr Top Bioenerg 16: 1–81

Aro EM, Tyystjärvi E and Nurmi A (1990) Temperature-dependent changes in photosystem II heterogeneity of attached leaves under high light. Physiol Plant 79: 585–592

Aro EM, Virgin I and Andersson B (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Aro EM, Suorsa M, Rokka A, Allahverdiyeva Y, Paakkarinen V, Saleem A, Battchikova N and Rintamaki E (2005) Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. J Exp Bot 56: 347–356

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50: 601–639

Bailey S, Silva P, Nixon P, Mullineaux C, Robinson C and Mann N (2001a) Auxiliary functions in photosynthesis: the role of the FtsH protease. Biochem Soc Trans 29: 455–459

Bailey S, Walters RG, Jansson S and Horton P (2001b) Acclimation of *Arabidopsis thaliana* to the light environment: the existence of separate low light and high light responses. Planta 213: 794–801

Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW, Robinson C and Mann NH (2002) A critical role for the Var2 FtsH homologue of *Arabidopsis thaliana* in the photosystem II repair cycle in vivo. J Biol Chem 277: 2006–2011

Ballottari M, Dall'Osto L, Morosinotto T and Bassi R (2007) Contrasting behavior of higher plant photosystem I and II antenna system during acclimation. J Biol Chem 282: 8947–8958

Barber J (2006) Photosystem II: an enzyme of global significance. Biochem Soc Trans 34: 619–631

Barber J and Andersson B (1992) Too much of a good thing – light can be bad for photosynthesis. Trends Biochem Sci 17: 61–66

Barth C and Krause GH (1999) Inhibition of photosystems I and II in chilling-sensitive and chilling-tolerant plants under light and low-temperature stress. Z Naturforsch 54c: 645–657

Barth C, Krause GH and Winter K (2001) Responses of photosystem I compared with photosystem II to high-light stress in tropical shade and sun leaves. Plant Cell Environ 24: 163–176

Bellafiore S, Barneche F, Peltier G and Rochaix JD (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895

Berry J and Björkman O (1980) Photosynthetic response and adaptation to temperature in higher plants. Annu Rev Plant Physiol 31: 491–543

Björkman O and Powles SB (1984) Inhibition of photosynthetic reactions under water stress: interaction with light level. Planta 161: 490–504

Bongi G and Long SP (1987) Light-dependent damage to photosynthesis in olive leaves during chilling and high temperature stress. Plant Cell Environ 10: 241–249

Bukhov NG, Govindachary S, Rajagopal S, Joly D and Carpentier R (2004) Enhanced rates of $P700^{+}$ dark-reduction in leaves of *Cucumis sativus* L. photoinhibited at chilling temperature. Planta 218: 852–861

Burrows PA, Sazanov LA, Svab Z, Maliga P and Nixon PJ (1998) Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid *ndh* genes. EMBO J 17: 868–876

Chaves MM (1991) Effects of water deficits on carbon assimilation. J Exp Bot 42:1–46

Chen GX, Kazimir J and Cheniae GM (1992) Photoinhibition of hydroxylamine-extracted photosystem II membranes: studies of the mechanism. Biochemistry 31:11072–11083

Chen M, Choi YD, Voytas DF and Rodermel S (2000) Mutations in the Arabidopsis VAR2 locus cause leaf variegation due to the loss of a chloroplast FtsH protease. Plant J 22: 303–313

Choi S, Jeong S, Jeong W, Kwon S, Chow W and Park Y (2002) Chloroplast Cu/Zn-superoxide dismutase is a highly sensitive site in cucumber leaves chilled in the light. Planta 216: 315–324

Chung SK and Jung J (1995) Inactivation of the acceptor side and degradation of the D1 protein of photosystem II by singlet oxygen photogenerated from the outside. Photochem Photobiol 61: 383–389

Clark RD, Hawkesford MJ, Coughlan SJ, Bennett J and Hind G (1984) Association of ferredoxin-$NADP^{+}$ oxidoreductase with the chloroplast cytochrome *b-f* complex. FEBS Lett 174: 137–142

Cornic G (2000) Drought stress inhibits photosynthesis by decreasing stomatal aperture – not by affecting ATP synthesis. Trends in Plant Sci 5: 187–188

Cornic G and Briantais JM (1991) Partitioning of photosynthetic electron flow between CO_2 and O_2 reduction in a C3 leaf (*Phaseolus vulgaris* L.) at different CO_2 concentrations and during drought stress. Planta 183: 178–184

Crafts-Brandner SJ, Van de Loo FJ and Salvucci ME (1997) The two forms of ribulose-1,5-bisphosphate carboxylase/oxygenase activase differ in sensitivity to elevated temperature. Plant Physiol 114: 439–444

Danielius RV, Satoh K, Van Kan PJM, Plijter JJ, Nuijs AM and Van Gorkom HJ (1987) The primary reaction of photosystem II in the D1-D2-cytochrome *b*-559 complex. FEBS Lett 213: 241–244

Debus RJ (2001) Amino acid residues that modulate the properties of tyrosine Y_Z and the manganese cluster in the water oxidizing complex of Photosystem II. Biochim Biophys Acta 1503: 164–186

Demmig-Adams B and Adams WW III (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1: 21–26

Demmig-Adams B and Adams WW III (2003) Photoinhibtion. In: Thomas B, Murphy D and Murray B (eds) Encyclopedia of Applied Plant Science, pp 707–717. Academics Press, London

Diner BA and Rappaport F (2002) Structure, dynamics, and energetics of the primary photochemistry of photosystem II of oxygenic photosynthesis. Annu Rev Plant Biol 53: 551–580

Durrant JR, Giorgi LB, Barber J, Klug DR and Porter G (1990) Characterization of triplet states in isolated photosystem II reaction centres: oxygen quenching as a mechanism for photodamage. Biochim Biophys Acta 1017: 167–175

Edge R and Truscott GT (1999) Carotenoid radicals and the interaction of carotenoids with active oxygen species. In: Frank HA, Young AJ, Britton G and Cogdell RJ (eds) The Photochemistry of Carotenoids, Advances in Photosynthesis, Vol 8, pp 223–234. Kluwer Academic Publishers, Dordrecht

Endo T, Shikanai T, Takabayashi A, Asada K and Sato F (1999) The role of chloroplastic NAD(P)H dehydrogenase in photoprotection. FEBS Lett 457: 5–8

Feller U, Crafts-Brandner SJ and Salvucci ME (1998) Moderately high temperatures inhibit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. Plant Physiol 116: 539–546

Forsberg J, Strom J, Kieselbach T, Larsson H, Alexciev K, Engstrom A and Akerlund HE (2005) Protease activities in the chloroplast capable of cleaving an LHCII N-terminal peptide. Physiol Plant 123: 21–29

Flexas J and Medrano H (2002) Drought-inhibition of photosynthesis in C_3 plants: stomatal and non-stomatal limitations revisited. Ann Bot 89: 183–189

Fufezan C, Gross CM, Sjodin M, Rutherford AW, Krieger-Liszkay A and Kirilovsky D (2007) Influence of the redox potential of the primary quinone electron acceptor on photoinhibition in photosystem II. J Biol Chem 282: 12492–12502

Garab G, Cseh Z, Kovacs L, Rajagopal S, Varkonyi Z, Wentworth M, Mustardy L, Der A, Ruban AV, Papp E, Holzenburg A and Horton P (2002) Light-induced trimer to monomer transition in the main light-harvesting antenna complex of plants: thermo-optic mechanism. Biochemistry 41: 15121–15129

Golding AJ and Johnson GN (2003) Down-regulation of linear and activation of cyclic electron transport during drought. Planta 218: 107–114

Hakala M, Tuominen I, Keränen M, Tyystjärvi T and Tyystjärvi E (2005) Evidence for the role of the oxygen-evolving manganese complex in photoinhibition of Photosystem II. Biochim Biophys Acta 1706: 68–80

Haldrup A, Jensen PE, Lunde C and Scheller HV (2001) Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci 6: 301–305

Hankamer B, Morris E; Nield J, Gerle C and Barber J (2001) Three-dimensional structure of the photosystem II core dimer of higher plants determined by electron microscopy. J Struct Biol 135: 262–269

Haussuhl K, Andersson B and Adamska I (2001) A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II. EMBO J 20: 713–722

Havaux M and Davaud A (1994) Photoinhibition of photosynthesis in chilled potato leaves is not correlated with a loss of photosystem-II activity. Photosynth Res 40: 75–92

Havaux M and Kloppstech K (2001) The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsis npq and tt mutants. Planta 213: 953–966

Havaux M and Tardy F (1996) Temperature-dependent adjustment of the thermal stability of photosystem II in vivo: possible involvement of xanthophyll-cycle pigments. Planta 198: 324–333

Havaux M, Dall'Osto L, Cuine S, Giuliano G and Bassi R (2004) The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana*. J Biol Chem 279: 13878–13888

Heddad M, Noren H, Reiser V, Dunaeva M, Andersson B and Adamska I (2006) Differential expression and localization of early light-induced proteins in Arabidopsis. Plant Physiol 142: 75–87

Herrig R and Falkowski PG (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). I. Photosynthetic energy conversion and growth efficiencies. J Phycol 25: 462–471

Hideg E and Vass I (1995) Singlet oxygen is not produced in photosystem I under photoinhibitory conditions. Photochem Photobiol 62: 949–952

Hideg E, Kalai T, Hideg K and Vass I (2000) Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition? Philos Trans R Soc Lond B Biol Sci 355: 1511–1516

Holt NE, Fleming GR and Niyogi KK (2004) Toward an understanding of the mechanism of nonphotochemical quenching in green plants. Biochemistry 43: 8281–8289

Huesgen PF, Schuhmann H and Adamska I (2006) Photodamaged D1 protein is degraded in *Arabidopsis* mutants lacking the Deg2 protease. FEBS Lett 580: 6929–6932

Huner NPA, Öquist G and Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3: 224–230

Hurry VM and Huner NP (1992) Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiol 100: 1283–1290

Hwang HJ, Kim JH, Eu YJ, Moon BY, Cho SH and Lee CH (2004) Photoinhibition of photosystem I is accelerated by dimethyldithiocarbamate, an inhibitor of superoxide dismutase, during light-chilling of spinach leaves. J Photochem Photobiol B: Biol 73: 79–85

Itzhaki H, Naveh L, Lindahl M, Cook M and Adam Z (1998) Identification and characterization of DegP, a serine protease associated with the lumenal side of the thylakoid membrane. J Biol Chem 273: 7094–7098

Ivanov AG, Morgan RM, Gray GR, Velitchkova MY and Huner NP (1998) Temperature/light dependent development of selective resistance to photoinhibition of photosystem I. FEBS Lett 430: 288–292

Iwai M, Takizawa K, Tokutsu R, Okamura A, Takahashi Y and Minagawa J (2010) Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. Nature 464: 1210–1213

Jegerschold C, Virgin I and Styring S (1990) Light-dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction. Biochemistry 29: 6179–6186

Jegerschold C and Styring S (1996) Spectroscopic characterization of intermediate steps involved in donor-side-induced photoinhibition of photosystem II. Biochemistry 35: 7794–7801

Joliot P, Joliot A (2006) Quantification of cyclic and linear flows in plants. Proc Natl Acad Sci USA 102: 4913–4918

Kanervo E, Suorsa M and Aro EM (2005) Functional flexibility and acclimation of the thylakoid membrane. Photochem Photobiol Sci 4: 1072–1080

Kapri-Pardes E, Naveh L and Adam Z (2007) The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in Arabidopsis. Plant Cell 19: 1039–1047

Keren N, Gong H and Ohad I (1995) Oscillations of reaction center II-D1 protein degradation in vivo induced by repetitive flashes. Correlation between the level of RCII-Q_B^- and protein degradation in low light. J Biol Chem 270: 806–814

Kingston-Smith AH, Harbinson J, Williams J and Foyer CH (1997) Effect of chilling on carbon assimilation, enzyme activation, and photosynthetic electron transport in the absence of photoinhibition in maize leaves. Plant Physiol 114: 1039–1046

Koivuniemi A, Swiezewska E, Aro EM, Styring S and Andersson B (1993) Reduced content of the quinone acceptor QA in photosystem II complexes isolated from thylakoid membranes after prolonged photoinhibition under anaerobic conditions. FEBS Lett 327: 343–346

Koivuniemi A, Aro EM and Andersson B (1995) Degradation of the D1- and D2-proteins of photosystem II in higher plants is regulated by reversible phosphorylation. Biochemistry 34: 16022–16029

Kozaki A and Takeba G (1996) Photorespiration protects C3 plants from photooxidation. Nature 384: 557–560

Komayama K, Khatoon M, Takenaka D, Horie J, Yamashita A, Yoshioka M, Nakayama Y, Yoshida M, Ohira S, Morita N, Velitchkova M, Enami I and Yamamoto Y (2007) Quality control of photosystem II: cleavage and aggregation of heat-damaged D1 protein in spinach thylakoids. Biochim Biophys Acta 1767: 838–846

Kok B (1956) On the inhibition of photosynthesis by intense light. Biochim Biophys Acta 21: 234–244

Krieger-Liszkay A and Rutherford AW (1998) Influence of herbicide binding on the redox potential of the quinone acceptor in photosystem II: relevance to photodamage and phytotoxicity. Biochemistry 37: 17339–17344

Krinsky NI (1979) Carotenoid protection against oxidation. Pure Appl Chem 51: 649–660

Kudoh H and Sonoike K (2002) Irreversible damage to photosystem I by chilling in the light: cause of the degradation of chlorophyll after returning to normal growth temperature. Planta 215: 541–548

Kulheim C, Agren J and Jansson S (2002) Rapid regulation of light harvesting and plant fitness in the field. Science 297: 91–93

Law RD and Crafts-Brandner SJ (1999) Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of rubilose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 120: 173–182

Li XP, Björkman O, Shih C, Grossman AR, Rosenquist M, Jansson S and Niyogi KK (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403: 391–395

Lindahl M, Yang DH and Andersson B (1995) Regulatory proteolysis of the major light-harvesting chlorophyll *a/b* protein of photosystem II by a light induced membrane-associated enzymic system. Eur J Biochem 231: 503–509

Lindahl M, Tabak S, Cseke L, Pichersky E, Andersson B and Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. J Biol Chem 271: 29329–29334

Lindahl M, Spetea C, Hundal T, Oppenheim AB, Adam Z and Andersson B (2000) The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein. Plant Cell 12: 419–431

Loggini B, Scartazza A, Brugnoli E and Navari-Izzo F (1999) Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol 119: 1091–1099

Long SP, Humphries S and Falkowski PG (1994) Photoinhibition of photosynthesis in nature. Annu Rev Plant Physiol Plant Mol Biol 45: 633–662

Matsubara S and Chow WS (2004) Populations of photoinactivated photosystem II reaction centers characterized by chlorophyll a fluorescence lifetime in vivo. Proc Natl Acad Sci USA 101: 18234–18239

Medrano H, Escalona JM, Bota J, Gulias J and Flexas J (2002) Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter. Ann Bot 89: 895–905

Mizusawa N, Tomo T, Satoh K and Miyao M (2003) Degradation of the D1 protein of photosystem II under illumination in vivo: Two different pathways involving cleavage or intermolecular cross-linking. Biochemistry 42: 10034–10044

Moon BY, Higashi S, Gombos Z and Murata N (1995) Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants. Proc Natl Acad Sci USA 92: 6219–6223

Montane MH and Kloppstech K (2000) The family of light-harvesting-related proteins (LHCs, ELIPs, HLIPs): was the harvesting of light their primary function? Gene 258: 1–8

Miyake C, Yonekura K, Kobayashi Y and Yokota A (2002) Cyclic electron flow within PS II functions in intact chloroplasts from spinach leaves. Plant Cell Physiol 43: 951–957

Muller P, Li XP and Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy. Plant Physiol 125: 1558–1566

Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M and Shikanai T (2002) *PGR5* Is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110: 361–371

Murchie EH and Horton P (1997) Acclimation of photosynthesis to irradiance and spectral quality in British plant species: chlorophyll content, photosynthetic capacity and habitat preference. Plant Cell Environ 20: 438–448

Nakano R, Ishida H, Makino A and Mae T (2006) In vivo fragmentation of the large subunit of Ribulose-1,5-bisphosphate carboxylase by reactive oxygen species in an intact leaf of cucumber under chilling-light conditions. Plant Cell Physiol 47: 270–276

Nelson N and Yocum CF (2006) Structure and function of photosystem I and II. Annu Rev Plant Biol 57: 521–565

Nixon PJ, Barker M, Boehm M, de Vries R and Komenda J (2005) FtsH-mediated repair of the photosystem II complex in response to light stress. J Exp Bot 56: 357–363

Nixon PJ, Michoux F, Yu J, Boehm M and Komenda J (2010) Recent advances in understanding the assembly and repair of photosystem II. Ann Bot 106: 1–16

Niyogi KK, Li XP, Rosenberg V and Jung HS (2005) Is PsbS the site of non-photochemical quenching in photosynthesis? J Exp Bot 56: 375–382

Ohad I, Kyle DJ and Arntzen CJ (1984) Membrane protein damage and repair: removal and replacement of inactivated 32-kilodalton polypeptide in chloroplast membranes. J Cell Biol 99: 481–485

Ohad I, Adir N, Koike H, Kyle DJ and Inoue Y (1990) Mechanism of photoinhibition *in vivo*. A reversible light-induced conformational change of reaction center II is related to an irreversible modification of the D1 protein. J Biol Chem 265: 1972–1979

Ohira S, Morita N, Suh HJ, Jung J and Yamamoto Y (2005) Quality control of photosystem II under light stress - turnover of aggregates of the D1 protein in vivo. Photosynth Res 84: 29–33

Ohnishi N, Allakhverdiev SI, Takahashi S, Higashi S, Watanabe M, Nishiyama Y and Murata N (2005) Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. Biochemistry 44: 8494–8499

Ortega JM, Roncel M and Losada M (1999) Light-induced degradation of cytochrome b_{559} during photoinhibition of the photosystem II reaction center. FEBS Lett 458: 87–92

Osmond CB (1981) Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. Biochim Biophys Acta 639: 77–98

Osmond B, Badger M, Maxwell K, Björkman O and Leegood R (1997) Too many photons: photorespiration, photoinhibition and photooxidation. Trends Plant Sci 2: 119–121

Osmond B and Förster B (2005) Photoinhibition: Then and now. In: Demmig-Adams B, Adams WW III and Mattoa AK (eds) Photoprotection, Photoinhibition, Gene Regulation, and Environment, Advances in Photosynthesis and Respiration, Vol 21, pp 11–22. Springer, Dordrecht

Ott T, Clarke J, Birks K and Johnson G (1999) Regulation of the photosynthetic electron transport chain. Planta 209: 250–258

Oquist G, Hurry VM and Huner NPA (1993) The temperature dependence of the redox state of Q_A and the susceptibility of photosynthesis to photoinhibition. Plant Physiol Biochem 31: 683–691

Park YII, Chow WS, Osmond CB and Anderson JM (1996) Electron transport to oxygen mitigates against the photoinactivation of photosystem II *in vivo*. Photosynth Res 50: 23–31

Peltier JB, Emanuelsson O, Kalume DE, Ytterberg J, Friso G, Rudella A, Liberles DA, Soderberg L, Roepstorff P, von Heijne G and Van Wijk KJ (2002) Central functions of the lumenal and peripheral thylakoid proteome of Arabidopsis determined by experimentation and genome-wide prediction. Plant Cell 14: 211–236

Peng L and Shikanai T (2011) Supercomplex formation with photosystem I is required for the stabilization of the chloroplast NADH dehydrogenase-like complex in Arabidopsis. Plant Physiol 155: 1629–1639

Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol 35: 15–44

Rajagopal S, Bukhov NG, Tajmir-Riahi HA and Carpentier R (2003) Control of energy dissipation and photochemical activity in photosystem I by NADP-dependent reversible conformational changes. Biochemistry 42: 11839–11845

Rappaport F, Guergova-Kuras M, Nixon PJ, Diner BA and Lavergne J (2002) Kinetics and pathways of charge recombination in Photosystem II. Biochemistry 41: 8518–8527

Renger G (2011) Light induced oxidative water splitting in photosynthesis: Energetics, kinetics and mechanism. J Photochem Photobiol B: Biol 104: 35–43

Renger G and Renger T (2008) Photosystem II: the machinery of photosynthetic water splitting. Photosynth Res 98: 53–80

Rintamaki E, Kettunen R and Aro EM (1996) Differential D1 dephosphorylation in functional and photodamaged photosystem II centers. Dephosphorylation is a prerequisite for degradation of damaged D1. J Biol Chem 271: 14870–14875

Rokka A, Suorsa M, Saleem A, Battchikova N and Aro EM (2005) Synthesis and assembly of thylakoid protein complexes: multiple assembly steps of photosystem II. Biochem J 388: 159–168

Rutherford AW and Krieger-Liszkay A (2001) Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648–653

Sakamoto W (2006) Protein degradation machineries in plastids. Annu Rev Plant Biol 57: 599–621

Sakamoto W, Tamura T, Hanba-Tomita Y, Sodmergen and Murata M (2002) The VAR1 locus of Arabidopsis encodes a chloroplastic FtsH and is responsible for leaf variegation in the mutant alleles. Genes Cells 7: 769–780

Sakamoto W, Zaltsman A, Adam Z and Takahashi Y (2003) Coordinated regulation and complex formation of yellow

variegated1 and yellow variegated2, chloroplastic FtsH metalloproteases involved in the repair cycle of photosystem II in Arabidopsis thylakoid membranes. Plant Cell 15: 2843–2855

Salonen M, Aro EM and Rintamäki E (1998) Reversible phosphorylation and turnover of the D1 protein under various redox states of photosystem II induced by low temperature photoinhibition. Photosynth Res 58: 143–151

Satoh K (1970) Mechanism of photoinactivation in photosynthetic systems III. Site and mode of photoinactivation in photosystem I. Plant Cell Physiol 11: 187–197

Satoh K and Fork DC (1982) Photoinhibition of reaction centers of photosystem I and II in intact *Bryopsis* chloroplasts under anaerobic conditions. Plant Physiol 70: 1004–1008

Scheller HV and Haldrup A (2005) Photoinhibition of photosystem I. Planta 221: 5–8

Schubert M, Petersson UA, Haas BJ, Funk C, Schroder WP and Kieselbach T (2002) Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. J Biol Chem 277: 8354–8365

Seemann JR, Sharkey TD, Wang J and Osmond CB (1987) Environmental effects on photosynthesis, nitrogen-use efficiency, and metabolite pools in leaves of sun and shade plants. Plant Physiol 84: 796–802

Silva P, Thompson E, Bailey S, Kruse O, Mullineaux CW, Robinson C, Mann NH and Nixon PJ (2003) FtsH is involved in the early stages of repair of photosystem II in *Synechocystis* sp. PCC 6803. Plant Cell 15: 2152–2164

Sonoike K (1996) Photoinhihition of photosystem I: Its physiological significance in the chilling sensitivity of plants. Plant Cell Physiol 37: 239–247

Sonoike K and Terashima I (1994) Mechanism of photosystem-I photoinhibition in leaves of *Cucumis sativus* L. Planta 194: 287–293

Sonoike K, Terashima I, Iwaki M and Itoh S (1995) Destruction of photosystem I iron-sulfur centers in leaves of *Cucumis sativus* L. by weak illumination at chilling temperatures. FEBS Lett 362: 235–238

Shikanai T, Endo T, Hashimoto T, Yamada Y, Asada K and Yokota A (1998) Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proc Natl Acad Sci USA 95: 9705–9709

Stitt M (1986) Limitation of photosynthesis by carbon metabolism: I. Evidence for excess electron transport capacity in leaves carrying out photosynthesis in saturating light and CO_2. Plant Physiol 81: 1115–1122

Styring S and Jegerschöld C (1994) Light-induced reactions impairing electron transfer through photosystem II. In: Baker NR and Bowyer J (eds) Photoinhibition of Photosynthesis; from Molecular Mechanisms to the Field, pp 51–74. BIOS Scientific Publishers, Oxford

Sun ZL, Lee HY, Matsubara S, Hope AB, Pogson BJ, Hong YN and Chow WS (2006) Photoprotection of residual functional photosystem II units that survive illumination in the absence of repair, and their critical role in subsequent recovery. Physiol Plant 128: 415–424

Takahashi S and Murata N (2006) Glycerate-3-phosphate, produced by CO_2 fixation in the Calvin cycle, is critical for the synthesis of the D1 protein of photosystem II. Biochim Biophys Acta 1757: 198–205

Takahashi S, Bauwe H and Badger M (2007) Impairment of the photorespiratory pathway accelerates photoinhibition of photosystem II by suppression of repair but not acceleration of damage processes in Arabidopsis. Plant Physiol 144: 487–494

Teardo E, de Laureto PP, Bergantino E, Dalla Vecchia F, Rigoni F, Szabo I and Giacometti GM (2007) Evidences for interaction of PsbS with photosynthetic complexes in maize thylakoids. Biochim Biophys Acta 1767: 703–711

Teicher HB, Møller BL and Scheller HV (2000) Photoinhibition of photosystem I in field-grown barley (*Hordeum vulgare* L.): Induction, recovery and acclimation. Photosynth Res 64: 53–61

Terashima I, Sonoike K, Kawazu T and Katoh S (1991) Exposure of leaves of *Cucumis sativus* L. to low temperatures in the light causes uncoupling of thylakoids II. Non-destructive measurements with intact leaves. Plant Cell Physiol 32: 1275–1283

Terashima I, Funayama S and Sonoike K (1994) The site of photoinhibition in leaves of *Cucumis sativus* L. at low temperatures is photosystem I, not photosystem II. Planta 193: 300–306

Tikkanen M, Piippo M, Suorsa M, Sirpiö S, Mulo P, Vainonen J, Vener AV, Allahverdiyeva Y and Aro EM (2006) State transitions revisited - a buffering system for dynamic low light acclimation of Arabidopsis. Plant Mol Biol 62: 779–793

Tjus SE, Moller BL and Scheller HV (1998) Photosystem I is an early target of photoinhibition in barley illuminated at chilling temperatures. Plant Physiol 116: 755–764

Tjus SE, Moller BL and Scheller HV (1999) Photoinhibition of photosystem I damages both reaction centre proteins PS I-A and PS I-B and acceptor-side located small photosystem I polypeptides. Photosynth Res 60: 75–86

Tjus SE, Scheller HV, Andersson B and Moller BL (2001) Active oxygen produced during selective excitation of photosystem I is damaging not only to photosystem I, but also to photosystem II. Plant Physiol 125: 2007–2015

Tourneux C and Peltier G (1995) Effect of water deficit on photosynthetic oxygen measured using $^{18}O_2$ and mass spectrometry in *Solanum tuberosum* L. leaf discs. Planta 195: 570–577

Tyystjärvi E (2008) Photoinhibition of photosystem II and photodamage of the oxygen-evolving manganese cluster. Coord Chem Rev 252: 361–376

Vass I (2011) Role of charge recombination processes in photodamage and photoprotection of the photosystem II complex. Physiol Plant 142: 6–16

Vass I and Aro EM (2007) Photoinhibition of photosystem II electron transport. In: Renger G (ed) Primary

Processes of Photosynthesis: Basic Principles and Apparatus. Comprehensive Series in Photochemical and Photobiological Sciences, pp 393–411. Royal Society Chemistry, Cambridge

Vass I, Styring S, Hundal T, Koivuniemi A, Aro E and Andersson B (1992) Reversible and irreversible intermediates during photoinhibition of photosystem II: Stable reduced Q_A species promote chlorophyll triplet formation. Proc Natl Acad Sci USA 89: 1408–1412

Wingler A, Lea PJ, Quick WP and Leegood RC (2000) Photorespiration: metabolic pathways and their role in stress protection. Philos Trans R Soc Lond Ser B Biol Sci 355: 1517–1529

Weis E (1981) The temperature sensitivity of dark-inactivation and light-activation of the ribulose-1,5-bisphosphate carboxylase in spinach chloroplasts. FEBS Lett 129: 197–200

Wollman FA (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J 20: 3623–3630

Yamamoto Y (2001) Quality control of photosystem II. Plant Cell Physiol 42: 121–128

Yamamoto Y, Ishikawa Y, Nakatani E, Yamada M, Zhang H and Wydrzynski T (1998) Role of an extrinsic 33 kilodalton protein of photosystem II in the turnover of the reaction center-binding protein D1 during photoinhibition. Biochemistry 37: 1565–1574

Yang DH, Paulsen H and Andersson B (2000) The N-terminal domain of the light-harvesting chlorophyll a/b-binding protein complexes (LHCII) is essential for its acclimative proteolysis. FEBS Lett 466: 385–388

Yoshioka M and Yamamoto Y (2011) Quality control of photosystem II: Where and how does the degradation of the D1 protein by FtsH protease start under light stress? Facts and hypotheses. J Photochem Photobiol B: Biol 104: 229–235

Zaltsman A, Feder A and Adam Z (2005a) Developmental and light effects on the accumulation of FtsH protease in Arabidopsis chloroplasts-implications for thylakoid formation and photosystem II maintenance. Plant J 42: 609–617

Zaltsman A, Ori N and Adam Z (2005b) Two types of FtsH protease subunits are required for chloroplast biogenesis and photosystem II repair in Arabidopsis. Plant Cell 17: 2782–2790

Zhang LX, Paakkarinen V, Van Wijk KJ and Aro EM (2000) Biogenesis of the chloroplast-encoded D1 protein: regulation of translation elongation, insertion, and assembly into photosystem II. Plant Cell 12: 1769–1782

Zhang H, Whitelegge JP and Cramer WA (2001) Ferredoxin:NADP+oxidoreductase is a subunit of the chloroplast cytochrome b_6f complex. J Biol Chem 276: 38159–38165

Zhang S and Scheller HV (2004) Photoinhibition of photosystem I at chilling temperature and subsequent recovery in *Arabidopsis thaliana*. Plant Cell Physiol 45: 1595–1602

Chapter 14

Light Stress Proteins in Viruses, Cyanobacteria and Photosynthetic Eukaryota

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Summary

Plants in Nature frequently perceive environmental extremes such as light stress. To maintain their physiological functions under light stress conditions plants have developed different protection strategies that operate at morphological, anatomical and subcellular levels. The accumulation of light stress proteins

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from the light-harvesting complex (LHC)-like protein family can be considered to be a part of such photoprotective responses. LHC-like proteins are distant relatives of the chlorophyll *a/b*-binding proteins of photosystem I and II that accumulate only transiently in thylakoid membranes under certain physiological stress conditions. Based on predicted secondary structure LHC-like family members are divided into three-helix ELIPs (early light-induced proteins), two-helix SEPs (stress-enhanced proteins) and one-helix OHPs (one-helix proteins), called also HLIPs (high light-induced proteins) or SCPs (small chlorophyll-binding-like proteins) in cyanobacteria and viruses. It is believed that these proteins play a protective role within the chloroplast under light stress conditions either by transient binding of free chlorophylls and preventing the formation of free radicals and/or by acting as sinks for excitation energy. Expanding functional genomics provided new tools for the identification of genes and proteins structurally and/or functionally related to LHC-like family members that escaped previous detection by classical molecular biology or biochemical methods. This chapter provides an overview of "classical" LHC-like family members, discusses their evolution and photoprotective functions and introduces novel types of LHC-like proteins in algae and land plants.

I. Introduction

In order to use light energy for photosynthesis plants and algae have molecules that act as light-harvesting antennas, which collect light quanta through chlorophyll (Chl) excitation and transfer the collected energy to photosynthetic reaction centers. However, in full sunlight, much of the energy absorbed is not needed and this energy excess can promote the formation of reactive oxygen species that in turn may lead to photooxidative damage of photosynthetic membranes. Therefore, under conditions of excessive light the efficient light-harvesting antenna is rapidly and reversibly switched into a photoprotected quenched state in which potentially harmful absorbed energy is dissipated as heat, a process referred to as non-photochemical quenching (NPQ) of Chl fluorescence (Pascal et al., 2005; Ruban et al., 2007; Horton et al., 2008; Pérez-Bueno et al., 2008; Barros et al., 2009). In addition, specific light stress proteins with photoprotective functions accumulate in the thylakoid membranes.

The Chl-binding (CB) protein superfamily gathers several protein families, where the most known are the light-harvesting complex (LHC) proteins, the LHC-like proteins and the photosystem II (PS II) subunit S (PSBS). While the primary function of LHC proteins is light harvesting, members of LHC-like and PSBS families are involved in photoprotection. In the era of functional genomics several other proteins from the CB superfamily were discovered in genomes of various algae, the moss *Physcomitrella patens* and higher plants: such as LHCz proteins (Koziol et al., 2007), LI818 proteins (Savard et al., 1996; Richard et al., 2000; Six et al., 2005; Koziol et al., 2007; Alboresi et al., 2008; Zhu and Green, 2008), red lineage Ch *a/b*-binding (CAB)-like proteins (RedCAP) (Engelken et al., 2010) and high intensity light-inducible LHC-like (LHL) (Teramoto et al., 2004, 2006) proteins. Except for their presence in genomes of various taxa there is limited knowledge about the localization, expression and physiological function of these distantly related LHC-like proteins. The aim of this chapter is to provide a short summary of the "classical" LHC-like family members, such as three-helix early light-induced proteins (ELIPs), two-helix stress-enhanced proteins (SEPs), and one-helix proteins/high light-induced proteins/small Chl-binding-like proteins (OHP/HLIP/SCPs), and report the progress in the characterization of novel, distantly related members of this family, such as RedCAP, LHL and LI818 proteins.

Abbreviations: CAB – Chlorophyll *a/b*-binding; CB – Chlorophyll-binding; Chl – Chlorophyll; ELIP – Early light-induced protein; HL – High light intensity; HLIP – High light-induced protein; LHC – Light-harvesting complex; LHL – High intensity light-inducible LHC-like; LIL – Light-harvesting-like; LL – Low light intensity; NPQ – Non-photochemical quenching; OHP – One-helix protein; PS – Photosystem; PSBS – The S subunit of photosystem II; RedCAP – Red lineage Chl *a/b*-binding-like proteins; SCP – Small chlorophyll-binding-like proteins; SEP – Stress-enhanced protein; TM – Transmembrane

II. Over 20 Years of Early Light-Induced Protein (ELIP) Research: A Historical Overview

The first LHC-like family members were discovered at the beginning of the 1980s during greening of etiolated pea (*Pisum sativum*) or barley (*Hordeum vulgare*) seedlings exposed to light (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987). The *ELIP* transcripts reached their maximal accumulation within 2–4 h after the transition from dark to light. This unusually rapid and transient accumulation of *ELIP* transcripts, as compared to other light-regulated genes, provided the basis for the name, "the early light-inducible proteins". The posttranslational in vitro import of radioactively labeled ELIP precursors into isolated intact chloroplasts revealed that mature ELIPs are integral thylakoid membrane proteins (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987; Grimm et al., 1989). Furthermore, it was shown for barley (Grimm and Kloppstech, 1987) and proposed for pea (Kolanus et al., 1987) that small multigene families encode ELIPs. Since no *ELIP* transcripts were detected in mature green plants grown under ambient light conditions, it was assumed that the induction of *ELIP* genes is restricted to the early stage of seedling development and that the function of these proteins is related to chloroplast differentiation. A role of ELIPs in: (i) the synchronization of nuclear and chloroplast gene expression, (ii) the structural rearrangement of the plastid membranes, such as the degradation of dark-specific proteins during transformation of etioplasts into chloroplasts, (iii) the facilitation of the membrane insertion of other light-induced proteins, or (iv) the regulation of lipid or Chl biosynthesis was discussed (Meyer and Kloppstech, 1984; Scharnhorst et al., 1985; Grimm and Kloppstech, 1987; Kolanus et al., 1987). After *ELIP* cDNA (Scharnhorst et al., 1985) and genomic clones (Kolanus et al., 1987) were isolated and sequenced it turned out that ELIPs have three predicted transmembrane (TM) domains, where domains I and III share amino acid sequence similarity with CAB proteins from photosystem I (PS I) and PS II (Grimm et al., 1989; Green et al., 1991; Green and Kühlbrandt, 1995). Biochemical fractionation of developing thylakoid membranes suggested first that ELIPs in pea might be associated with both photosystems (Cronshagen and Herzfeld, 1990). However, detailed crosslinking studies revealed the location of these proteins in the vicinity of the D1-protein from the PS II reaction center (Adamska and Kloppstech, 1991). Expression studies demonstrated that, similar to *CAB* transcripts (Kloppstech, 1985; Piechulla and Gruissem, 1987), the level of *ELIP* transcripts in pea and barley seedlings (Kloppstech, 1985; Green et al., 1991) was controlled by a circadian oscillator and varied by a factor of 20 between the maximum in the morning and the minimum during the middle of the night. Also the level of ELIP protein oscillated during the day (Adamska et al., 1991). Based on structural similarities, localization and expression pattern it was proposed that ELIPs are distant relatives of the CAB family and might represent pigment-free substitutes for CAB proteins during the assembly of photosynthetic complexes in developing thylakoids. The discovery of the *ELIP*-like gene induced in parallel with accelerated carotenoid biosynthesis in the green alga *Dunaliella bardawil* suggested that this protein (called CBR, for carotene biosynthesis-related) might play a role in the synthesis, transfer and/or integration of carotenoids during the assembly of Chl-protein complexes (Lers et al., 1991). Such a function of ELIPs was also consistent with their location in the non-appressed regions of thylakoid membranes (Adamska and Kloppstech, 1991), where assembly/disassembly of pigment-protein complexes occur (Mulo et al., 2008).

The breakthrough in ELIP research started with the discovery that the ELIP induction is not restricted to the early stages of chloroplast development. The exposure of mature green leaves to light stress led to the transient accumulation of these proteins in pea (Adamska et al., 1992a, b), barley (Pötter and Kloppstech 1993) and *Arabidopsis thaliana* (Heddad and Adamska, 2000) plants. The accumulation of ELIPs correlated with the progression of photoinhibition of PS II and changes in the level of pigments (Adamska et al., 1992b, 1993; Heddad et al., 2006).

Progress in genome sequencing revealed the presence of ELIPs and ELIP-like proteins in all oxygenic photosynthetic organisms investigated so far, which suggests their conserved physiological role (Heddad and Adamska, 2002; Engelken et al., 2010). Isolation of ELIP from light-stressed pea leaves and analysis of bound pigments revealed

that this protein binds Chl *a* and lutein with unusual characteristics that exclude the light-harvesting function (Adamska et al., 1999). Based on our current knowledge it is believed that ELIPs fulfill a protective role within the thylakoids under stress conditions either by transient binding of released free Chl molecules and/or by acting as sinks for excitation energy. Binding of free Chls by ELIPs and prevention of the formation of singlet oxygen would be essential for the protection of thylakoid membrane components against photooxidative damage. The following paragraphs provide a short summary of our current knowledge on the intriguing members of the ELIP family.

III. Division and Characteristics of LHC-Like Family Members

Based on predicted secondary structure and expression pattern the members of the LHC-like family are divided into three groups (Fig. 14.1): three-helix ELIPs (Montané and Kloppstech, 2000; Adamska, 2001), two-helix SEPs (Heddad and Adamska, 2000), also called light-harvesting-like (LIL) proteins (Jansson, 1999) and one-helix OHPs (Jansson et al., 2000; Andersson et al., 2003). In cyanobacteria the latter are also called HLIPs or SCPs (Dolganov et al., 1995; Funk and Vermaas, 1999). While ELIPs are not detected in thylakoid membranes under low light (LL) conditions and accumulate in response to illumination

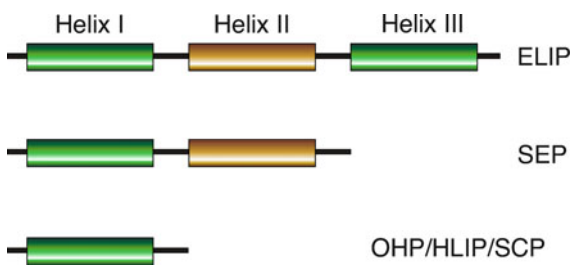

Fig. 14.1. Schematic representation of LHC-like family members. Based on predicted secondary structure the LHC-like family members can be divided into three-helix ELIPs, two-helix SEPs (called also LILs) and one-helix OHPs, called also HLIPs or SCPs in cyanobacteria. The transmembrane alpha-helices I and III are highly conserved and they contain ELIP consensus motifs, the helix II is polymorphic.

with high light (HL), significant amounts of SEPs and OHPs are present in the absence of light stress but their amounts increase during HL exposure (Heddad and Adamska, 2000; Andersson et al., 2003). Two different ELIPs, six SEPs and two OHPs are encoded by the genome of *A. thaliana* (Table 14.1).

The transmembrane alpha-helices I and III of ELIPs are related to each other and carry ELIP consensus motifs, ERINGRLAMIGFVAALAVE and ELWNGRFAMLGLVALAFTE, respectively. Helices I of SEPs and OHPs are related to helix I of ELIPs. The helix II is polymorphic and differs in its amino acid composition between and within various ELIPs and SEPs (Fig. 14.1). Based on the homology between LHC and LHC-like family members it is expected that all these proteins will have a similar three-dimensional structure. The first atomic structures were resolved for the major LHC of PS II (LHCII) in spinach (*Spinacia oleracea*) (Kühlbrandt et al., 1994; Liu et al., 2004) and pea (Standfuss et al., 2005). The monomeric LHCII protein contains three TM helices A-C and one C-terminally-located amphipathic helix D exposed at the surface on the lumenal side of the thylakoid membrane. The two central helices A and B are in close contact with each other, held together by two inter-helix ionic pairs, forming a left-handed supercoil with two-fold symmetry structure (Kühlbrandt et al., 1994; Liu et al., 2004). Eight Chl *a*, six Chl *b*, four carotenoids and two lipids are bound to LHCII monomers (Kühlbrandt et al., 1994; Liu et al., 2004; Standfuss et al., 2005). Based on homology it is expected that helices I and III of ELIPs and their relatives will also form a similar two-fold symmetry structure. However, the amphiphatic helix D present in CAB proteins is missing in ELIPs and the soluble connectors between the helices A-C are much shorter (Grimm et al., 1989; Green et al., 1991). In the case of SEPs and OHPs a similar two-fold symmetry structure can be formed by homo- or heterodimers (Dolganov et al., 1995; Funk and Vermaas, 1999).

Despite several similar structural features shared by LHC-like family members and CAB proteins there are also very pronounced differences. The CAB proteins are constitutively expressed and represent structural components of PS I and PS II (Blankenship, 2001; Green, 2003),

Table 14.1. The members of LHC-like family in the genome of *Arabidopsis thaliana*. Transcript sizes according to the TAIR database (http://www.arabidopsis.org)

Name	Chromosomal accession	Gene size (bp)	Transcript size (nt)	Protein size (aa)	References
ELIP1	At3g22840	1,094	867	p195-m151	(1)
ELIP2	At4g14690	978	806	p193-m152	(1)
SEP1	At4g34190	1,234	772	p146-m103	(1)
SEP2	At2g21970	931	856	p202-m181	(1)
SEP3-1	At4g17600	1,135	971	p262-m223	
SEP3-2	At5g47110	1,327	1,086	p258-m216	
SEP4	At3g12345	813	813	p186-m154	
SEP5	At4g28025	1,905	874	p157-m96	
OHP1	At5g02120	680	526	p110-m69	(2)
OHP2	At1g34000	1,599	1,369	p172-m130	(3)

Genomic size (*bp* base pair), transcript/cDNA size (*nt* nucleotide), protein size (*aa* amino acids). *References*: (1) Heddad and Adamska (2000), (2) Jansson et al. (2000), (3) Andersson et al. (2003)

whereas LHC-like family members accumulate only transiently under certain physiological conditions (Adamska, 1997, 2001). In contrast to LHC proteins, which are the most abundant membrane proteins in algae and plants (Jansson, 1999), LHC-like proteins accumulate in the membrane in substoichiometric amounts (Adamska and Kloppstech, 1991; Adamska, 1997, 2001). Finally, a different expression pattern and pigment binding characteristics were assayed for LHC-like and CAB proteins (Adamska, 2001).

A. Three-Helix ELIPs

Alignments of ELIP sequences with LHC family members revealed that these proteins share conserved amino acid residues capable of binding Chls. Four potential Chl ligands are present in helices I and III of ELIPs. The pigment binding by ELIPs is supported by the requirement for Chl *a* during the insertion of in vitro translated radioactively labeled ELIP precursors into etioplast membranes of barley (Adamska et al., 2001). The direct experimental evidence of pigment binding was obtained by the purification of ELIPs from HL-stressed pea leaves (Adamska et al., 1999). Chl *a* and lutein were identified in purified ELIP fractions. However, a very weak excitonic coupling was measured between bound Chl *a* molecules suggesting that these Chls are not involved in energy transfer to photosynthetic reaction centers but fulfill non-light-harvesting functions. In agreement with such a function is a high lutein content assayed for ELIPs that was twice as high as reported for CAB proteins (Adamska et al., 1999).

Localization studies revealed that pea ELIPs are located in the non-appressed regions of thylakoid membranes in the vicinity of PS II (Adamska and Kloppstech, 1991). Based on in vivo labeling studies it has been calculated that one ELIP molecule accumulated per 10–20 PS II reaction centers, when mature green pea leaves were exposed to light stress (Adamska, 1997). A spontaneous insertion mechanism into thylakoid membrane was reported for ELIP2 in *A. thaliana* (Kim et al., 1999) and low molecular mass ELIPs in barley (Kruse and Kloppstech, 1992). The latter proteins are inserted into the thylakoid membranes without the requirement for membrane surface proteins or stroma factors. Formation of high molecular-mass oligomeric complexes of over 100 kDa of unknown composition was reported for low molecular mass ELIPs of barley under combined light and cold stress conditions (Montané et al., 1999). Similar complexes with unidentified polypeptides of 24–26 kDa were found in pea during light stress (Adamska et al., 1999). The formation of a large protein complex with the minor LHCII proteins was proposed for ELIP homolog CBR in the green alga *D. bardawil* (Lers et al., 1991; Levy et al., 1992, 1993). Similar complexes described from *Dunaliella salina* were highly enriched in xanthophylls zeaxanthin and lutein (Jin et al., 2001). Recently, it was demonstrated

that ELIP1 and ELIP2 in *A. thaliana* are located in monomeric and trimeric LHCII under light stress conditions (Heddad et al., 2006). Longer exposure to light stress resulted in higher ELIP amounts in the trimeric LHCII population as compared to monomers. Different distribution of ELIP1 and ELIP2 in the sucrose gradient fractions suggested their co-isolation with different LHCII subpopulations (Heddad et al., 2006).

Regulation of *ELIP* gene expression by different stress conditions, different developmental stages and endogenous rhythms was reported. While in *A. thaliana* ELIP induction is strictly regulated by light stress (Heddad and Adamska, 2000) different stress conditions, such as cyclic heat shock, cold stress, desiccation, osmotic stress, salt stress, oxidative stress and combinations of them, were reported to trigger the induction of these proteins in higher plants (Bartels et al., 1992; Beator et al., 1992; Beator and Kloppstech, 1993; Adamska and Kloppstech, 1994; Montané et al., 1996; Ouvrard et al., 1996; Król et al., 1997; Lindahl et al., 1997; Shimosaka et al., 1999; Peng et al., 2008) or green algae (Lers et al., 1991; Król et al., 1997). Induction of ELIP during exposure of pea plants to low levels of UV-B radiation was also reported (Sävenstrand et al., 2004). A cryprochrome-like receptor, absorbing blue and UV-A light, was proposed to control ELIP expression in mature green plants exposed to light stress (Adamska et al., 1992a, b; Kleine et al., 2007). The accumulation of ELIPs under light stress conditions occurred in a light intensity-dependent manner (Adamska et al., 1992b, 1993; Heddad et al., 2006; Kleine et al., 2007) and correlated with the degree of photoinactivation and photodamage of the PS II reaction center (Adamska et al., 1992b; Pötter and Kloppstech, 1993; Heddad et al., 2006). The different induction kinetics were reported for ELIP1 and ELIP2 in *A. thaliana* exposed to light stress (Heddad et al., 2006). While ELIP1 accumulated almost linearly with increasing light intensities, the induction of ELIP2 occurred stepwise when 40% of PS II reaction centers became photodamaged.

ELIPs are induced also during various developmental stages including de-etiolation (Meyer and Kloppstech, 1984; Grimm and Kloppstech, 1987; Grimm et al., 1989; Beator and Kloppstech, 1993), senescence (Binyamin et al., 2001; Bhalerao et al., 2003; Norén et al., 2003; Heddad et al., 2006) or ripening of tomato fruits (Bruno and Wetzel, 2004). Endogenous regulation of ELIP gene expression by circadian rhythms has also been reported (Kloppstech, 1985; Adamska et al., 1991; Norén et al., 2003).

B. Two-Helix SEPs

Our knowledge about localization and expression of SEPs is very sparse. Also pigment binding has not yet been proven for these proteins. Recently, it was demonstrated that the SEP3 homolog in barley (called LIL3) assembles as pigment-protein complexes during de-etiolation of dark-grown seedlings (Reisinger et al., 2008). Chl *a*, protochlorophyll *a* and carotenoids were identified as components of these complexes. According to molecular masses of 160–180 kDa and 210–250 kDa it was suggested that these complexes constitute a homo-multimeric assembly state composed of 7 and 9 protein subunits, respectively (Reisinger et al., 2008). Accumulation of *A. thaliana* SEP1 and SEP2 in response to light stress was reported (Heddad and Adamska, 2000). Other physiological stress conditions, such as cold, heat, desiccation, salt, wounding or oxidative stress did not significantly influence the expression of *SEP* genes.

C. One-Helix OHP/HLIP/SCPs

Two types of OHPs can be distinguished: the OHP1/HLIP/SCP-type present in cyanophages, cyanobacteria and photosynthetic eukaryota (Dolganov et al., 1995; Funk and Vermaas, 1999; Jansson et al., 2000; Mann et al., 2003; Lindell et al., 2004) and the OHP2-type restricted to eukaryotic organisms (Andersson et al., 2003) (Fig. 14.2). It was reported that accumulation of OHP1 (Jansson et al., 2000) and OHP2 (Andersson et al., 2003) in *A. thaliana* is triggered by light stress and occurs in a light intensity-dependent manner. Location in PS I was reported for OHP2 (Andersson et al., 2003).

While very limited information exists about OHP1 and OHP2 in higher plants and algae, the cyanobacterial homologues HLIP/SCPs are better characterized. Recent experimental data showed that four HLIP/SCPs from *Synechocystis* sp. PCC 6803 form dimers that could be reconstituted with Chl and carotenoids in vitro confirming

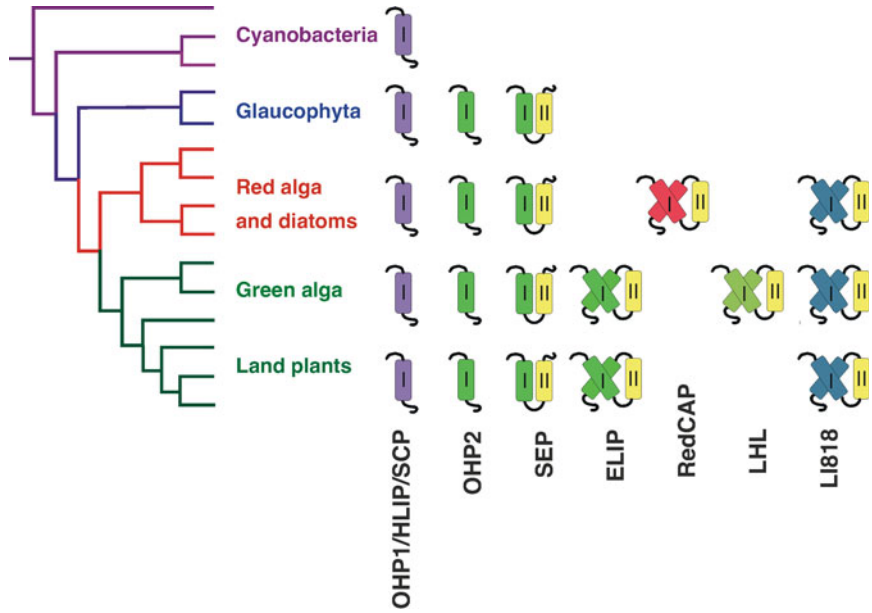

Fig. 14.2. Distribution of LHC-like family members in photosynthetic organisms.

the ability of these proteins for pigment binding (Storm et al., 2008).

Originally, it was shown that *HLIP/SCP* transcripts in *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 accumulated in response to HL exposure (Dolganov et al., 1995; Funk and Vermaas, 1999; He et al., 2001). A different accumulation kinetic was reported for each of these proteins. The HLIA/SCPC, HLIB/SCPD and HLIC/SCPB proteins accumulated rapidly under HL conditions but while the level of HLIC/SCPB remained high for at least 24 h, the amount of HLIA/SCPC and HLIB/SCPD declined after 9–12 h of HL exposure. The HLID/SCPE protein was only transiently expressed under HL conditions and was no longer detectable after 24 h of light stress (He et al., 2001). Similar to higher plants' ELIPs, the expression of *HLIP/SCP* genes was controlled by blue and UV-A light (Dolganov et al., 1995; Salem and Van Waasbergen, 2004). Some years later the induction of HLIP/SCPs under nitrogen or sulfur starvation (He et al., 2001; Osanai et al., 2006), cold stress (Mikami et al., 2002), low pH (Ohta et al., 2005), salt and hyperosmotic stresses (Allakhverdiev et al., 2002; Mikami et al., 2002; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005), presence of hydrogen peroxide (Li et al., 2004), and inhibitors of photosynthetic electron flow (Hihara et al., 2003) was demonstrated for *Synechocystis* sp. PCC 6803. Biochemical as well as immunological studies localized HLIP/SCPs in PS II of *Synechocystis* sp. PCC 6803 (Promnares et al., 2006; Yao et al., 2007; Kufryk et al., 2008). The HLID/SCPE was associated with the monomeric and dimeric PS II core complexes that were suggested to be in the process of assembly and/or repair (Promnares et al., 2006; Yao et al., 2007). No association of HLIP/SCPs with PS I was detected (Yao et al., 2007), although contradictory reports exist. Recently, it was demonstrated that HLIA/SCPC and HLIB/SCPD proteins were associated with trimeric PS I complexes in *Synechocystis* sp. PCC 6803, whereas two others, HLIC/SCPB and HLID/SCPE, were associated with the PSAL subunit of PS I or with a partially dissociated PS I complex, respectively (Wang et al., 2008).

IV. The Era of Functional Genomics

Thanks to the massive genome sequencing projects of photosynthetic organisms ranging from cyanobacteria to higher plants (http://www.jcvi.org), and to the integration of the genome sequence analysis with the different available profiling

expression data (http://www.jcvi.org/cms/research/groups/plant-genomics/resources), attempts to assess gene function through functional genomics have become a lot easier. Plant transcript assemblies are available for more than 257 species (http://plantta.jcvi.org/cgi-bin/plantta_release.pl), allowing the identification of new members of the LHC-like family. Nowadays, functional genomic analysis is eased by the increasing number of available genetic resources, such as The European Arabidopsis Stock Centre (NASC, http://arabidopsis.info), The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org), The Chlamydomonas Center (http://www.chlamy.org) or The Genome Database for Cyanobacteria (http://bacteria-genome.kazusa.or.jp/cyanobase), making the function of a putative gene assessable. Additional tools such as proteomics, structural genomics and bioinformatics are increasing the chances to identify and further characterize new putative light stress proteins.

Completely sequenced genomes of various photosynthetic pro- and eukaryota are available in the age of comparative genomics. Among them are several marine phage genomes (http://www.jgi.doe.gov), over 30 cyanobacterial genomes (http://bacteria.kazusa.or.jp/cyanobase), several algal genomes including those from Glaucophyta, diatoms, Rhodophyta (red alga), Chlorophyta (green alga), the moss *P. patens* and higher plant genomes. The analysis of these genomes allows new insights into the taxonomical distribution of LHC-like family members and provides evolutionary implication of conservation and heterogeneity of various LHC-like subfamilies.

A. Conservation of HLIP Genes in Viral, Cyanobacterial and Eukaryotic Genomes

Currently, more than several hundred different LHC-like family members and related proteins have been found in various photosynthetic organisms (summarized in Fig. 14.2) starting from cyanophages up to higher plants. A short overview of these proteins is provided below.

1. HLIP/SCPs in Viral Genomes

Emerging marine phage genomics revealed the presence of genes central to oxygenic photosynthesis, including homologs encoding the PS II reaction center D1 and D2 proteins and HLIP/SCPs in the genomes of several cyanophages from two families of double-stranded DNA viruses (*Myoviride* and *Podoviride*) that infect marine cyanobacteria (Mann et al., 2003; Lindell et al., 2004; Millard et al., 2004; Mann et al., 2005; Sullivan et al., 2005). Between 1 and 6 *HLIP* genes were found in various *Myovirus* and *Podovirus* strains. It was suggested that the presence of HLIPs in cyanophages might help to maintain photosynthetic activity of the host during phage infection (Lindell et al., 2004, 2005). Phylogenetic analysis revealed that D1, D2 and HLIP/SCPs cluster with those from the cyanobacterium *Prochlorococcus marinus*, indicating that they are of cyanobacterial origin (Lindell et al., 2004). It was proposed that phage have evolved to use up-regulated host genes, leading to their stable incorporation into phage genomes and their subsequent transfer back to the host in genome islands (Lindell et al., 2004, 2007).

2. Cyanobacterial HLIP/SCPs

Searches in the database of cyanobacteria (http://genome.kazusa.or.jp/cyanobase/) revealed that multigene HLIP/SCPs families are present in genomes of marine and fresh water cyanobacteria (Bhaya et al., 2002; Heddad and Adamska, 2002). Eight different members are listed in *Nostoc* sp. PCC 7120, seven in the *Anabaena variabilis* strain ATCC 29413 and six in the atypical cyanobacterium *Gloeobacter violaceus* PCC 7421. The latter cyanobacterium totally lacks thylakoid membranes (Nakamura et al., 2003; Steiger et al., 2005) and genes for PsaI, PsaJ, PsaK, and PsaX subunits of PS I and for PsbY, PsbZ and Psb27 subunits of PS II (Jürgens and Schneider, 1991). Surprisingly, it still harbors *HLIP/SCP* genes. In *Synechocystis* sp. PCC 6803, four HLIP/SCPs were identified (Funk and Vermaas, 1999). These genes encode for HLIA-D, corresponding to SCPC, SCPD, SCPB and SCPE, respectively. Eight HLIP/SCP members are present in the genome of *Synechococcus* sp. PCC 7942. Depending on photoadaptative processes different numbers of *HLIP/SCP* genes were found in genomes of *P. marinus* strains. *P. marinus* strain SS120 represents an ecotype adapted to extreme LL, strain MIT9313 is a LL-adapted ecotype (both strains are most abundant in deeper water) and strain

MED4 is a HL-adapted ecotype dominating well-illuminated water surfaces. The physiological adaptation to LL or HL intensities can be linked to genetic adaptations expressed in the efficiency of photoprotective systems. The number of *HLIP* genes present in the genomes *P. marinus* strains correlates with the adaptation to LL or HL intensity and supports the role of these proteins in photoprotection. The HL-adapted MED4 strain has almost twice as many *HLIP* genes (22 compared with 9 or 13) as are detected in the genomes of LL-adapted strains SS120 or MIT 9313, respectively (Rocap et al., 2003; Hess, 2004). The *Acaryochloris marina* strain MBIC 11017 harbors only three putative HLIP/SCP members. This unique cyanobacterium contains Chl *d* as the major (95%) and Chl *a* as the minor (5%) photosynthetic pigments in addition to phycocyanin and traces of Chl *c* (Swingley et al., 2008). The *Trichodesmium erythraeum* strain IMS101 is a filamentous marine cyanobacterium able to perform nitrogen fixation that contains only two HLIP/SCPs. This organism gives the Red Sea its name when large blooms appear and is one of the organisms most often associated with large blooms in marine waters (http://www.expasy.ch/sprot/hamap/TRIEI.html).

3. Eukaryotic LHC-Like Proteins

The occurrence of three-helix ELIPs is restricted to the green algal lineage and land plants (Heddad and Adamska, 2002; Engelken et al., 2010), as summarized in Fig. 14.2. Two-helix SEPs (Heddad and Adamska, 2000), also called LIL proteins (Jansson, 1999), are ubiquitously distributed in photosynthetic eukaryotes from Glaucophyta, red and green algal lineages, to land plants (Fig. 14.2). One *SEP* sequence was found in the red alga *Galdieria sulphuraria*, six in *A. thaliana*, and a maximal number of nine in the moss *P. patens* (Engelken et al., 2010). The expansion in the number of paralogous *SEP* genes in *P. patens* was proposed to represent an evolutionary strategy to avoid photooxidative damage in the new terrestrial environment (Rensing et al., 2008).

A recently reported protein with two predicted TM helices in a *Synechococcus* strain hli5OS-B' (Kilian et al., 2008) is not a SEP family member since the CB motif is located in the second, instead of first, TM helix (Engelken et al., 2010).

4. Novel Types of LHC-Like Proteins in Algae and Land Plants

The progress in algal and moss genomics has allowed the identification of novel sequences related to LHC-like family members (Fig. 14.2 and Table 14.2). For some of these proteins basic expression analysis and localization studies have been performed. The paragraphs below summarized these studies.

a. RedCAP in the Red Algal Lineage

Recently, nuclear-encoded RedCAP sequences distantly related to LHC-like family members were found in the red algal lineage, including Rhodophyta, Heterokontophyta, and Cryptophyta (Engelken et al., 2010) (Fig. 14.2 and Table 14.2). In contrast to ELIPs from the green algal lineage, the second helix of RedCAPs is also conserved. Apart from their three-helix structure with ELIP consensus motifs, RedCAPs do not share any specific similarity with ELIPs from the green algal lineage nor are they closely related to LHC proteins.

b. LHL in Green Algae

Four *LHL* genes were identified in the genome of *Chlamydomonas reinhardtii* (Teramoto et al., 2004). The work by Teramoto and colleagues showed the expression of three of these genes was induced in response to HL exposure. Homology searches revealed that while LHL1, LHL2 and LHL3 proteins were homologous to ELIPs, OHP/HLIP/SCPs and SEPs, respectively, LHL4 was a novel type of HL-induced protein (Teramoto et al., 2004). Further, this work has revealed that the accumulation of *LHL4* transcripts occurs in a light intensity-dependent manner (Teramoto et al., 2006). The action spectrum for *LHL4* gene expression showed a main peak in the blue light region and a shoulder in the UV-A suggesting that similarly to ELIPs, a flavin-based receptor of cryptochrome/phototropin type participates in these responses. Searches in the sequenced genomes revealed that *LHL4* genes are present in *Mesostigma viridae* and *Volvox carteri*, in addition to already reported *LHL4* from *C. reinhardtii* (Teramoto et al., 2004, 2006; Engelken et al., 2010).

Table 14.2. Novel types of LHC-like proteins in algae and land plants

Name	Organism	Accession	Database	Transcript size (nt)	Protein size (aa)	References
RedCAP	*Galdieria sulphuraria* (Rhodophyta)	Gs47790.1	http://genomics.msu.edu/galdieria/	1125	219	(1)
RedCAP	*Thalassiosira pseudonana* (Heterokontophyta)	*Thaps3:270215*	JGI	1114	221	(1)
RedCAP	*Guillardia theta* (Cryptophyta)	Q5K264	Uniprot	n.a.	189 (partial) (2)	
RedCAP	*Emiliania huxleyi* (Haptophyta)	Emihu1:463191	JGI	n.a.	303 (partial)	(2)
LHL4	*Chlamydomonas reinhardtii* (Chlorophyta)	Chlre3:139895	JGI	858	285	(3)
LHL4	*Mesostigma viride* (Streptophyta)	A3QQP2	Uniprot	n.a.	n.a.	(2)
LI818	*Karlodinium micrum* (Dinophyta)	KML00004375	TBestDB	Partial	Partial	(4)
LI818(Lhcx1)	*Phaeodactylun tricornutum* (Heterokontophyta)	Phatr2: 27278	JGI	630	209	(5)
LI818 (Lhcf1)	*Isochrysis galbana* (Haptophyta)	Q2IA70	Uniprot	n.a.	224	(5)
LI818	*Chlamydomonas reinhardtii* (Chlorophyta)	P93664	Uniprot	n.a.	253	(6)
LI818-1	*Bigelowiella natans* (Chlorarachniophyta)	BK005987	NCBI TPA	n.a.	185	(5)
LI818	*Euglena gracilis* (Euglenophyta)	EC668852	NCBI EST	n.a.	n.a.	(4)
LI818	*Physcomitrella patens* (Streptophyta)	A9SNV6	Uniprot	n.a.	244	(7)
LI818	*Picea glauca* (Streptophyta)	DR591434	NCBI EST	n.a.	n.a.	(2)

Accession numbers for EST sequences and JGI gene models are given. Abbreviations: *aa* amino acid; *n.a.* not available; nt, nucleotide. *References*: (1) Engelken et al., 2010, (2) this review; (3) Teramoto et al. (2006), (4) O'Brien et al. (2007), (5) Koziol et al. (2007), (6) Elrad and Grossman (2004), (7) Alboresi et al. (2008)

Therefore, it seems that the presence of LHL4 proteins is restricted to green algae (Fig. 14.2 and Table 14.2).

c. LI818 in Algae and Land Plants

The first LI818 protein was reported from *C. reinhardtii* and found to be a distant relative of the CAB proteins although it exhibited a different expression pattern (Savard et al., 1996). In synchronized 12 h light/12 h dark *C. reinhardtii* cultures, *LI818* transcripts were expressed in a light-dependent and photosynthesis-independent manner before the accumulation of *CAB* transcripts (Savard et al., 1996; Richard et al., 2000). Phylogenetic analysis and protein sequence comparisons suggested that LI818 proteins might represent a distinct type of CAB proteins (Richard et al., 2000). In the meantime, LI818 proteins were identified in the major groups of algae, including several other green algae (*Ostreococcus tauri*, *Micromonas viride*), Chlorarachniophyta (*Bigelowiella natans*), Heterokontophyta (*Cyclotella cryptica, Thalassiosira pseudonana* and *Phaeodactylum tricornutum)*, Haptophyta (*Isochrysis galbana*) and the moss *P. patens* (Table 14.1 and Fig. 14.2; Savard et al., 1996; Richard et al., 2000; Six et al., 2005; Koziol et al., 2007; Alboresi et al., 2008; Zhu and Green, 2008). Interestingly, no LI818 proteins were found in angiosperms investigated so far.

According to predictions based on the amino acid sequences, LI818 proteins possess three TM helices and several conserved residues involved in Chl binding. It has been shown for *C. reinhardtii* that LI818 protein is not tightly embedded in the stroma region of the thylakoid membranes and can be extracted with chaotropic agents and extreme alkaline pH treatment (Richard et al., 2000). Therefore, it was concluded that both hydrophobic and electrostatic interactions participate in anchoring LI818 polypeptides to the thylakoid membrane.

Analysis of the expression pattern revealed that in addition to HL induction in green cells (Savard et al., 1996; Richard et al., 2000) LI818 proteins accumulated very early during the greening of dark-grown *C. reinhardtii* cultures and several hours before the other CAB proteins could be detected (Richard et al., 2000). However, other stress conditions also triggered the induction of *LI818* genes. Microarray analysis revealed that *LI818* transcripts in *C. reinhardtii* increased 20-fold following sulfur deprivation, where *LHC* transcripts generally declined (Elrad and Grossman, 2004). In the diatom *C. cryptica* transcripts for LI818 proteins, called here *FCP6/LHCf6, FCP7/LHCf7* and *FCP12/LHCf12,* accumulated in response to illumination with HL (Eppard et al., 2000; Oeltjen et al., 2002; Koziol et al., 2007). Similar results were also reported for the diatom *T. pseudonana* (Zhu and Green, 2008). This suggests that similarly to LHC-like family members, LI818 proteins may also have a photoprotective function.

Although LI818 proteins seem to be phylogentically more closely related to LHC than to LHC-like proteins (Elrad and Grossman, 2004) their expression pattern resembles those of LHC-like family members. Since a photoprotective function was proposed for LI818 we included this group of proteins in our review.

B. Evolution of LHC-Like Family Members

With regard to the evolution of the CB superfamily, different models have been proposed (Green and Pichersky, 1994; Durnford et al., 1999; Montané and Kloppstech, 2000; Heddad and Adamska, 2002; Garczarek et al., 2003; Green, 2003; Six et al., 2005; Jansson, 2006; Koziol et al., 2007). A "four-helix common ancestor" of LHC/LHC-like and PSBS family members was postulated by Green and Pichersky (1994). According to this model (Fig. 14.3a) two *HLIP/SCP*-type genes fused during evolution, resulting in the generation of a two-helix ancestor. This model was supported by the discovery of two-helix SEPs in *A. thaliana* and green and red algal lineages (Engelken et al., 2010). The SEPs were proposed to be a missing link between one-helix HLIP/SCPs of cyanobacteria and three-helix ELIPs and LHC proteins of higher plants and algae. An internal gene duplication of a two-helix SEP-like protein would lead to a four-helix intermediate, the PSBS protein. This theory was supported by the fact that TM helices I and III and II and IV of the PSBS protein are closely related (Funk, 2001). Finally the deletion of the forth helix of the PSBS protein appears to have given rise to the three-helix ancestor of LHC-like and LHC proteins.

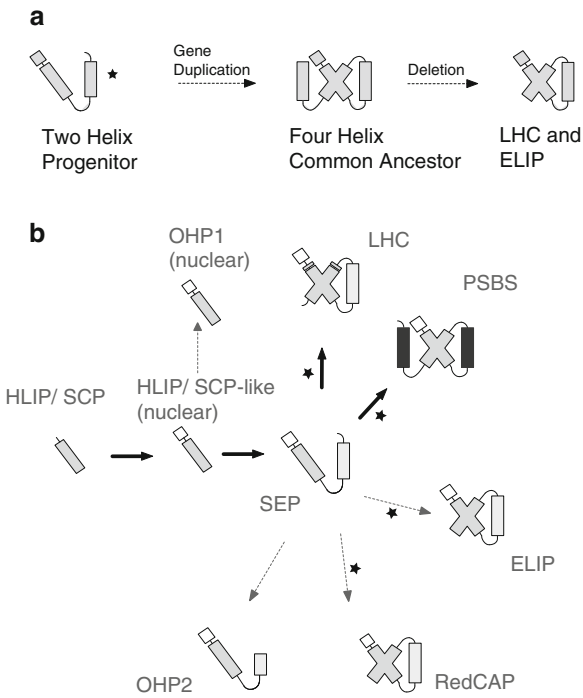

Fig. 14.3. Proposed models for the evolution of LHC-like family members. (**a**) Model presented by Green and Pichersky (1994). One-helix HLIP/SCP-like protein acquired a second helix resulting in a two-helix SEP-like ancestor. An internal gene duplication led to a four-helix PSBS-like protein. After loss of the forth helix, three-helix ELIPs and LHC proteins evolved. (**b**) Model proposed by Engelken and colleagues (2010). This model suggests a stepwise evolution from the cyanobacterial HLIP/SCPs to a central group of SEPs. Independent origins of LHC-like proteins, LHC proteins and PSBS from a two-helix SEP are proposed. Internal gene duplication events are indicated by *stars*.

Such a scenario would impose the presence of an ancestral PSBS-like protein in the red/green algal ancestor. This would also imply that the PSBS-like branch would have been lost secondarily in the red lineage but would have persisted as highly conserved in the green lineage.

Based on information coming from the available algal genomes a novel model for the evolution of the CB superfamily has recently been proposed (Engelken et al., 2010). Similar to previous models, this model (Fig. 14.3b) also suggests a stepwise evolution from the cyanobacterial HLIP/SCPs to the central group of SEPs. According to this scenario the monophyletic group of OHP2 sequences might represent intermediates between nuclear HLIP/SCPs and SEPs, or alternatively, they could be degenerated SEP sequences with their putative membrane anchors leftover from the second SEP helix. However, the novelty of this model is the postulation of independent origins of three-helix LHC-like, LHC and four-helix PSBS proteins. It was suggested that early LHC proteins arose in the red/green ancestor from a SEP and subsequently diversified into different antenna proteins in the red (Chl *a*-binding and Chl *a/c*-binding proteins) and green (CAB) lineages. In this model, PSBS would arise early in the green lineage from a different SEP. This implies that ELIPs are neither ancestral to PSBS nor to LHC, but likely evolved independently from still other SEPs. Similarly, RedCAP sequences would evolve independently in the red algal lineage.

V. Photoprotective Functions of LHC-Like Proteins

Photoprotective functions were proposed for LHC-like family members. It has been suggested that ELIPs may participate in transient binding of Chls released from photodamaged CB proteins and thus prevent the formation of singlet oxygen generated by reaction of free excited Chls in the triplet state with molecular oxygen (Adamska, 1997; Montané and Kloppstech, 2000; Adamska, 2001). Past data on the expression of ELIPs in higher plants showed a clear correlation between their induction/enhancement and the HL-induced degradation of the D1 protein of the PS II reaction center and a massive reduction of the antenna size of PS II in higher plants (Adamska et al., 1993; Lindahl et al., 1997, Heddad et al., 2006) and the green alga *D. salina* (Jin et al., 2001, 2003). A low excitonic coupling between Chl molecules bound to ELIPs (Adamska et al., 1999), the localization of these proteins in the non-appressed regions of thylakoid membranes (Adamska and Kloppstech, 1991), where the assembly/disassembly of photosynthetic complexes occur (Mulo et al., 2008) and their interactions with monomeric and trimeric LHCII (Heddad et al., 2006) are in agreement with such as a function. The discovery that OHP2 from *A. thaliana* is located in PS I during light stress (Andersson et al., 2003) suggests that the photoprotective function of LHC-like family members might be

extended also to this photosystem. The PS I complex has long been believed to be resistant to photoinhibition; however, it was lately found that PS I is sensitive to light especially under chilling conditions (Hihara and Sonoike, 2001).

Additionally, a function of ELIPs in NPQ was reported (Adamska et al., 1999; Montané and Kloppstech, 2000; Adamska, 2001; Funk, 2001; Havaux et al., 2003; Hutin et al., 2003). This function is supported by a high lutein content present in ELIP proteins (Adamska et al., 1999) and field studies in the Colorado Rocky Mountains showing that decreases in the amount of LHC-like family members in conifers from winter through spring paralleled disengagement of sustained zeaxanthin-dependent photoprotection (Zarter et al., 2006a, b). Use of the *A. thaliana chaos* mutant, affected in the posttranslational targeting of LHC proteins to the thylakoids and suppressed in the rapid accumulation of ELIPs during HL, confirmed a photoprotective function of ELIPs (Hutin et al., 2003). Exposure of such a mutant to HL resulted in leaf bleaching and extensive photooxidative damage. Constitutive expression of *ELIP* genes in the *chaos* mutant before light stress restored the phototolerance of plants (Hutin et al., 2003). While the photoprotective function of ELIPs has been experimentally proven in the *chaos* mutant the suppression of *ELIP1*, *ELIP2* or both in wild-type *A. thaliana* plants did not affect tolerance to photoinhibition and photooxidative stress (Casazza et al., 2005; Rossini et al., 2006), probably due to a high redundancy of members of the extended LHC-like family.

Similarly to higher plant ELIPs, HLIP/SCPs in cyanobacteria were also proposed to prevent the formation of reactive oxygen species by serving as transient carriers of Chl and/or to participate in NPQ (Havaux et al., 2003). The quadruple HLIP/SCP mutant of *Synechocystis* sp. PCC 6803 showed a bleached phenotype under HL conditions and died as light intensities exceeded 500 μmol m^{-2} s^{-1} (Havaux et al., 2003). Immunoblot analysis revealed that subunits of PS I, but not of PS II, were strongly reduced in such a mutant. Additionally, HLIP/SCPs seem to stabilize the pigment-binding proteins of PS II (He et al., 2001; Xu et al., 2002a, b, 2004; Vavilin et al., 2007) and trigger trimerization of PS I (Wang et al., 2008).

Recently, a constitutive expression of the *ELIP2* gene in wild-type *A. thaliana* plants suggested the involvement of this protein in the Chl biosynthesis pathway (Tzvetkova-Chevolleau et al., 2007). It was proposed the ELIPs might be a Chl sensor that modulates Chl synthesis to prevent accumulation of free Chl and thereby lower the risk of photooxidative damage. Also cyanobacterial HLIP/SCPs appear to participate in tetrapyrrole biosynthesis and to regulate pigment availability (Xu et al., 2002a, b, 2004). Stable isotope labeling revealed that HLIP/SCPs prevent degradation of PS II-associated Chl molecules (Vavilin et al., 2007). The lifetime of Chls associated with PS I was not significantly affected. The proposed functions of LHC-like family members in higher plants and cyanobacteria are currently under active investigation.

VI. Concluding Remarks

Although enormous progress has been made during the last several years to understand the function of LHC-like family members in cyanobacteria, algae and land plants there are still many open questions concerning this point. One problem is a possible functional redundancy of LHC-like family members or a functional complementation by other photoprotective mechanisms. The majority of analyzed knockout mutants for LHC-like family members in higher plants and cyanobacteria have no obvious phenotype (Casazza et al., 2005; Rossini et al., 2006; I. Adamska, unpublished). Simultaneous inactivation of several LHC-like genes is therefore required. This can be achieved by suppression of gene expression through RNAi technology that has the advantage that several members of the LHC-like family can be targeted in parallel. The physiological role of LHC-like proteins might become evident in the field, under highly variable environmental conditions as was reported for the PSBS protein (Külheim et al., 2002). In a controlled light environment the PSBS protein does not appear to be essential for photoprotection since other photoprotective mechanisms based on antioxidant molecules take on this function (Li et al., 2002; Dall'Osto et al., 2006; Golan et al., 2006). Therefore, the contribution of LHC-like family members to plant fitness in the field and acclimative processes should be proven in future work. The latter point can be investigated using available

A. thaliana ecotypes (accessions). Over 750 natural accessions of *A. thaliana* have been collected from around the world and are available from the two major stock centers, ABRC and NASC. Similarly, HL- (strain MED4) and LL- (strains SS120 and MIT9313) adapted ecotypes (Hess, 2004) of the marine cyanobacterium *P. marinus* can be used for such studies.

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References

Adamska I (1997) Elips-Light-induced stress proteins. Physiol Plant 100: 794–805

Adamska I (2001) The Elip family of stress proteins in the thylakoid membranes of pro- and eukaryota. In: Aro E-M and Andersson B (eds) Regulation of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 11, pp 487–505. Kluwer Academic Publishers, Dordrecht

Adamska I and Kloppstech K (1991) Evidence for an association of the early light-inducible protein (Elip) of pea with photosystem II. Plant Mol Biol 16: 209–223

Adamska I and Kloppstech K (1994) Low temperature increases the abundance of early light-inducible transcript under light stress conditions. J Biol Chem 269: 30221–30226

Adamska I, Scheel B and Kloppstech K (1991) Circadian oscillations of nuclear-encoded chloroplast proteins in pea (*Pisum sativum*). Plant Mol Biol 14: 1055–1065

Adamska I, Kloppstech K and Ohad I (1992a) UV light stress induces the synthesis of the early light-inducible protein and prevents its degradation. J Biol Chem 267: 24732–24737

Adamska I, Ohad I and Kloppstech K (1992b) Synthesis of early light-inducible protein is controlled by blue light and related to light stress. Proc Natl Acad Sci USA 89: 2610–2613

Adamska I, Kloppstech K and Ohad I (1993) Early light-inducible protein in pea is stable during light stress but is degraded during recovery at low light intensity. J Biol Chem 268: 5438–5444

Adamska I, Roobol-Boza M, Lindahl M and Andersson B (1999) Isolation of pigment-binding early light-inducible proteins from pea. Eur J Biochem 260: 453–460

Adamska I, Kruse E and Kloppstech K (2001) Stable insertion of the early light-induced proteins into etioplast membranes requires chlorophyll *a*. J Biol Chem 276: 8582–8587

Alboresi AS, Caffarri S, Nogue F, Bassi R and Morosinotto T (2008) *In silico* and biochemical analysis of *Physcomitrella patens* photosynthetic antenna: identification of subunits which evolved upon land adaptation. PLoS ONE 3: e2033

Allakhverdiev SI, Nishiyama Y, Miyairi S, Yamamoto H, Inagaki N, Kanesaki Y and Murata N (2002) Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*. Plant Physiol 130: 1443–1453

Andersson U, Heddad M and Adamska I (2003) Light stress-induced one helix protein of the chlorophyll *a/b*-binding family associated with photosystem I. Plant Physiol 132: 811–820

Barros T, Royant A, Standfuss J, Dreuw A and Kühlbrandt W (2009) Crystal structure of plant light-harvesting complex shows the active, energy-transmitting state. EMBO J 28: 298–306

Bartels D, Hanke C, Schneider K, Michel D and Salamini F (1992) A desiccation-related *ELIP*-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. EMBO J 11: 2771–2778

Beator J and Kloppstech K (1993) The circadian oscillator coordinates the synthesis of apoproteins and their pigments during chloroplast development. Plant Physiol 103: 191–196

Beator J, Pötter E and Kloppstech K (1992) The effect of heat shock on morphogenesis in barley: Coordinated circadian regulation of mRNA levels for light-regulated genes and of the capacity for accumulation of chlorophyll protein complexes. Plant Physiol 100: 1780–1786

Bhalerao R, Keskitalo J, Sterky F, Erlandsson R, Björkbacka H, Birve SJ, Karlsson J, Gardeström P, Gustafsson P, Lundeberg J and Jansson S (2003) Gene expression in autumn leaves. Plant Physiol 131: 430–442

Bhaya D, Dufresne A, Vaulot D and Grossman A (2002) Analysis of the *hli* gene family in marine and freshwater cyanobacteria. FEMS Microbiol Lett 215: 209–219

Binyamin L, Falah M, Portnoy V, Soudry E and Gepstein S (2001) The early light-induced protein is also produced during leaf senescence of *Nicotiana tabacum*. Planta 212: 591–597

Blankenship RE (2001) Molecular evidence for the evolution of photosynthesis. Trends Plant Sci 6: 4–6

Bruno AK and Wetzel CM (2004) The early light-inducible protein (ELIP) gene is expressed during the chloroplast-to-chromoplast transition in ripening tomato fruit. J Exp Bot 55: 2541–2548

Casazza AP, Tarantino D and Soave C (2005) Preparation and functional characterization of thylakoids from *Arabidopsis thaliana*. Photosynth Res 68: 175–180

Cronshagen U and Herzfeld F (1990) Distribution of early light-inducible proteins in the thylakoids of developing pea chloroplasts. Eur J Biochem 193: 361–366

Dall'Osto L, Lico C, Alric J, Giuliano G, Havaux M and Bassi R (2006) Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection *in vivo* under strong light. BMC Plant Biol 6: 32

Dolganov NA, Bhaya D and Grossman AR (1995) Cyanobacterial protein with similarity to the chlorophyll *a/b* binding proteins of higher plants: evolution and regulation. Proc Natl Acad Sci USA 92: 636–640

Durnford DG, Deane JA, Tan S, McFadden GI, Gantt E and Green BR (1999) A phylogenetic assessment of the eukaryotic light-harvesting antenna proteins, with implications for plastid evolution. J Mol Evol 48: 59–68

Elrad D and Grossman A (2004) A genome´s-eye view of the light-harvesting polypeptides of *Chlamydomonas reinhardtii*. Curr Genet 45: 61–75

Engelken J, Brinkmann H and Adamska I (2011) Taxonomic distribution and origins of the extended LHC (light-harvesting complex) antenna protein superfamily. BMC Evol Biol 10: 233

Eppard M, Krumbein WE, von Haeseler A and Rhiel E (2000) Characterization of *fcp4* and *fcp12*, two additional genes encoding light-harvesting proteins of *Cyclotella cryptica* (Bacillariophyceae) and phylogenetic analysis of this complex gene family. Plant Biol 2: 283–289

Funk C (2001) The PsbS protein: A Cab-protein with a function of its own. In: Aro E-M and Andersson B (eds) Regulation of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 11, pp 453–467. Kluwer Academic Publishers, Dordrecht

Funk C and Vermaas W (1999) A cyanobacterial gene family coding for single-helix proteins resembling part of the light-harvesting proteins from higher plants. Biochemistry 38: 9397–9404

Garczarek L, Poupon A and Partensky F (2003) Origin and evolution of transmembrane chl-binding proteins: hydrophobic cluster analysis suggests a common one-helix ancestor for prokaryotic (Pcb) and eukaryotic (LHC) antenna protein superfamilies. FEMS Microbiol Lett 222: 59–68

Golan T, Müller-Moulé P and Niyogi KK (2006) Photoprotection mutants of *Arabidopsis thaliana* acclimate to high light by increasing photosynthesis and specific antioxidants. Plant Cell Environ 29: 879–887

Green BR (2003) The evolution of light-harvesting antennas. In: Green BR and Parson WW (eds) Light-Harvesting Antenna in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 129–168. Kluwer Academic Publishers, Dordrecht

Green BR and Kühlbrandt W (1995) Sequence conservation of light-harvesting and stress-response proteins in relation to the three-dimensional molecular structure of LHCII. Photosynth Res 44: 149–162

Green BR and Pichersky E (1994) Hypothesis for the evolution of three-helix chl *a/b* and chl *a/c* light-harvesting antenna proteins from two-helix and four-helix ancestors. Photosynth Res 39: 149–162

Green BR, Pichersky E and Kloppstech K (1991) Chlorophyll *a/b*-binding proteins: An extended family. Trends Biochem Sci 16: 493–499

Grimm B and Kloppstech K (1987) The early light-inducible proteins of barley. Characterization of two families of 2-h-specific nuclear-encoded chloroplast proteins. Eur J Biochem 167: 493–499

Grimm B, Kruse E and Kloppstech K (1989) Transiently expressed early light-inducible thylakoid proteins share transmembrane domains with light-harvesting chlorophyll-binding proteins. Plant Mol Biol 13: 583–593

Havaux M, Guedeney G, He Q and Grossman AR (2003) Elimination of high-light-inducible polypeptides related to eukaryotic chlorophyll *a/b*-binding proteins results in aberrant photoacclimation in *Synechocystis* PCC6803. Biochim Biophys Acta 1557: 21–33

He Q, Dolganov N, Björkman O and Grossman AR (2001) The high light-inducible polypeptides in *Synechocystis* PCC6803. Expression and function in high light. J Biol Chem 276: 306–314

Heddad M, and Adamska I (2000) Light stress-regulated two-helix proteins in *Arabidopsis thaliana* related to the chlorophyll *a/b*-binding gene family. Proc Natl Acad Sci USA 97: 3741–3746

Heddad M and Adamska I (2002) The evolution of light stress proteins in photosynthetic organisms. Comp Funct Genom 3: 504–510

Heddad M, Norén H, Reiser V, Dunaeva M, Andersson B and Adamska I (2006) Differential expression and localization of early light-induced proteins in Arabidopsis. Plant Physiol 142: 75–87

Hess WR (2004) Genome analysis of marine photosynthetic microbes and their global role. Curr Opin Biotechnol 15: 191–198

Hihara Y and Sonoike K (2001) Regulation, inhibition and protection of photosystem I. In: Aro E-M and Andersson B (eds) Regulation of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 11, pp 507–531. Kluwer Academic Publishers, Dordrecht

Hihara Y, Sonoike K, Kanehisa M and Ikeuchi M (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 185: 1719–1725

Horton P, Johnson MP, Pérez-Bueno ML, Kiss AZ and Ruban AV (2008) Photosynthetic acclimation: Does the dynamic structure and macro-organization of photosystem II in higher plant grana membranes regulate light harvesting states? FEBS J 275: 1069–1079

Hutin C, Nussaume L, Moise N, Moya I, Kloppstech K and Havaux M (2003) Early light-induced proteins protect *Arabidopsi*s from photooxidative stress. Proc Natl Acad Sci USA 100: 4921–4926

Jansson S (1999) A guide to the identification of the *Lhc* genes and their relatives in Arabidopsis. Trends Plant Sci 4: 236–240

Jansson S (2006) A protein family saga: From photoprotection to light-harvesting (and back?). In: Demmig-Adams B, William W, Adams III and Mattoo AK (eds) Photoprotection, Photoinhibition, Gene Regulation and Environment, Advances in Photosynthesis and Respiration, Vol 21, pp 145–153. Springer, Dordrecht

Jansson S, Andersson J, Kim SJ and Jackowski G (2000) An *Arabidopsis thaliana* protein homologous to cyanobacterial high-light-inducible protein. Plant Mol Biol 42: 345–351

Jin ES, Polle JWE and Melis A (2001) Involvement of zeaxanthin and of the Cbr protein in the repair of photosystem II from photoinhibition in the green alga *Dunaliella salina*. Biochim Biophys Acta 227: 680–685

Jin ES, Yokthongwattana K, Polle JEW and Melis A (2003) Role of the reversible xanthophyll cycle in the photosystem II damage and repair cycle in *Dunaliella salina*. Plant Physiol 132: 352–364

Jürgens U and Schneider S (1991) Cell wall and sheath constituents of the cyanobacterium *Gloeobacter violaceus*. Arch Microbiol 155: 312–318

Kilian O, Soisig Steunou A, Grossman AR and Bhaya D (2008) A novel two-domain fusion protein in cyanobacteria with similarity to the CAB/ELIP/HLIP superfamily: Evolutionary implications and regulation. Mol Plant 1: 155–166

Kim SJ, Jansson S, Hoffman NE, Robinson C and Mant A (1999) Distinct "assisted" and "spontaneous" mechanisms for the insertion of polytopic chlorophyll-binding proteins into the thylakoid membranes. J Biol Chem 274: 4715–4721

Kleine T, Kindgren P, Benedict C, Hendrickson L and Strand Å (2007) Genome-wide gene expression analysis reveals a critical role for CRYPTOCHROME1 in the response of Arabidopsis to high irradiance. Plant Physiol 144: 1391–1406

Kloppstech K (1985) Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. Planta 165: 502–506

Kolanus W, Scharnhorst C, Kühne U and Herzfeld F (1987) The structure and light-dependent transient expression of a nuclear-encoded chloroplast protein gene from pea (*Pisum sativum* L.). Mol Gen Genet 209: 234–239

Koziol AG, Borza T, Ishida K, Keeling P, Lee RW and Durnford DG (2007) Tracing the evolution of the light-harvesting antennae in chlorophyll *a/b*-containing organisms. Plant Physiol 143: 1802–1816

Król M, Maxwell DP and Huner NPA (1997) Exposure of *Dunaliella salina* to low temperature mimics the high light-induced accumulation of carotenoids and the carotene-binding protein (Cbr). Plant Cell Physiol 38: 213–216

Kruse E and Kloppstech K (1992) Integration of early light-inducible proteins into isolated thylakoid membranes. Eur J Biochem 208: 195–202

Kufryk G, Hernandez-Prieto MA, Kieselbach T, Miranda H, Vermaas W and Funk C (2008) Association of small CAB-like proteins (SCPs) of *Synechocystis* sp. PCC 6803 with photosystem II. Photosynth Res 95: 135–145

Kühlbrandt W, Wang DN and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Külheim C, Agren J and Jansson S (2002) Rapid regulation of light harvesting and plant fitness in the field. Science 297: 91–93

Lers A, Levy H and Zamir A (1991) Co-regulation of a gene homologous to early light-induced genes in higher plants and β-carotene biosynthesis in the alga *Dunaliella bardawil*. J Biol Chem 266: 13698–13705

Levy H, Gokhman I and Zamir A (1992) Regulation and light-harvesting complex II association of a *Dunaliella* protein homologous to early light-induced proteins in higher plants. J Biol Chem 267: 18831–18836

Levy H, Tal T, Shaish A and Zamir A (1993) Cbr, an algal homolog of plant early light-induced proteins is a putative zeaxanthin binding protein. J Biol Chem 268: 20892–20896

Li H, Singh AK, McIntyre LM and Sherman LA (2004) Differential gene expression in response to hydrogen peroxide and the putative PerR regulon of *Synechocystis* sp. strain PCC 6803. J Bacteriol 186: 3331–3345

Li XP, Muller-Moule P, Gilmore AM and Niyogi KK (2002) PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. Proc Natl Acad Sci USA 99: 15222–15227

Lindahl M, Funk C, Webster J, Bingsmark S, Adamska I and Andersson B (1997) Expression of Elips and Psb-S protein in spinach during acclimative reduction of the photosystem II antenna in response to increased light intensities. Photosynth Res 54: 227–236

Lindell D, Sullivan MB, Johnson ZI, Tolonen AC, Rohwer F and Chisholm SW (2004) Transfer of photosynthesis genes to and from *Prochlorococcus* viruses. Proc Natl Acad Sci USA 101: 11013–11018

Lindell D, Jaffe JD, Johnson ZI, Church GM and Chisholm SW (2005) Photosynthesis genes in marine viruses yield proteins during host infection. Nature 438: 86–89

Lindell D, Jaffe JD, Coleman ML, Futschik ME, Axmann IM, Rector T, Kettler G, Sullivan MB, Steen R, Hess WR et al. (2007) Genome-wide expression dynamics of a marine virus and host reveal features of co-evolution. Nature 449: 83–86

Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X and Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. Nature 428: 287–292

Mann NH, Cook A, Millard A, Bailey S and Clokie M (2003) Marine ecosystems: bacterial photosynthesis genes in a virus. Nature 424: 741

Mann NH, Clokie MR, Millard A, Cook A, Wilson WH, Wheatley PJ, Letarov A and Krisch HM (2005) The genome of S-PM2, a 'photosynthetic' T4-type bacteriophage that infects marine *Synechococcus* strains. J Bacteriol 187: 188–200

Meyer G and Kloppstech K (1984) A rapidly light-induced chloroplast protein with a high turnover coded for by pea nuclear DNA. Eur J Biochem 138: 201–207

Mikami K, Kanesaki Y, Suzuki I and Murata N (2002) The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp. PCC6803. Mol Microbiol 46: 905–915

Millard A, Clokie MR, Shub DA and Mann NH (2004) Genetic organization of the *psbAD* region in phages infecting marine *Synechococcus* strains. Proc Natl Acad Sci USA 101: 11007–11012

Montané MH and Kloppstech K (2000) The family of light-harvesting-related proteins (LHCs, ELIPs, HLIPs): was the harvesting of light their primary function? Gene 258: 1–8

Montané MH, Dreyer S and Kloppstech K (1996) Posttranslational stabilization of Elips and regulation of other light stress genes under prolonged light- and cold-stress in barley. In: Grilo S and Leone A (eds) Physical stresses in plants: Genes and their products for tolerance, pp 210–222. Springer, Berlin

Montané MH, Petzold B and Kloppstech K (1999) Formation of early light-inducible protein complexes and status of xanthophyll levels under high light and cold stress in barley (*Hordeum vulgare* L.). Planta 208: 519–527

Mulo P, Sirpiö S, Suorsa M and Aro EM (2008) Auxiliary proteins involved in the assembly and sustenance of photosystem II. Photosynth Res 98: 489–501

Nakamura Y, Kaneko T, Sato S, Mimuro M, Miyashita T, Tsuchiya T, Sasamoto S, Watanabe A, Kawashima K, Kishida Y at al. (2003) Complete genome structure of *Gloeobacter violaceus* PCC 7421, a cyanobacterium that lacks thylakoids. DNA Res 10: 137–145

Norén H, Svensson P, Stegmark R, Funk C, Adamska I and Andersson B (2003) Expression of the early light-induced protein but not the PsbS protein is influenced by low temperature and depends on the developmental stage of the plant in field-grown pea cultivars. Plant Cell Environ 26: 245–253

O'Brien EA, Koski LB, Zhang Y, Yang L, Wang E, Gray MW, Burger G and Lang BF (2007) TBestDB: a taxonomically broad database of expressed sequence tags (ESTs). Nucleic Acids Res 35: D445–451

Oeltjen A, Krumbein WE and Rhiel E (2002) Investigations on transcript sizes, steady-state mRNA concentrations and diurnal expression of genes encoding fucoxanthin chlorophyll *a/c* light-harvesting polypeptides in the centric diatom *Cyclotella cryptica*. Plant Biol 4: 250–257

Ohta H, Shibata Y, Haseyama Y, Yoshino Y, Suzuki T, Kagasawa T, Kamei A, Ikeuchi M and Enami I (2005) Identification of genes expressed in response to acid stress in *Synechocystis* sp. PCC 6803 using DNA microarrays. Photosynth Res 84: 225–230

Osanai T, Imamura S, Asayama M, Shirai M, Suzuki I, Murata N and Tanaka K (2006) Nitrogen induction of sugar catabolic gene expression in *Synechocystis* sp. PCC 6803. DNA Res 13: 185–195

Otto B, Ohad I and Kloppstech K (1992) Temperature treatments of dark-grown pea seedlings cause an accelerated greening in the light at different levels of gene expression. Plant Mol Biol 18: 887–896

Ouvrard O, Cellier F, Ferrare K, Tousch D, Lamaze T, Dupuis JM and Casse-Delbart F (1996) Identification and expression of water stress- and abscisic acid-regulated genes in a drought-tolerant sunflower genotype. Plant Mol Biol 31: 819–829

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I and Murata N (2004) Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. J Biol Chem 279: 53078–53086

Pascal AA, Liu Z, Broess K, Van Oort B, Van Amerongen H, Wang C, Horton P, Robert B, Chang W and Ruban A (2005) Molecular basis of photoprotection and control of photosynthetic light-harvesting. Nature 436: 134–137

Peng Y, Lin W, Wei H, Krebs SL and Arora R (2008) Phylogenetic analysis and seasonal cold acclimation-associated expression of early light-induced protein genes of *Rhododendron catawbiense*. Physiol Plant 132: 44–52

Pérez-Bueno ML, Johnson MP, Zia A, Ruban AV and Horton P (2008) The Lhcb protein and xanthophyll composition of the light harvesting antenna controls the delta-pH-dependency of non-photochemical quenching in *Arabidopsis thaliana*. FEBS Lett 582: 1477–1482

Piechulla B and Gruissem W (1987) Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. EMBO J 6: 3593–3599

Promnares K, Komenda J, Bumba L, Nebesarova J, Vacha F and Tichy M (2006) Cyanobacterial small chlorophyll-binding protein ScpD (HliB) is located on the periphery of photosystem II in the vicinity of PsbH and CP47 subunits. J Biol Chem 281: 32705–32713

Pötter E and Kloppstech K (1993) Effect of light stress on the expression of early light-inducible proteins in barley. Eur J Biochem 214: 779–786

Reisinger V, Ploscher M and Eichacker LA (2008) Lil3 assembles as chlorophyll-binding protein complex during deetiolation. FEBS Lett 582: 1547–1551

Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud PF, Lindquist EA, Kamisugi Y et al. (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science 319: 64–69

Richard C, Ouellet H and Guertin M (2000) Characterization of the LI818 polypeptide from the green unicellular alga *Chlamydomonas reinhardtii*. Plant Mol Biol 42: 303–316

Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, Ahlgren NA, Arellano A, Coleman M, Hauser L, Hess WR et al. (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature 424: 1042–1047

Rossini S, Casazza AP, Engelmann EC, Havaux M, Jennings RC and Soave C (2006) Suppression of both ELIP1 and ELIP2 in *Arabidopsis* does not affect tolerance to photoinhibition and photooxidative stress. Plant Physiol 141: 1264–1273

Ruban AV, Berera R, Ilioaia C, Van Stokkum IH, Kennis JT, Pascal AA, Van Amerongen H, Robert B, Horton P and Van Grondelle R (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature 450: 575–578

Salem K and Van Waasbergen LG (2004) Light control of *hliA* transcription and transcript stability in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. Plant Cell Physiol 45: 651–658

Savard F, Richard C and Guertin M (1996) The *Chlamydomonas reinhardtii* LI818 gene represents a distant relative of the *cab I/II* genes that is regulated during the cell cycle and in response to illumination. Plant Mol Biol 32: 461–473

Sävenstrand H, Olofsson M, Samuelsson M and Strid Å (2004) Induction of early light-inducible protein gene expression in *Pisum sativum* after exposure to low levels of UV-B irradiation and other environmental stresses. Plant Cell Rep 22: 532–536

Scharnhorst C, Heinze H, Meyer G, Kolanus W, Bartsch K, Heinrichs S, Gudschun T, Möller M and Herzfeld F (1985) Molecular cloning of a pea mRNA encoding an early light-induced, nuclear coded chloroplast protein. Plant Mol Biol 4: 241–245

Shimosaka E, Sasanuma T and Handa H (1999) A wheat cold-regulated cDNA encoding an early light-inducible protein (ELIP): Its structure, expression and chromosomal localization. Plant Cell Physiol 40: 319–325

Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV, Tanticharoen M, Suzuki I and Murata N (2005) Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in *Synechocystis*. J Biol Chem 280: 21531–21586

Six C, Worden AZ, Rodriguez F, Moreau H and Partensky F (2005) New insights into the nature and phylogeny of prasinophyte antenna proteins: *Ostreococcus tauri*, a case study. Mol Biol Evol 22: 2217–2230

Standfuss J, Terwisscha Van Scheltinga AC, Lamborghini M and Kühlbrandt W (2005) Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. EMBO J 24: 919–928

Steiger S, Jackisch Y and Sandmann G (2005) Carotenoid biosynthesis in *Gloeobacter violaceus* PCC4721 involves a single crtI-type phytoene desaturase instead of typical cyanobacterial enzymes. Arch Microbiol 184: 207–214

Storm P, Hernandez-Prieto MA, Eggink LL, Hoober JK and Funk C (2008) The small CAB-like proteins of *Synechocystis* sp. PCC 6803 bind chlorophyll: *In vitro* pigment reconstitution studies on one-helix light-harvesting-like proteins. Photosynth Res 98: 479–488

Sullivan MB, Coleman ML, Weigele P, Rohwer F and Chisholm SW (2005) Three *Prochlorococcus* cyanophage genomes: signature features and ecological interpretations. PLoS Biol 3: e144

Swingley WD, Chen M, Cheung PC, Conrad AL, Dejesa LC, Hao J, Honchak BM, Karbach LE, Kurdoglu A, Lahiri S et al. (2008) Niche adaptation and genome expansion in the chlorophyll *d*-producing cyanobacterium *Acaryochloris marina*. Proc Natl Acad Sci USA 105: 2005–2010

Teramoto H, Itoh T and Ono T (2004) High-intensity-light-dependent and transient expression of new genes encoding distant relatives of light-harvesting chlorophyll-*a/b* proteins in *Chlamydomonas reinhardtii*. Plant Cell Physiol 45: 1221–1232

Teramoto H, Ishii A, Kimura Y, Hasegawa K, Nakazawa S, Nakamura T, Higashi S, Watanabe M and Ono TA (2006) Action spectrum for expression of the high intensity light-inducible Lhc-like gene *Lhl4* in the green alga *Chlamydomonas reinhardtii.* Plant Cell Physiol 47: 419–425

Tzvetkova-Chevolleau T, Franck F, Alawady AE, Dall'Osto L, Carrière F, Bassi R, Grimm B, Nussaume L and Havaux M (2007) The light stress-induced protein ELIP2 is a regulator of chlorophyll synthesis in *Arabidopsis thaliana*. Plant J 50: 795–709

Vavilin D, Yao D and Vermaas W (2007) Small Cab-like proteins retard degradation of photosystem II-associated chlorophyll in *Synechocystis* sp. PCC 6803: kinetic analysis of pigment labeling with ^{15}N and ^{13}C. J Biol Chem 282: 37660–37668

Wang Q, Jantaro S, Lu B, Majeed W, Bailey M and He Q (2008) The high light-inducible polypeptides stabilize trimeric photosystem I complex under high light conditions in *Synechocystis* PCC 6803. Plant Physiol 147: 1239–1250

Xu H, Vavilin D, Funk C and Vermaas W (2002a) Small Cab-like proteins regulating tetrapyrrole biosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Mol Biol 49: 149–160

Xu H, Vavilin D and Vermaas W (2002b) The presence of chlorophyll *b* in *Synechocystis* sp. PCC 6803 disturbs tetrapyrrole biosynthesis and enhances chlorophyll degradation. J Biol Chem 277: 42726–42732

Xu H, Vavilin D, Funk C and Vermaas W (2004) Multiple deletions of small Cab-like proteins in the cyanobacterium *Synechocystis sp*. PCC 6803: consequences for pigment biosynthesis and accumulation. J Biol Chem 272: 27971–27979

Yao D, Kieselbach T, Komenda J, Promnares K, Prieto MA, Tichy M, Vermaas W and Funk C (2007) Localization of the small CAB-like proteins in photosystem II. J Biol Chem 282: 267–276

Zarter CR, Adams III WW, Ebbert V, Cuthbertson DJ, Adamska I and Demmig-Adams B (2006a) Winter down-regulation of intrinsic photosynthetic capacity coupled with up-regulation of ELIP-like proteins and persistent energy dissipation in a subalpine forest. New Phytol 172: 272–282

Zarter CR, Demmig-Adams B, Ebbert V, Adamska I and Adams III WW (2006b) Photosynthetic capacity and light harvesting efficiency during the winter-to-spring transition in subalpine conifers. New Phytol 172: 283–292

Zhu SH and Green BR (2008) Light-harvesting and photoprotection in diatoms: identification and expression of LI818-like proteins. In: Allen JF, Gantt E, Golbeck JH and Osmond B (eds) Photosynthesis. Energy from the Sun, 14th International Congress on Photosynthesis, Chapter 6, pp 261–264. Springer, Netherlands

Chapter 15

Environmentally-Induced Oxidative Stress and Its Signaling

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Summary

Several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism. Chloroplasts are a major site of ROS generation in plants. Many environmental changes such as light, temperature, drought, pathogens or nutrient stresses affect the efficiency of photosynthesis and result in ROS production. Over the last few years, a wide range of plant responses have been found to be triggered by ROS. Several examples of how changes in ROS concentrations are perceived and transferred into signals that change the transcription of genes have been described. Moreover, the chemical identity of a given ROS seems to affect the specificity of its biological activity, thus increasing further the complexity of ROS signaling within the plant. Although the mechanisms that control ROS signaling remain to be established more precisely, we are beginning to understand how ROS signals are integrated to finally regulate biological processes such as development, acclimation, hypersensitive reactions and programmed cell death.

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I. Introduction

Photosynthesis, as an aerobic process, unavoidably leads to the production of reactive oxygen species (ROS) in chloroplasts. Ground state oxygen may be converted to singlet oxygen by energy transfer or by electron transfer reactions to superoxide, hydrogen peroxide, and hydroxyl radicals (Klotz, 2002; Apel and Hirt, 2004; Gechev et al., 2006). The production of ROS in plants might be enhanced by environmental changes such as light, temperature, drought, pathogens or nutrient stresses (Apel and Hirt, 2004; Laloi et al., 2004; Gechev et al., 2006). The relative contribution of the different ROS that are produced may vary depending on the actual environmental stress to which plants are exposed. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. Furthermore, increasing evidence indicates that ROS also function as signaling molecules involved in regulating plant development, acclimation, hypersensitive reaction (HR) and programmed cell death (PCD) (Foyer and Noctor, 2009). The ROS signaling function depends on their chemical identity. In this chapter, we describe the different ROS that might be generated, the mechanism of their production in plastids and their biological effects in plants.

II. Chemistry of the Major Reactive Oxygen Species (ROS) Encountered in Plants

In plants, ROS are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments (Foyer and Noctor, 2003; Apel and Hirt, 2004). ROS are generated in plants mostly by pathways that involve electron transfer reactions, although the generation of radicals can also take place by direct cleavage of chemical bonds when plants are exposed to energetic radiation (Hideg and Vass, 1996; Hawkins and Davies, 2001).

The relatively stable ground state of oxygen is a triplet state with two unpaired electrons of identical spin quantum number, each located in different antibonding orbitals (Fig. 15.1). To oxidize a nonradical (singlet) atom or molecule, triplet oxygen would need to react with a partner that provides a pair of electrons with parallel spins that fit into its free electron orbitals. However, pairs of electrons typically have opposite spins, and thus luckily impose a restriction on the reaction of triplet molecular oxygen with most organic molecules (Cadenas, 1989; Apel and Hirt, 2004). Nevertheless, ground state oxygen maybe converted to the much more reactive singlet oxygen ($^1O_2$) by energy transfer; in this state, the spin restriction is removed and therefore the oxidizing ability of the oxygen is greatly increased. (Apel and Hirt, 2004; Krieger-Liszkay, 2005; Ledford and Niyogi, 2005) (Fig. 15.1). Singlet oxygen, a non-radical ROS, has a short halftime of about 2×10^{-7} s in cells (Gorman and Rodgers, 1992), and its possible diffusion distance has been estimated to be up to 1×10^{-8} m in a physiologically relevant environment (Sies and Menck, 1992). Thus singlet oxygen only reacts with lipids, nucleic acids and proteins in their immediate neighborhood (Martinez et al., 2003; Davies, 2004; Girotti and Kriska, 2004).

Ground state oxygen may also be converted to ROS by electron transfer reactions. The first step during oxygen reduction leads to the formation of a superoxide anion ($O_2^{\bullet -}$) (Fig. 15.1). A superoxide anion is a very highly reactive intermediate that has a very short half-life of 2×10^{-6} to 4×10^{-6} s (Gechev et al., 2006). The second step during O_2 reduction leads to the formation of hydrogen peroxide (H_2O_2). In plastids, superoxide anions are rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD, Fig. 15.1). Unlike singlet oxygen and superoxide anions, hydrogen peroxide is a relatively stable molecule with a 1×10^{-3} s half-life (Henzler and Steudle, 2000; Gechev and Hille, 2005). Therefore, hydrogen peroxide can migrate from its cellular

Abbreviations: $^1O_2$ – Singlet oxygen; ABA – Abscisic acid; ANP – Arabidopsis npk1-related protein kinase; APX – Ascorbate peroxidase; EEE – Excess excitation energy; *flu* – Fluorescent; GPX – Glutathione peroxidase; HOTE – Hydroxy octadecatrieonic acid; HR – Hypersensitive reaction; JA – Jasmonic acid; LH – Lipid hydroperoxide; LOX – Lipoxygenase; MAPK – Mitogen-activated protein kinase; NDPK – Nucleotide diphosphate kinase; ONOO- – Peroxynitrite; $O_2^{\bullet -}$ – Superoxide anion; OPDA – 12-oxo-phytodienoic acid; PCD – Programmed cell death; PI3P – Phosphatidylinositol 3-phosphate; PS – Photosystem; PTP – Protein tyrosine phosphatases; Rboh – Respiratory burst oxidase homologue; ROS – Reactive oxygen species; SA – Salicylic acid; SAR – Systemic acquired resistance; SOD – Superoxide dismutase; TF – Transcription factor

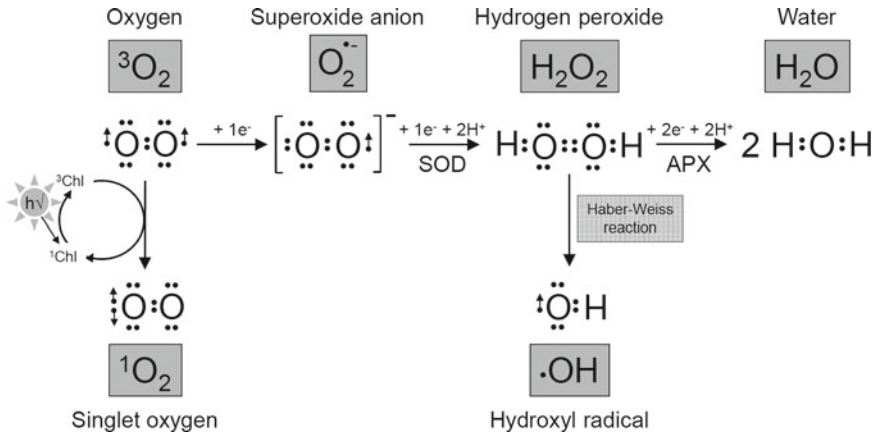

Fig. 15.1. Structure and generation of the major reactive oxygen species in plants. Ground state oxygen maybe converted to the much more reactive singlet oxygen ($^1O_2$) by energy transfer, or it may also be converted to a superoxide anion ($O_2^{\bullet-}$) by electron transfer reactions. In most biological systems, superoxide anions are rapidly converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase (*SOD*). In plastids, H_2O_2 can be converted to water (H_2O) by the enzyme ascorbate peroxidase (*APX*). The concomitant presence of hydrogen peroxide and superoxide anions can lead to the production of highly reactive hydroxyl radicals ($HO^{\bullet}$), via the Haber-Weiss reaction.

synthesis site to adjacent compartments and even neighboring cells (Henzler and Steudle, 2000). The high reactivity of $O_2^{\bullet-}$ and H_2O_2 makes them harmful molecules for the surrounding cellular environment where they can inactivate important proteins such as metabolic enzymes containing Fe-S clusters (Gechev et al., 2006; Halliwell, 2006). The concomitant presence of hydrogen peroxide and superoxide anions in a cell might have the potential to bring about even more destructive consequences when they are in the company of metal ions. In the Haber-Weiss reaction, hydrogen peroxide can be transformed into highly reactive hydroxyl radicals ($HO^{\bullet}$; Fig. 15.1), using divalent iron ions as catalysts (Kehrer, 2000). A hydroxyl radical can react with and damage virtually everything with which it comes into contact and causes oxidative damage by oxidizing fatty acids and/or amino acids, which can impair membrane structure or protein function (Halliwell, 2006). Since hydroxyl radicals are extremely reactive, cells cannot detoxify them using enzymatic mechanisms and they must rely on mechanisms that avoid their formation such as the elimination of $O_2^{\bullet-}$ and H_2O_2 and the sequestering of divalent iron ions necessary for the Haber-Weiss reaction, using metal-binding proteins, such as ferritins or metallothioneins (Mittler et al., 2004; Gechev et al., 2006; Hintze and Theil, 2006).

III. Environmental Stresses and ROS Production

Many environmental changes such as light, temperature, drought or nutrient stresses affect the efficiency of photosynthetic electron transport and result in changes of the redox state of chloroplasts (Foyer and Noctor, 2003; Pfannschmidt, 2003). As a result of these disturbances, intracellular levels of ROS may rapidly rise (Fig. 15.2). For instance, in the chloroplasts, singlet oxygen is produced by Photosystem II (PS II) at high light intensities, when the absorption of light energy exceeds the capacity for CO_2 assimilation via the Calvin-Benson cycle (Fig. 15.2). Excited triplet chlorophyll molecules in PS II interact with O_2 to generate $^1O_2$ (Fryer et al., 2002; Hideg et al., 2002) (Fig. 15.2). At the same time, hyper-reduction of the photosynthetic electron transfer chain favors the direct reduction of O_2 by Photosystem I and the subsequent production of superoxide and H_2O_2 (Foyer and Noctor, 2003; Pfannschmidt, 2003) (Fig. 15.2). Since under natural stress conditions different ROS may be generated simultaneously in chloroplasts, it is difficult to test the possible biological activity of each ROS separately. For instance, singlet oxygen, superoxide anion and hydrogen peroxide are released simultaneously in plants exposed to excess excitation energy (EEE)

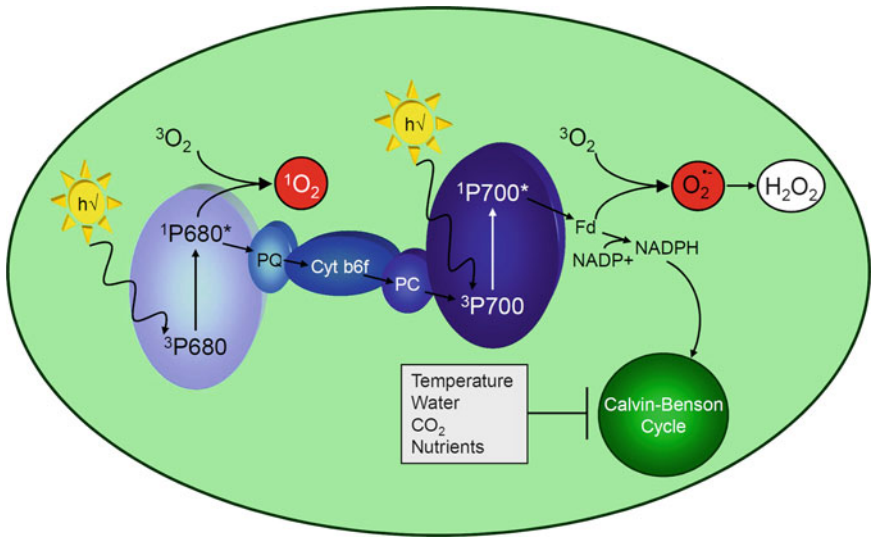

Fig. 15.2. Factors in photosynthetic redox chemistry that influence ROS production. The electron transfer chain of a chloroplast is drawn schematically according to the current Z scheme and the electron flow is represented by *black arrows*. During photosynthesis, water is split into protons, electrons and oxygen by a light-induced charge separation in the reaction center (*P680/P680**) of Photosystem II (PS II). Excess excited triplet chlorophyll molecules in PS II can interact with molecular oxygen (O_2) to generate singlet oxygen ($^1O_2$). Electrons are then transferred to the cytochrome b_6f (*Cyt* b_6f) complex by the mobile electron carrier plastoquinone (*PQ*). Electrons from the cytochrome b_6f complex are transferred to Photosystem I (PS I) by plastocyanin (*PC*). After activation through a second light reaction (*P700/P700**), they are accepted by the electron terminal acceptor *NADPH*. Under extreme conditions (e.g., high light intensity or low temperatures), electrons of PS I can also be transferred to oxygen, which results in the generation of superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) species. When various environmental influences inhibit (—) the capacity for CO_2 assimilation by the Calvin-Benson cycle, the different ROS described above might be produced.

(Fryer et al., 2002), whereas a stronger EEE leads to excited triplet chlorophyll molecules in PS II that mainly lead to singlet oxygen production (Hideg et al., 1998; Laloi et al., 2007). In spinach leaves under very strong EEE, singlet oxygen is generated while hardly any superoxide anions are detected (Hideg et al., 2002). On the other hand, treatments such as chilling stress and drought, that both restrict CO_2 assimilation by the Calvin–Benson cycle (Fig. 15.2), induce either singlet oxygen (Torres et al., 2002), or superoxide anions and hydroperoxide (Laloi et al., 2004; Laloi et al., 2007). Thus, the relative contribution of each ROS generated in plastids depends on the environmental stress to which the plants are exposed, thereby making it difficult to study the impact of a specific ROS on plant signaling. The selective production of chemically distinct ROS under specific stress conditions might enable plants to adjust their responses to the needs imposed by different environmental stresses (Apel and Hirt, 2004; Laloi et al., 2004).

IV. The Chemical Identity of a Given ROS Affects Its Signaling Function

The recent development of experimental strategies that allow the biological activity of only one ROS to be studied at a given time has opened the door to investigate specific signaling pathways that might be induced by singlet oxygen and hydroperoxide and/or the superoxide anion (op den Camp et al., 2003; Danon et al., 2005; Gadjev et al., 2006; Gechev et al., 2006; Laloi et al., 2007).

A. Gene Expression and ROS Specificity

Depending on the quality of the stimulus, plants differentially enhance the release of chemically distinct ROS. For instance, during an incompatible plant-pathogen interaction superoxide and hydrogen peroxide are produced enzymatically (Overmyer et al., 2003; Laloi et al., 2004), whereas plants under an abiotic stress such as high light,

drought and high or low temperatures not only generate these two ROS but singlet oxygen is also generated in chloroplasts (Hideg et al., 1998; Fryer et al., 2002; Hideg et al., 2002). Stress reactions induced by pathogens differ from those induced by these abiotic stress conditions. If ROS act as signals that evoke these different stress responses, their biological activities should exhibit a high degree of specificity that could also be derived from their chemical identities. In animals, $^1O_2$ production has been shown to lead to the activation of distinct signaling pathways (Klotz et al., 2003), some of which are selectively activated by $^1O_2$ and not by superoxide (Godar, 1999) or hydrogen peroxide (Zhang and Klessig, 2000).

In plants, experimental strategies have been described that allow the analysis of the biological activity of a specific ROS at a given time. For instance, paraquat is a herbicide that specifically generates superoxide and H_2O_2 within chloroplasts in the light (Mehler, 1951). In *Arabidopsis thaliana*, the fluorescent (*flu*) mutant has been used to study the specific impact of singlet oxygen production in chloroplasts. In angiosperms kept in the dark, the chlorophyll biosynthesis pathway is blocked after the formation of protochlorophyllide, the immediate precursor of chlorophyllide, because the reduction of protochlorophyllide to chlorophyllide requires light (Reinbothe et al., 1996; Cornah et al., 2003). The *flu* mutant of *A. thaliana* is defective in this metabolic feedback control. As a result of the mutation, the *flu* mutant accumulates protochlorophyllide in the dark (Meskauskiene et al., 2001). Upon irradiation, photosensitizing molecules such as protochlorophyllide are capable of generating singlet oxygen by transferring light energy to ground-state molecular oxygen, thereby elevating it to the excited singlet state (Apel and Hirt, 2004; Krieger-Liszkay, 2005). Thus, after a dark-to-light shift, singlet oxygen is generated in the *flu* mutant, within the first minute of illumination and this is confined to the plastids (op den Camp et al., 2003) .

The ability of the *flu* mutant to generate singlet oxygen upon a dark-to-light shift has been exploited to identify and analyze singlet oxygen-controlled gene expression (op den Camp et al., 2003; Laloi et al., 2007). The total number of genes activated differentially after the release of singlet oxygen or superoxide/hydrogen peroxide was assessed by comparing global changes in the gene expression of *flu* plants subjected to a dark/light shift with those of paraquat-treated *flu* plants (op den Camp et al., 2003). In this study, 70 genes were identified as being up-regulated by singlet oxygen in *flu* plants but not by superoxide/hydrogen peroxide during paraquat treatment. A group of 9 genes were found to be up-regulated in paraquat-treated plants but not in the *flu* mutant, whereas a last group consisted of 31 genes up-regulated under both experimental conditions (op den Camp et al., 2003). These results demonstrate that chemical differences between the two ROS contribute to the selectivity of the induced stress responses and that genes induced by superoxide/hydrogen peroxide are often different from those activated by $^1O_2$ in the *flu* mutant. Selective gene induction was confirmed in another study in which specific transcriptomic signatures triggered by different ROS were identified (Gadjev et al., 2006).

B. Lipid Peroxidation

The chloroplast membranes are particularly susceptible to ROS-induced lipid peroxidation (Rusterucci et al., 1999; op den Camp et al., 2003), since approximately 90% of the fatty acids of the thylakoid glycolipids, phospholipids and sulpholipids consist of the unsaturated fatty acid α-linolenic acid (Knox and Dodge, 1985). The enzymatic peroxidation of linolenic acid is catalyzed mainly by lipoxygenases (LOX), leading to different lipid hydroperoxide (LH; Fig. 15.3) derivatives resulting from regio-specific and stereo-specific oxygenations (Berger et al., 2001; Feussner and Wasternack, 2002).

The singlet-oxygen-mediated peroxidation of lipids was monitored in *flu* plants by measuring changes in the amount of the oxygenation products of linolenic acid, and hydroxy octadecatrieonic acid (HOTE). The free regio-specific isomer 13-HOTE accumulated rapidly in *flu* plants after singlet oxygen production (Fig. 15.3), exceeding that of the wild-type control by 10- to 20-fold (op den Camp et al., 2003). The concentration of the free form of other regio-isomers, 9-HOTE and 12-HOTE, remained at similar low levels throughout the experiment in both wild-type and *flu* plants. Therefore in the *flu* mutant, only the 13LOX but not the 9LOX pathway was activated rapidly after singlet oxygen production.

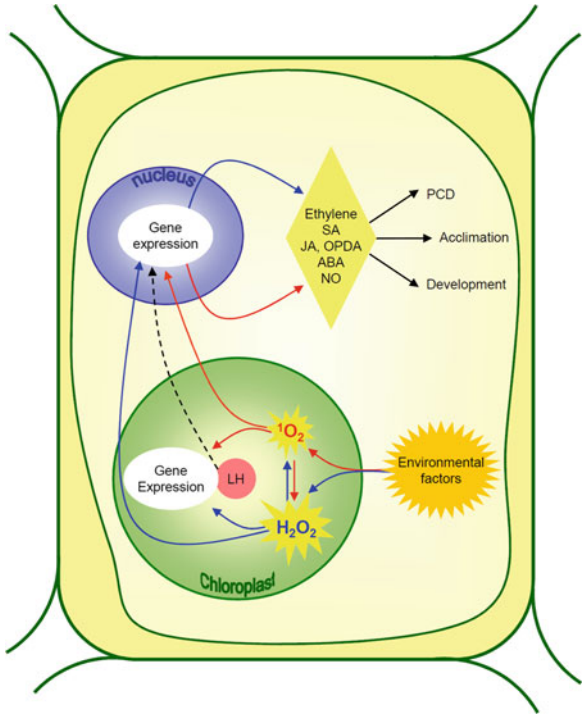

Fig. 15.3. Schematic representation of the regulation of ROS-induced responses. Many environmental changes such as light, temperature, drought or nutrient stresses affect the efficiency of photosynthetic electron transfer and result in the production of singlet oxygen ($^1O_2$, symbolized using *black arrows*) and/or superoxide anions ($O_2^{\bullet -}$)/hydrogen peroxide (H_2O_2, symbolized using *gray arrows*). The generation of these particular ROS influences the expression of distinct sets of plastidic and nuclear genes (symbolized with *gray arrows* for H_2O_2, and *black arrows* for $^1O_2$) and leads to the production of specific lipid hydroperoxides (*LH*). *Dotted lines* indicate the putative relationship that might exist between LH production, plastidic gene expression and nuclear gene expression. As a result, key signaling molecules such as abscisic acid (*ABA*), ethylene, jasmonic acid (*JA*), 12-oxo-phytodienoic acid (*OPDA*) and nitric oxide (*NO*) might be differentially produced and have an influence on a range of phenomena such as programmed cell death (*PCD*), acclimation and/or plant development.

Programmed cell death (PCD) during incom patible plant–pathogen interactions is a response attributed to the production of superoxide radical/hydrogen peroxide (Beers and McDowell, 2001; Hoeberichts and Woltering, 2003; Overmyer et al., 2003). Lipoxygenases also play an important role in the response of plants to wounding and pathogen attack (Feussner and Wasternack, 2002), that results in the production of specific lipid hydroperoxides (Fig. 15.3). The 9LOX pathway had been reported to be specifically up-regulated during the hypersensitive response (Rusterucci et al., 1999; Stumpe et al., 2001), a plant defense reaction triggered by hydrogen peroxide (Alvarez et al., 1998) that does not affect the 13LOX pathway.

The above results indicate that chemical differences between superoxide/hydrogen peroxide and singlet oxygen might induce specific lipoxygenase pathways that lead to the production of the corresponding lipid hydroperoxides (Fig. 15.3). Whether these different lipoxygenase pathways induce specific lipid-derived second messengers remains to be clearly established (Fig. 15.3).

C. Mitogen-Activated Protein (MAP) Kinases/Ca^{2+} and Transcription Factors

ROS produced in different subcellular compartments have been shown to influence the expression of a large number of genes (Neill et al., 2002; Gechev et al., 2006). This observation suggests that cells have evolved strategies to utilize ROS as biological signals that control various genetic stress programs. As discussed above, this interpretation is based on the assumption that a given ROS may selectively modify gene expression. Such a change in transcriptional activity may be achieved by oxidation of components of signaling pathways that subsequently activate transcription factors (TFs) or by modifying directly a redox-sensitive TF.

An indirect TF activation is illustrated by the effects of ROS on components of the Mitogen-Activated Protein kinase (MAPK) cascade. In *A. thaliana (At)*, H_2O_2 activates the MAPKs: AtMPK3 and AtMPK6 via the Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK) Arabidopsis NPK1-related Protein Kinase 1 (ANP1) (Kovtun et al., 2000) and strongly induces Nucleotide Diphosphate 2 Kinase (NDPK2) (Moon et al., 2003). Plants overexpressing *AtNDPK2* have reduced levels of ROS and an enhanced tolerance to cold, salt and ROS stress, whereas the *atndpk2* knock-out mutant is more sensitive to oxidative stress. *AtNDPK2* over-expression also leads to an increased expression of antioxidant genes. Since AtNDPK2 specifically interacts with AtMPK3 and AtMPK6 and enhances the activity of AtMPK3 in vitro, a model has been proposed in which oxidative stress conditions activate AtNDPK2 that regulates the cellular redox-state by signaling the

transient expression of antioxidant genes, possibly through activation of the MAPK cascade. The activation of the MAPK cascade may be initiated by redox-controlled protein tyrosine phosphatases (PTPs). Indeed, AtPTP1 is reversibly inactivated by H_2O_2 and can inactivate the *A, thaliana* AtMPK6 (Fig. 15.2) (Gupta and Luan, 2003). Whether or not the inactivation of AtMPK6 is direct or mediated by AtNDPK2 is not known yet. The activation of AtPTP1 by H_2O_2 may be due to the oxidation state of the active-site cysteine, as described for the human PTP1B (Van Montfort et al., 2003).

V. Consequences of ROS Production

A. Programmed Cell Death/Hypersensitive Reaction (HR) and Hormonal Regulation

Generation of hydrogen peroxide and superoxide during photosynthesis has been implicated in the induction of the HR (Willekens et al., 1997; Mateo et al., 2004). An early HR event is the generation of superoxide anions and the accumulation of hydrogen peroxide, which have been proposed to be part of the mechanism that controls plant defenses during an incompatible plant pathogen interaction (Beers and McDowell, 2001; Hoeberichts and Woltering, 2003; Overmyer et al., 2003). In addition, the initiation, spreading and containment of HR cell death reactions is controlled by a complex network of signaling pathways, some of which are activated by the phytohormones; ethylene, salicylic acid (SA) and jasmonic acid (JA). Ethylene and SA have been associated with the initiation of PCD, whereas JA has been implicated in the suppression and containment of PCD (Overmyer et al., 2000; Devadas et al., 2002; Rao et al., 2002; Overmyer et al., 2003).

Only a very few case studies have been performed that suggest a selective signaling effect of a given ROS in PCD induction in plants. This may in part be due to the fact that most of these studies have focused on analyzing the signaling role of H_2O_2 and/or superoxide radicals, whereas other ROS such as hydroxyl radicals and singlet oxygen have been largely ignored (Apel and Hirt, 2004). The *flu* mutant of *A. thaliana* has been used to analyze the impact of singlet oxygen, generated within the plastid, on cell death (op den Camp et al., 2003). Singlet oxygen production in the *flu* mutant induces a cell death that exhibits the hallmarks of PCD at the cellular level (Danon et al., 2005) and which is genetically controlled (Wagner et al., 2004). The role of the phytohormones involved in the control of HR-induced PCD has been analyzed during singlet-oxygen-mediated cell death reactions of the *flu* mutant, in order to assess the extent of commonalities and differences between cell death reactions triggered by singlet oxygen or superoxide radical/hydrogen peroxide molecules (Danon et al., 2005). Whereas the effects of SA and ethylene were very similar, the influence of JA and 12-oxo-phytodienoic acid (OPDA) and dinor OPDA, intermediates of its biosynthetic pathway, on singlet-oxygen-mediated cell death in *flu* was distinct from that reported earlier for PCD triggered by hydrogen peroxide/superoxide in the wild type (Danon et al., 2005). Thus, in contrast to earlier studies that have identified JA as a suppressor of PCD induced by H_2O_2 and/or superoxide (Overmyer et al., 2000; Devadas et al., 2002; Rao et al., 2002), in *flu* it promotes the singlet-oxygen-mediated cell death reaction (Danon et al., 2005).

Another feature of ROS signaling during incompatible plant pathogen interactions had been the identification of the role that peroxynitrite (ONOO-) might play in this phenomenon (Delledonne et al., 2001). Peroxynitrite is a long lived and highly reactive oxidant species that freely crosses membranes; it is generated by the reaction between superoxide anions and nitric oxide radicals (NO) (Marla et al., 1997). It appears that the generation of superoxide anions and the accumulation of hydrogen peroxide during a HR is necessary but not sufficient to trigger host cell death, and that peroxynitrite cooperates with ROS in the activation of hypersensitive cell death (Delledonne et al., 1998; Delledonne et al., 2003).

B. Acclimation

Acclimation is regarded as an environmentally induced short-term response leading to an improved tolerance of plants to subsequent stresses. A regulatory role for ROS as signaling molecules involved in plants acclimation has emerged from several reports (Prasad et al., 1994; Dat et al., 1998; Lopez-Delgado et al., 1998; Karpinski et al., 1999). Changes in the redox state of chloroplastic

components regulate the expression of both plastome- and nuclear-encoded chloroplast proteins (Pfannschmidt, 2003; Laloi et al., 2004), (Fig. 15.3). Thus, the chloroplast not only provides energy but also represents a sensor of environmental information, and chloroplast redox signals help acclimate the plant to environmental stresses. Redox changes in chloroplasts, hydrogen peroxide, salicylic acid and the induction of antioxidant defenses are key determinants of this mechanism of systemic acquired acclimation. In particular it has been shown that modulating hydrogen peroxide levels in plants has an impact on their viability and on the activation of nuclear genes, after a subsequent treatment leading to ROS production (Lopez-Delgado et al., 1998; Karpinski et al., 1999; Laloi et al., 2007). A cross-talk between hydrogen peroxide- and singlet oxygen-dependent signaling pathways (Fig. 15.3) might contribute to the overall stability and robustness of plants exposed to adverse environmental stress conditions (Laloi et al., 2007). Nevertheless, even if some genes have been found to be induced (Vranova et al., 2002; Oono et al., 2006) or involved (Moon et al., 2003; Rizhsky et al., 2004; Davletova et al., 2005) in acclimation, the mechanism leading to the establishment of this physiological response in plants remains to be established in more detail.

C. Systemic Acquired Resistance

Concomitant with the appearance of a HR, a secondary resistance response known as systemic acquired resistance (SAR) is induced in the uninfected tissues (Sticher et al., 1997). SAR provides long-lasting resistance throughout the plant to subsequent infections by a broad range of pathogens and can therefore also be considered as a form of acclimation to pathogens.

A direct activation of signal transduction pathways by redox-sensitive TFs like OxyR in *Escherichia coli* or Yap1 in yeast has been well established in prokaryotes and animals. In plants, the redox regulation of NPR1 (Nonexpressor of Pathogenesis Related genes), an essential regulator of plant SAR has been reported (Mou et al., 2003). In an uninduced state, NPR1 is present in the cytosol as an oligomer formed through intermolecular disulfide bonds (Fig. 15.2). During SAR, a biphasic change in cellular reduction potential occurs and results in the reduction of NPR1 to a monomeric form that accumulates in the nucleus and activates gene expression. At the same time, prior to the induction of SAR and in the absence of salicylic acid (SA), the TF TGA1 (so called because their associated DNA binding elements contain the core sequence TGACG), that is responsible for the transcriptional control of genes involved in mediating SAR is present in an oxidized state that is manifested by the formation of a disulfide bridge between two conserved Cys residues (Despres et al., 2003). During SAR, SA accumulation causes the reduction of these Cys residues, and the reduced form of TGA1 is capable of interacting with the monomeric form of NPR1 that is localized in the nucleus. The interaction with NPR1 enhances the DNA binding capacity of the reduced TGA1 which binds specifically to SA-regulatory sequences found in the promoters of numerous PR genes in an NPR1-dependent manner. Perception of ROS in prokaryotes and fungi relies in part on two-component histidine kinase and thiol peroxidase systems (Delaunay et al., 2002). Plants contain a large range of two component histidine kinases as well as thiol peroxidases, whether some of these proteins can also function as ROS sensors is not yet known.

D. Development

ROS production by plasma membrane NADPH oxidases, encoded by *RBOH* (respiratory burst oxidase homologue) genes, seems not to be limited to the control of stomatal opening and the generation of the oxidative burst but may be of a more general importance during plant signaling and development (Torres and Dangl, 2005). For instance, abscisic acid (ABA) inhibition of root elongation is reduced in *atrbohD/F* and *atrbohF* mutants (Kwak et al., 2003). Furthermore, the *atrbohD/F*-double mutant also shows reduced ABA inhibition of seed germination and a reduction of ROS production induced by bacterial and fungal infection (Torres et al., 2002), which suggests pleiotropic functions of ROS produced by NADPH oxidases and a functional redundancy amongst different members of the *Atrboh* family. Finally, the *atrbohC*/*rhd2* mutant of *A. thaliana* with a defect in a third NADPH oxidase catalytic subunit contains reduced ROS levels in growing root hairs that lead to a distortion of Ca^{2+} uptake and a disruption of root cell expansion (Foreman

et al., 2003). Direct quenching of root ROS also inhibits root elongation (Demidchik et al., 2003).

Collectively, these results indicate that ROS production by catalytic subunits of several plasma membrane NADPH oxidases may control plant development by activating Ca^{2+} channels. Since *A. thaliana* possesses 10 *Atrboh* genes, which are differentially expressed (Kwak et al., 2003), it is conceivable that different *Rboh* either alone or in various combinations may contribute to the release of signals in a variety of different cell types, and in response to numerous endogenous and/or exogenous cues (Pnueli et al., 2003).

The regulation of stomatal closure involves various controls that help the plant to adapt to a variety of environmental changes (Hetherington and Woodward, 2003). Among the components of this complex regulatory network, ROS are essential signals that mediate stomatal closure induced by ABA via the activation of plasma membrane calcium-permeable channels (Pei et al., 2000; Neill et al., 2002). In *A. thaliana*, it has been shown that ROS production involves at least two different catalytic subunits of plasma-membrane-bound NADPH oxidases, AtrbohD and AtrbohF (Kwak et al., 2003). The phenotypes of the *ost1* protein kinase mutant, disrupted in ABA-induced ROS production but still capable to close stomata in response to H_2O_2, and the *abi1-1* mutant that no longer shows ABA-induced activation of the OST1 (open stomata1) kinase, suggest that protein phosphorylation by OST1 functions between ABI1 and ROS production in the ABA signaling cascade (Mustilli et al., 2002). Whether OST1 regulates ROS production directly via an NADPH oxidase remains unknown. Phosphatidylinositol 3-phosphate (PI3P), that has been implicated in regulating stomatal closure (Mustilli et al., 2002), may also act via an ABA-induced ROS generation (Park et al., 2003). In neutrophils, PI3P regulates H_2O_2 production by binding to the noncatalytic component $p40^{phox}$ of the NADPH oxidase (Ellson et al., 2001). However, so far no plant homolog of $p40^{phox}$ has been identified.

VI. Concluding Remarks

During the evolution of organisms adapted to aerobic life conditions, the biological activities associated with ROS appear to have undergone several modifications. The continuous production of ROS is an unavoidable consequence of aerobic metabolic processes such as respiration and photosynthesis that has required the evolution of ROS scavengers to minimize their cytotoxic effects within the cell. At the same time the sensing of changing ROS concentrations that result from metabolic disturbances has been used by plants to activate stress responses that help the plant to cope with environmental changes.

Several studies have now clearly demonstrated that chemical differences between ROS, such as superoxide/hydrogen peroxide and singlet oxygen, contribute to the selectivity of the induced stress responses. Details of how these different reactive oxygen species interact and how they are sensed are still scarce (Foyer and Noctor, 2009; Foyer and Shigeoka, 2011). Key issues to be addressed in the future concern the questions of how ROS are integrated in the general signaling network of a cell, how the chemical identity of a given ROS and/or the intracellular production sites affect its signaling capacity, and which factors determine the specificity of their biological activities.

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References

Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA and Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92: 773–784

Apel K and Hirt H (2004) Reactive Oxygen Species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Physiol Plant Mol Biol 55: 373–399

Beers EP and McDowell JM (2001) Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. Curr Opin Plant Biol 4: 561–567

Berger S, Weichert H, Porzel A, Wasternack C, Kuhn H and Feussner I (2001) Enzymatic and non-enzymatic lipid peroxidation in leaf development. Biochim Biophys Acta 1533: 266–276

Cadenas E (1989) Biochemistry of oxygen toxicity. Annu Rev Biochem 58: 79–110

Cornah JE, Terry MJ and Smith AG (2003) Green or red: what stops the traffic in the tetrapyrrole pathway? Trends Plant Sci 8: 224–230

Danon A, Miersch O, Felix G, Camp RG and Apel K (2005) Concurrent activation of cell death-regulating signaling pathways by singlet oxygen in *Arabidopsis thaliana*. Plant J 41: 68–80

Dat JF, Lopez-Delgado H, Foyer CH and Scott IM (1998) Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol 116: 1351–1357

Davies MJ (2004) Reactive species formed on proteins exposed to singlet oxygen. Photochem Photobiol Sci 3: 17–25

Davletova S, Schlauch K, Coutu J and Mittler R (2005) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. Plant Physiol 139: 847–856

Delaunay A, Pflieger D, Barrault MB, Vinh J and Toledano MB (2002) A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell 111: 471–481

Delledonne M, Polverari A and Murgia I (2003) The functions of nitric oxide-mediated signaling and changes in gene expression during the hypersensitive response. Antioxid Redox Signal 5: 33–41

Delledonne M, Xia Y, Dixon RA and Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394: 585–588

Delledonne M, Zeier J, Marocco A and Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci USA 98: 13454–13459

Demidchik V, Shabala SN, Coutts KB, Tester MA and Davies JM (2003) Free oxygen radicals regulate plasma membrane Ca^{2+}- and K^+-permeable channels in plant root cells. J Cell Sci 116: 81–88

Despres C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D and Fobert PR (2003) The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell 15: 2181–2191

Devadas SK, Enyedi A and Raina R (2002) The Arabidopsis hrl1 mutation reveals novel overlapping roles for salicylic acid, jasmonic acid and ethylene signalling in cell death and defence against pathogens. Plant J 30: 467–480

Ellson CD, Gobert-Gosse S, Anderson KE, Davidson K, Erdjument-Bromage H, Tempst P, Thuring JW, Cooper MA, Lim ZY, Holmes AB, Gaffney PR, Coadwell J, Chilvers ER, Hawkins PT and Stephens LR (2001) PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40(phox). Nat Cell Biol 3: 679–682

Feussner I and Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53: 275–297

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM and Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422: 442–446

Foyer CH and Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119: 355–364

Foyer CH and Noctor G (2009) Redox regulation in photosynthetic organisms: Signaling, acclimation, and practical implications. Antioxid Redox Signal 11: 861–905

Foyer CH and Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155: 93–100

Fryer MJ, Oxborough K, Mullineaux PM and Baker NR (2002) Imaging of photo-oxidative stress responses in leaves. J Exp Bot 53: 1249–1254

Gadjev I, Vanderauwera S, Gechev TS, Laloi C, Minkov IN, Shulaev V, Apel K, Inze D, Mittler R and Van Breusegem F (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiol 141: 436–445

Gechev TS and Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol 168: 17–20

Gechev TS, Van Breusegem F, Stone JM, Denev I and Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. Bioessays 28: 1091–1101

Girotti AW and Kriska T (2004) Role of lipid hydroperoxides in photo-oxidative stress signaling. Antioxid Redox Signal 6: 301–310

Godar DE (1999) Light and death: photons and apoptosis. J Investig Dermatol Symp Proc 4: 17–23

Gorman AA and Rodgers MA (1992) Current perspectives of singlet oxygen detection in biological environments. J Photochem Photobiol B 14: 159–176

Gupta R and Luan S (2003) Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants. Plant Physiol 132: 1149–1152

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141: 312–322

Hawkins CL and Davies MJ (2001) Generation and propagation of radical reactions on proteins. Biochim Biophys Acta 1504: 196–219

Henzler T and Steudle E (2000) Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. J Exp Bot 51: 2053–2066

Hetherington AM and Woodward FI (2003) The role of stomata in sensing and driving environmental change. Nature 424: 901–908

Hideg E and Vass I (1996) UV-B induced free radical production in plant leaves and isolated thylakoid membranes. Plant Science 115: 251–260

Hideg E, Kalai T, Hideg K and Vass I (1998) Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry 37: 11405–11411

Hideg E, Barta C, Kalai T, Vass I, Hideg K and Asada K (2002) Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photoinhibition or UV radiation. Plant Cell Physiol 43: 1154–1164

Hintze KJ and Theil EC (2006) Cellular regulation and molecular interactions of the ferritins. Cell Mol Life Sci 63: 591–600

Hoeberichts FA and Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays 25: 47–57

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G and Mullineaux P (1999) Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. Science 284: 654–657

Kehrer JP (2000) The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149: 43–50

Klotz LO (2002) Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen. Biol Chem 383: 443–456

Klotz LO, Kroncke KD and Sies H (2003) Singlet oxygen-induced signaling effects in mammalian cells. Photochem Photobiol Sci 2: 88–94

Knox JP and Dodge AD (1985) Singlet oxygen and plants. Phytochemistry 24: 889–896

Kovtun Y, Chiu WL, Tena G and Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA 97: 2940–2945

Krieger-Liszkay A (2005) Singlet oxygen production in photosynthesis. J Exp Bot 56: 337–346

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG and Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J 22: 2623–2633

Laloi C, Apel K and Danon A (2004) Reactive oxygen signalling: the latest news. Curr Opin Plant Biol 7: 323–328

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I and Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104: 672–677

Ledford HK and Niyogi KK (2005) Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell Environ 28: 1037–1045

Lopez-Delgado H, Dat J, Foyer C and Scott I (1998) Induction of thermotolerance in potato microplants by acetylsalicylic acid and H_2O_2. J Exp Bot 49: 713–720

Marla SS, Lee J and Groves JT (1997) Peroxynitrite rapidly permeates phospholipid membranes. Proc Natl Acad Sci USA 94: 14243–14248

Martinez GR, Loureiro AP, Marques SA, Miyamoto S, Yamaguchi LF, Onuki J, Almeida EA, Garcia CC, Barbosa LF, Medeiros MH and Di Mascio P (2003) Oxidative and alkylating damage in DNA. Mutat Res 544: 115–127

Mateo A, Muhlenbock P, Rusterucci C, Chang CC, Miszalski Z, Karpinska B, Parker JE, Mullineaux PM and Karpinski S (2004) LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiol 136: 2818–2830

Mehler AH (1951) Studies on reactions of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. Arch Biochem 34: 339–351

Meskauskiene R, Nater M, Goslings D, Kessler F, op den Camp R and Apel K (2001) FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 98: 12826–12831

Mittler R, Vanderauwera S, Gollery M and Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9: 490–498

Moon H, Lee B, Choi G, Shin D, Prasad DT, Lee O, Kwak SS, Kim DH, Nam J, Bahk J, Hong JC, Lee SY, Cho MJ, Lim CO and Yun DJ (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci USA 100: 358–363

Mou Z, Fan WH and Dong XN (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113: 935–944

Mustilli AC, Merlot S, Vavasseur A, Fenzi F and Giraudat J (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell 14: 3089–3099

Neill S, Desikan R and Hancock J (2002) Hydrogen peroxide signalling. Curr Opin Plant Biol 5: 388–395

Oono Y, Seki M, Satou M, Iida K, Akiyama K, Sakurai T, Fujita M, Yamaguchi-Shinozaki K and Shinozaki K (2006) Monitoring expression profiles of *Arabidopsis* genes during cold acclimation and deacclimation using DNA microarrays. Funct Integr Genomics 6: 212–234

op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M and Apel K (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15: 2320–2332

Overmyer K, Brosche M and Kangasjarvi J (2003) Reactive oxygen species and hormonal control of cell death. Trends Plant Sci 8: 335–342

Overmyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann H, Jr. and Kangasjarvi J (2000) Ozone-Sensitive Arabidopsis rcd1 Mutant Reveals Opposite

Roles for Ethylene and Jasmonate Signaling Pathways in Regulating Superoxide-Dependent Cell Death. Plant Cell 12: 1849–1862

Park KY, Jung JY, Park J, Hwang JU, Kim YW, Hwang I and Lee Y (2003) A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiol 132: 92–98

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E and Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406: 731–734

Pfannschmidt T (2003) Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33–41

Pnueli L, Liang H, Rozenberg M and Mittler R (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient *Arabidopsis* plants. Plant J 34: 187–203

Prasad TK, Anderson MD, Martin BA and Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6: 65–74

Rao MV, Lee HI and Davis KR (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant J 32: 447–456

Reinbothe S, Reinbothe C, Apel K and Lebedev N (1996) Evolution of chlorophyll biosynthesis—the challenge to survive photooxidation. Cell 86: 703–705

Rizhsky L, Davletova S, Liang H and Mittler R (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. J Biol Chem 279: 11736–11743

Rusterucci C, Montillet JL, Agnel JP, Battesti C, Alonso B, Knoll A, Bessoule JJ, Etienne P, Suty L, Blein JP and Triantaphylides C (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J Biol Chem 274: 36446–36455

Sies H and Menck CF (1992) Singlet oxygen induced DNA damage. Mutant Res 275: 367–375

Sticher L, Mauch-Mani B and Metraux JP (1997) Systemic acquired resistance. Annu Rev Phytopathol 35: 235–270

Stumpe M, Kandzia R, Gobel C, Rosahl S and Feussner I (2001) A pathogen-inducible divinyl ether synthase (CYP74D) from elicitor-treated potato suspension cells. FEBS Lett 507: 371–376

Torres MA and Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 8: 397–403

Torres MA, Dangl JL and Jones JD (2002) *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci USA 99: 517–522

Van Montfort RL, Congreve M, Tisi D, Carr R and Jhoti H (2003) Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature 423: 773–777

Vranova E, Atichartpongkul S, Villarroel R, Van Montagu M, Inze D and Van Camp W (2002) Comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. Proc Natl Acad Sci USA 99: 10870–10875

Wagner D, Przybyla D, Op den Camp R, Kim C, Landgraf F, Lee KP, Wursch M, Laloi C, Nater M, Hideg E and Apel K (2004) The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. Science 306: 1183–1185

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inze D and Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C_3 plants. EMBO J 16: 4806–4816

Zhang S and Klessig DF (2000) Pathogen-induced MAP kinases in tobacco. Results Probl Cell Differ 27: 65–84

Part IV

Energy Conversion

Chapter 16

The Characteristics of Specific Chlorophylls and Their Roles in Biogenesis of the Photosynthetic Apparatus

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Summary

This chapter promotes a concept that provides insight into unique biological functions of the accessory chlorophylls *b* and *c*. The basis for the concept is the nature of atoms and molecules as malleable clouds of electrons. The distribution of electron density in the chlorophyll macrocycle, which is readily probed by absorption of light energy, is affected by functional groups on the periphery of the structure. Spectroscopic and molecular orbital data support the proposal that the central Mg ion in chlorophylls *b* and *c* is less shielded by the molecular electron cloud than in chlorophylls *a* and *d*. Consequently, the extent to which the Mg ion is exposed causes it to interact differently with ligands. Chlorophylls *a* and *d* are versatile Lewis acids and form coordination bonds with a range of Lewis bases, from electron-rich neutral structures such as the imidazole group of histidine and the charge-compensated ion-pair of the carboxyl group of glutamate with the guanidinium group of arginine, to those with a strong dipole

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moment such as amide groups and water. In contrast, chlorophyll *b* (and probably chlorophyll *c*) is found only with "hard" Lewis bases that have a strongly electronegative, oxygen-containing dipole moment. For a coordination bond to form between chlorophyll *b* and a protein, the potential ligand apparently must have a sufficiently strong dipole to displace a strongly bound water molecule. Chlorophyll *b* is required for accumulation of light-harvesting complexes in chloroplasts, a role that is attributed to interaction with a backbone peptide bond carbonyl group near the N-terminus of the apoproteins. Experiments on chloroplast development with the alga *Chlamydomonas reinhardtii* show that synthesis of chlorophyll *b* in the chloroplast envelope inner membrane is required to retain the proteins in this membrane during import into the organelle.

I. Introduction

Life on the surface of the Earth is sustained by absorption of light energy from the Sun. Organic molecules that efficiently absorb light have extensively conjugated, often aromatic, structures. Although many biological compounds exist that absorb light, the chlorophylls absorb most of the energy that productively enters the biosphere. An essential feature of productive absorption of light by chlorophyll molecules is their ability to pass on the energy to other molecules. Chlorophylls bind to proteins in light-harvesting complexes and the core reaction complexes of photosynthesis, and the flow of absorbed energy through these complexes achieves synthesis of high-energy chemical products that drive anabolic processes.

II. Atoms and Molecules

The following discussion will take an intuitive approach to the properties of the chlorophylls but with reference to molecular orbital calculations that underlie an understanding of their functions. These calculations have been done for all the chlorophyll and bacteriochlorophyll molecules by Linnanto and Korppi-Tommola (2004), and thus a formal comparative analysis of the properties of the different species of the chlorophylls is possible. First, however, a less formal presentation may allow greater biological insight.

Earlier attempts to describe our nascent concepts about the purposes for which the variety of species of chlorophyll, in particular Chl *b*, are synthesized by photosynthetic organisms (Hoober and Eggink, 1999, 2001) were embryonic and in some respects incorrect. This chapter is an attempt to describe the approaches that I consider necessary to address questions such as the mechanisms involved in the assembly of chlorophyll-protein complexes and thylakoid membrane biogenesis. These approaches require knowledge of the physicochemical properties of the chlorophylls as well as the biology of the photosynthetic systems. Earlier versions of this discussion are presented in previous volumes in this series (Hoober and Arygroudi-Akoyunoglou, 2004; Hoober, 2006) and in a review (Hoober et al., 2007), which include additional aspects not covered in this chapter.

It is important to realize that atoms and molecules are clouds of electron density that surround tiny specks of matter, the nuclei. Atoms are mostly a vacuum, a space within which electrons move around the nucleus in orbitals at defined energy levels. As a simple illustration, were the nucleus of a hydrogen atom (which is the smallest and contains only a proton but is 1,830 times more massive than an electron) the size of a grain of sand, a football field would easily fit within the diameter of the shell in which the single electron occurs (Ferris, 1988). The number of electrons in an atom is determined by the number of protons, which is the key to the chemical identity of the atom. Atoms of the heavier elements have more protons, along with neutrons, in the nucleus, and the electrons occur in multiple orbitals at distances from the nucleus several-fold greater than that of the single electron in the hydrogen atom.

Abbreviations: BChl – Bacteriochlorophyll; Chl – Chlorophyll; Chlide – Chlorophyllide; D – Debye; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHCI – Light-harvesting complex associated with photosystem I; LHCII – Light-harvesting complex associated with photosystem II; LHCP – Light-harvesting complex (apo)protein; Pchlide – Protochlorophyllide *a*; PS I – Photosystem I; PS II – Photosystem II

The energy of an electron in an atom is correlated with its radial distribution from the nucleus (Cox, 2000). Only two electrons can occur in a single orbital. Bonds form when atomic nuclei share pairs of electrons and create molecules. The specific distribution of the electron cloud in larger molecules is determined by their relationship to the atomic nuclei that make up the molecule and the type of bonding between the atoms. Nevertheless, a molecule is still mostly a vacuum. The structures that we write on paper are only convenient representations that allow us to communicate chemical concepts.

III. Absorption of Light Energy

The absorption of light is a sensitive and powerful probe of the structure of a molecule and its environment. The probability that a molecule will absorb a photon is described by its absorbance spectrum, whether free or in a complex with proteins and other molecules. The efficiency of absorption of light, referred to as the extinction coefficient or absorbance coefficient (in the absence of light scattering) is a measure of the rate at which transmission of light is reduced in intensity by absorption of energy as it passes through a substance. Whether a photon that encounters an electron cloud will be absorbed depends on the energy content of the photon and its directional orientation vis-à-vis the electron cloud. The energy of a photon, E, is a function of its wavelength, according to the equation, $E = h\nu = hc/\lambda$, where h is Planck's constant (6.626×10^{-27} erg s), ν is the frequency of the absorbed radiation (s^{-1}), c is the velocity of light (2.997×10^{10} cm s^{-1}) and λ is the wavelength.

The electron orbitals within a molecule are separated by discrete energy gaps. Absorption of a photon causes an electron in an outermost orbital to achieve a higher energy level. For pigments that we perceive as colored, such as chlorophylls, the energy gaps correspond to the energy of photons with wavelengths within the visible spectrum of light (400–700 nm). When the energy content of a photon matches the energy gap between the highest occupied molecular orbital (HOMO) and the next highest but lowest unoccupied molecular orbital (LUMO), the probability that the energy will be absorbed is at a maximum (Hanson, 1991). When the electron is displaced into an orbital of higher energy, the molecule is in an excited state, designated the "singlet excited state." The magnitude of the energy gap is determined by the arrangement of conjugated double bonds within the molecule. Molecules with a more extensive series of conjugated double bonds (a longer "resonance pathway", smaller energy gap) absorb longer wavelengths (lower energy) of light. A short series of double bonds has a greater energy gap between the HOMO and LUMO orbitals and thus absorbs light with shorter (higher energy) wavelengths. (See Rabinowitch and Govindjee, 1969; Clayton, 1973; and Blankenship, 2002, for discussions on the absorption of light).

In productive photosynthesis, an excited chlorophyll molecule transfers the packet of energy (an exciton) to other chlorophyll molecules in a process called Förster resonance energy transfer, which occurs over a distance of only a few nanometers (usually less than 5) on a time scale of femto- to picoseconds (Wu and Brand, 1994; Gadella et al., 1999; Clegg, 2004). When the exciton reaches a reaction center, the singlet excited state of a chlorophyll molecule has a sufficiently long lifetime to allow the energized electron, now farther from the nuclei than in the ground state, to be lost to an initial acceptor in the electron transport chain. Whether electron exchange occurs from the excited molecule depends on the nearness of the other molecule and the reduction/oxidation potential of the acceptor, which is a measure of the "willingness" of the molecule to accept the electron. Acceptor molecules generally have a more positive (downhill) oxidation potential than the donor and have unfilled orbitals into which the electron can enter.

When exciton or electron transfer does not occur, one possible fate for an excited molecule is internal, vibrational decay to the ground state with the loss of energy as heat. The electron regains pairing with another electron in the original orbital. With some pigments, in particular the chlorophylls, decay of the excited state occurs by a quantal drop to the ground state accompanied by emission of energy as a photon from the lowest excited state. This is the process of fluorescence, a rapid process that has a lifetime of pico- to nanoseconds (Blankenship, 2002; Van Grondelle and Gobets, 2004).

Alternatively, some of the energy in the excited state can be used to reverse the direction of spin of the excited electron to achieve the "triplet excited state". In the triplet excited state the two unpaired electrons, now in separate orbitals, cannot regain pairing because they have parallel spins and thus are repelled by their parallel magnetic fields. The triplet excited state is much more stable than the singlet excited state and has a lifetime of milliseconds to minutes. A delayed emission of light, sometimes long after the excitation light has been removed, occurs, which is the process of phosphorescence (Hanson, 1991). An electron can be lost from the triplet excited state, but the electron commonly returns to its original spin orientation in the ground state by transferring its extra energy to another molecule with a lower energy level. When the acceptor molecule is oxygen, the additional energy allows one of the two unpaired, parallel-spin electrons in the ground-state, "triplet" oxygen molecule to reverse spin and pair with the other electron (see Chapter 15). The result is excited, "singlet" oxygen, which now has an empty orbital and readily oxidizes other compounds by two-electron reactions (Ledford and Niyogi, 2005). Carotenoids in thylakoid membranes, in particular zeaxanthin, efficiently absorb the energy of triplet state chlorophyll and dissipate the energy as heat. This process is a major protective mechanism provided by the carotenoids in photosynthetic systems (Cuttriss et al., 2006).

IV. Chlorophyll Structure: Effect of Modifications on the Distribution of Electrons

Figure 16.1 shows the structures of the four chlorophylls that are considered in this discussion. Each is a tetrapyrrole, with an additional ring formed by cyclization of the C13 propionate side chain present on the precursor, Mg-protoporphyrin IX methyl ester. Three of the structures, Chls *a, b* and *d*, are designated "chlorins" because the double bond between C17 and C18 in the precursor protochlorophyllide (Pchlide) is reduced to a single bond. Chl *c retains* the C17-C18 double bond and is a "porphyrin". Each of the chlorophylls has characteristic structural modifications on the periphery of the molecule, which will be the focus of the following analysis. Reduction of the C17-C18 double bond, which converts the porphyrin ring structure to the chlorin system, strongly affects absorption of light (Belanger and Rebeiz, 1984). Plants nearly always also reduce the 8-vinyl group in newly synthesized chlorophylls to the 8-ethyl group (Adra and Rebeiz, 1998; Kolossov and Rebeiz, 2001; Nagata et al., 2005). Several of the bacteriochlorophylls have in addition the double bond between C7-C8 reduced to a single bond, which dramatically affects absorption of light (Hoff and Amesz, 1991). The various species of chlorophyll and bacteriochlorophyll are distinguished by their spectral characteristics caused by modifications of the peripheral side chains. (For further details on chlorophylls and bacteriochlorophylls, see Grimm et al., 2006.)

A. Spectral Transitions: The Q_x and Q_y Transition Moments

Figure 16.2 shows absorption spectra of several chlorophylls. All chlorophylls have strong absorption bands in the blue region of the visible range, between 400 and 470 nm, and absorption bands of variable strength in the red region of the spectrum between 600 and 700 nm. The major peak in the red region for Chl *a,* at 660–665 nm (the specific wavelength depends upon the solvent) is referred to as the Q_y dipole-allowed transition moment (Sauer et al., 1966; Houssier and Sauer, 1970). The Q_y transition provides the strongest absorption band in the red region of the spectrum of Chl *a*. It defines the photons of lowest energy that are absorbed and is the transition from which energy leaves the molecule as fluorescence.

The primary geometrical coordinate of the molecular framework of Chl *a* is the X axis, which transects the molecule from the position of C17 to C7, as shown in Fig. 16.1. The Y axis transects the molecule from C2 to C12 (Fragata et al., 1988; Van Zandvoort et al., 1995). The experimentally determined electronic Q_x transition-moment direction is very near the geometrical X axis. The experimentally determined Q_y transition-moment direction is rotated only 70° from the X axis, rather than 90° along the geometrical Y axis, which defines a vector from near C1 to near C11 (Sundholm, 2003; see

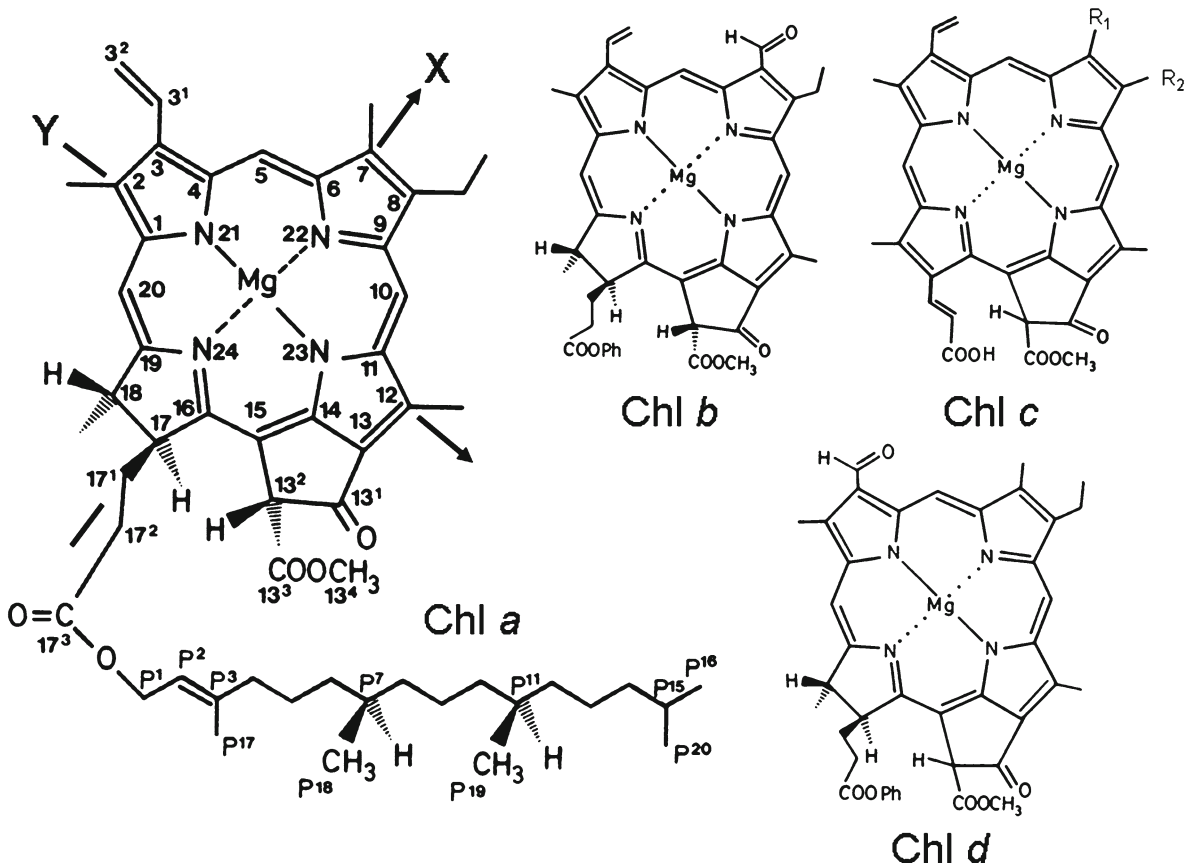

Fig. 16.1. Structures of the major chlorophylls. Except for the oxidation of the 7-methyl group in Chl *a* to the formyl group in Chl *b*, Chls *a* and *b* are identical. Chl *d* contains a formyl group at position 3. Chls *a, b* and *d* include the 20-carbon isoprene alcohol, phytol (Ph), esterified to the carboxyl group at position 17^3. This carboxyl group usually remains unesterified in Chl *c*, which also contains double bonds in the side chain between positions 17^1 and 17^2 and in the macrocycle between carbons 17 and 18. These additional double bonds extend conjugation of the macrocyclic π system to the free carboxyl group. Chl *c* species differ at positions 7 and 8; c_1: R_1 = -CH_3, R_2 = -C_2H_5; c_2: R_1 = -CH_3, R_2 = -C_2H_3; c_3: R_1 = -$COOCH_3$, R_2 = -C_2H_3.

Hoober et al., 2007, for additional discussion and references). The Q_x transition occurs at shorter wavelengths, is much weaker, and in some cases is not clearly distinguished from subvibronic transitions of the stronger Q_y vector (Umetsu et al., 1999; Knox and Spring, 2003). Umetsu et al. (1999) concluded from magnetic circular dichroism that the negative maximum at 615–619 nm in the spectrum (in diethyl ether) describes absorption along the Q_x axis of Chl *a* rather than the assignment of 582 nm, also from magnetic circular dichroism spectral studies, proposed by Frackowiak et al. (1987) and the 578 nm assignment proposed earlier by Sauer et al. (1966). The strong absorption bands in blue light, referred to as the B or Soret bands, also occur along these vectors, with the absorbance band for Chl *a* at 430–434 nm assigned as the B_x band and at 410–415 nm as the B_y band (Sauer, 1975).

B. Effect of Structural Modifications on the Q_x and Q_y Dipole Strengths

The different features in the spectra of the various chlorophyll species, particularly the wavelengths of maximal absorbance, result from structural differences around the periphery of the molecules, which allow their chemical identification. Reduction of the C17-C18 double bond at one end of the X axis of the molecule and of the C8-vinyl group on the other end generates

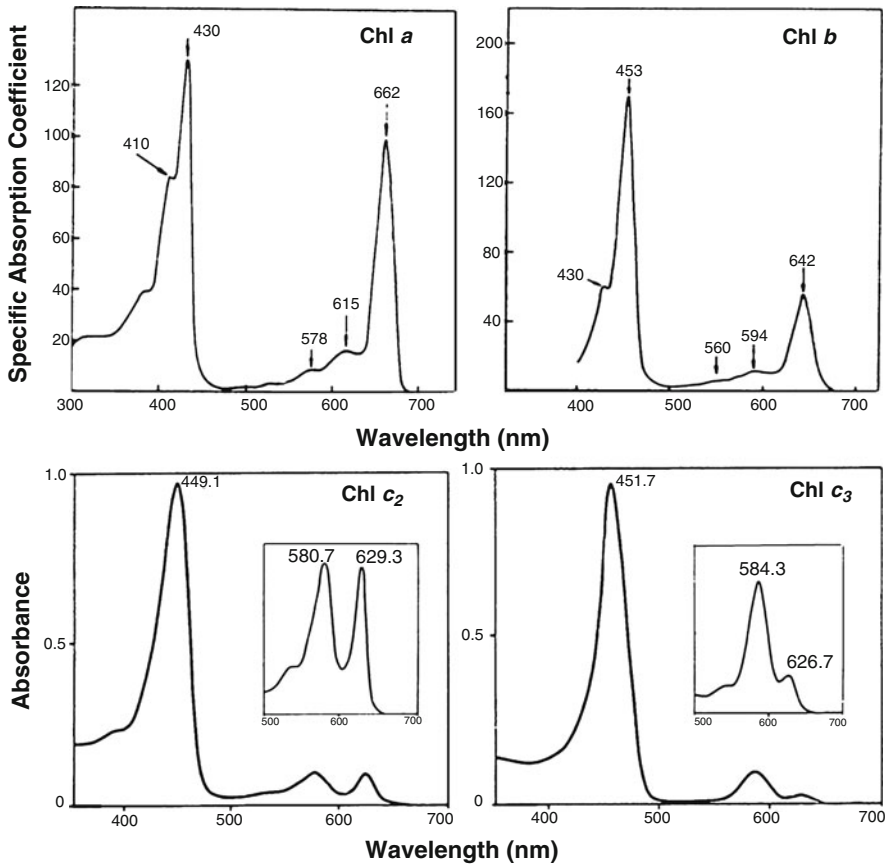

Fig. 16.2. Absorption spectra of chlorophylls. The spectra of Chl *a* and Chl *b* were obtained with the pigments dissolved in diethyl ether. The spectra for Chls c_2 and c_3 were obtained with acetone as the solvent, plotted as relative absorbance. The spectra of Chl c_2 and Chl c_3 between 500 and 700 nm were expanded to provide the insets. The wavelengths of absorbance maxima are indicated. Additional spectral information and spectra, including those of bacteriochlorophylls, are found in Jeffrey (1969), Jeffrey and Wright (1987) and Hoff and Amesz (1991), from which these spectra were adapted.

electron-donating groups on Chl *a* that shift electron density of the conjugated π system toward the center of the molecule. The result is an elliptical electron cloud, oriented along the Q_y vector, in which electron density encroaches on the Mg atom. Photons that encounter the molecule along the elongated Q_y axis are efficiently absorbed, and thus Chl *a* has a strong Q_y transition moment with a large extinction coefficient. The Q_y dipole strength is further enhanced in Chl *d* by oxidation of the 3-vinyl group to the electronegative formyl group (see Fig. 16.1). As a result, Chl *d* has an even greater extinction coefficient, at a longer (lower energy) wavelength, than Chl *a* (Miyashita et al., 1997).

Inspection of the spectra in Fig. 16.2 shows that the Q_y transition moments of Chl *b* and *c* are weaker than that of Chl *a*. Responsible for the redistribution of electrons in these chlorophylls is the introduction of the electron-withdrawing groups along the Q_x axis. For example, in Chl *b*, the 7-methyl group of Chl *a* is oxidized to the 7-formyl group, which contains the electronegative oxygen atom (Serlin et al., 1975; Eggink et al., 2004). Thus electron density is withdrawn toward the periphery of the molecule and away from the Mg ion along the X axis. This structural change strengthens the Q_x but weakens the Q_y transition moments. The Q_y transition-moment direction is displaced further from the Y axis than in Chl *a* and is at an angle of only 61° clockwise from the X axis (Simonetto et al., 1999). The extinction coefficient of the B_x transition of Chl *b* (158 l $mmol^{-1}$ cm^{-1} at 453 nm) also increases compared with that of Chl *a* (118 l $mmol^{-1}$ cm^{-1} at 430 nm) (Sauer et al., 1966).

This effect becomes particularly pronounced in the Chl *c* series. In Chl c_1, a double bond is introduced into the C17-propionic side chain to generate a *trans*-acrylate group. This oxidation is accompanied by two remarkable non-events: the C17-C18 double bond in Pchlide, which in other chlorophyll species is reduced to a single bond, is retained in Chl *c*, and the C17[3] side chain carboxyl group, which again in other chlorophyll species is esterified to an isoprenoid alcohol, usually remains free. Conversion of the propionate side chain of Pchlide to the *trans*-acrylate side chain of Chl *c* inhibits reduction of the C17-C18 double bond catalyzed by NADPH:Pchlide oxidoreductase (Helfrich et al., 2003). The consequence of these features is an extension of the conjugated π system of the macrocycle to the electronegative carboxyl group (Dougherty et al., 1970). In Chl c_2 the 8-vinyl group is not reduced to the more common 8-ethyl group and thus the more electronegative structure present in the precursor is retained (Jeffrey and Wright, 1987). Chl c_3 also retains the divinyl feature and in addition contains an unusual modification at the 7-position, in which the methyl group is oxidized to a carboxyl group that is then converted to the methyl ester (Scheer, 1991; see Porra, 1997, for biosynthetic pathways). The introduction of oxygen atoms at this position, combined with the modifications in the side chain on the opposite side of the molecule at position 17, exerts a strong electronegative pull at both ends of the X axis. The strengthening of the Q_x dipole moment is apparent in the higher extinction coefficient of the Q_x transition (580–584 nm) and weakening of the Q_y transition (627–629 nm) (Fig. 16.2).

Linnanto and Korppi-Tommola (2001, 2004) performed molecular orbital calculations to determine oscillator strengths of the Q_x and Q_y transitions for all chlorophylls and bacteriochlorophylls, which allowed a formal comparison of the various species. Oscillator strength, a measure of the integrated intensity of electronic transitions, is a useful means for comparing transition strengths between different types of quantum mechanical systems (IUPAC Compendium of Chemical Terminology, 1997; Thiel, 1999). The calculations of Linnanto and Korppi-Tommola (2001, 2004) led to assignments that were consistent with the earlier conclusions (Houssier and Sauer, 1970; Hoff and Amesz, 1991) for the Q_x transition of Chl *a* and Chl *b* being 578 and 549 nm, respectively. However, from the usual correspondence of a stronger transition moment with a longer wavelength absorption maximum, the strong negative transition signal at 625 nm observed in the magnetic circular dichroism spectrum of Chl *b* suggests that this wavelength describes the Q_x transition moment (Frackowiak et al., 1987). In Fig. 16.3a, a simple expression of the Q_x/Q_y ratio of oscillator strengths is plotted vs. the oscillator strength (*f*) of the long-wavelength Q_y transition, which is a function of the strength of the dipole moment (Knox and Spring, 2003). The decrease in strength of the Q_y transition and increase in the Q_x/Q_y ratio are obvious in this analysis. For this graph, the *ab initio* calculations for the molecules in a vacuum were used to exclude perturbations that may occur by interaction with solvent. However, similar plots were also obtained with values from calculations for chlorophylls in a 1:1

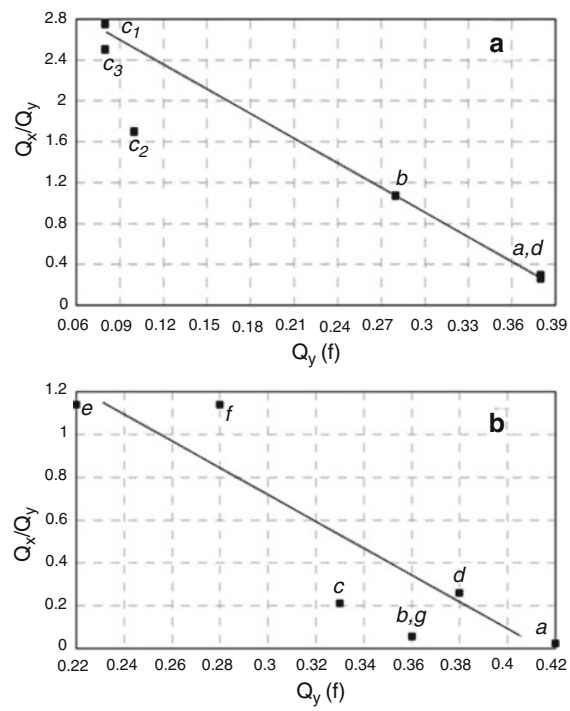

Fig. 16.3. Plot of the ratios of the Q_x and Q_y oscillator strengths vs. the Q_y oscillator strengths (*f*) for (**a**) chlorophylls and (**b**) bacteriochlorophylls. The data were obtained from Linnanto and Korppi-Tommola (2004). The letters are placed next to the data point for each type of chlorophyll. The line was inserted to show relationships between the different species of (bacterio)chlorophylls, but no other significance is intended.

complex with acetone (a solvent). The Q_x/Q_y ratio for Chl *b* is near unity, which suggests that the electron distribution is nearly circular.

The calculations made by Linnanto and Korppi-Tommola (2001, 2004) for bacteriochlorophylls are also of interest in this context. Figure 16.3b presents an analysis of oscillator strengths of bacteriochlorophylls that can be compared with those of the chlorophylls. The highest Q_x/Q_y ratios for this series, expressed by BChl *e* and BChl *f*, reach values of approximately 1.1, very similar to that of Chl *b*. These species contain a 7-formyl group and a double bond between C7 and C8, the same as in Chl *b*. BChl *e* contains an 8-vinyl group whereas BChl *f* has an 8-ethyl group. BChls *c* and *d* also contain a C7-C8 double bond but have no strong electronegative group on these carbons and thus are similar to Chls *a* and *d*. BChls *a, b, g* and *h* have a C7-C8 single bond and electronegative groups at C3 and thus exhibit strong Q_y transition moments (Linnanto and Korppi-Tommola, 2001).

The above analysis shows a consistent relationship among the chlorophylls and bacteriochlorophylls for the structural modifications at the periphery of the molecules and the consequent electronic density distributions, which determine specific spectral characteristics. In the discussion that follows, I propose that the differences in electronic distribution also affect the interactions between these molecules and the ligands with which they coordinate.

V. Influence of Structure on Binding of Chlorophylls to Ligands

A question of importance is whether the structural modifications and resulting electronic distortions have biological significance. In other words, is there a biological reason for the existence of the different chlorophylls? A traditional explanation for the additional, secondary or accessory chlorophylls has been the broadening of the overall absorption spectrum of an organism and thus an increased efficiency of light harvesting. This explanation may pertain to the various bacteriochlorophylls, which have Q_y absorption maxima with high extinction coefficients that span a range of over 100 nm (Hoff and Amesz, 1991). In contrast, the extinction coefficients in the red region of the spectrum of the 'secondary' chlorophylls are considerably less than that of Chl *a* (Fig. 16.3a), and the absorption maxima are within a narrow wavelength range (Hoff and Amesz, 1991; Porra, 1991). Moreover, the spectral features of Chl *a* itself can be broadened by binding to proteins (Nishigaki et al., 2001). Thus, a spectral advantage provided by the accessory chlorophylls in the red region of the spectrum seems trivial, although a significant enhancement of light absorption occurs in the blue region of the spectrum (Fig. 16.2). An alternate explanation is based on the effect that electronic configurations have on the properties of the central Mg ion and the resulting interactions of the chlorophylls with other molecules.

An essential interaction of chlorophylls with proteins is via coordination bonds between the Mg ion and ligands provided by proteins (Noy et al, 2000; Balaban et al., 2002; Balaban, 2005). The Mg in chlorophyll is pentacoordinate, with four bonds provided by the four pyrrole nitrogens of the tetrapyrrole macrocycle (Chow et al., 1975; Serlin et al., 1975; Smith, 1975). The fifth, axial bond is formed with an amino acid side chain in the protein or with the solvent (e.g., water or acetone). Coordination bonds are formed by the sharing of a pair of electrons between a Lewis base (the ligand) that has an unshared pair of electrons and a Lewis acid (Mg) with an unoccupied orbital. This bond may have significant orbital overlap typical of a covalent bond. Alternatively, because the Mg in chlorophyll has a positive atomic charge of about 1 (+0.7 to +1.3) (Linnanto and Korppi-Tommola, 2004), interactions with oxygen-containing polar ligands have a distinctly electrostatic character.

Mg is normally considered a "hard" Lewis acid because it prefers "hard" Lewis bases such as oxygen atoms. In Chl *a*, the electronic cloud, distributed mainly along the Q_y axis, partially shields the metal. The Mg consequently interacts preferably with ligands that are electron-rich but neutral structures, i.e., not repelled by the electron density of the more centralized π system. The most common ligand in Chl *a*-protein complexes is a nitrogen atom in the imidazole group of histidine (Nishigaki et al., 2001; Balaban et al., 2002; Oba and Tamiaki, 2002). In an environment of low dielectric constant, such as in a protein or membrane, the imidazole

group displaces a loosely-bound, polar water ligand, whose oxygen atom is probably somewhat repelled by the electron cloud surrounding the Mg. Less frequent as a ligand, but not uncommon, is the charge-compensated ion-pair between a negatively charged side chain carboxyl group of glutamate and the positively charged guanidinium group of arginine. The versatile Chl *a* also forms coordination bonds with the amide group of asparagine or glutamine and water (Kühlbrandt et al., 1994; Balaban et al., 2002; Liu et al., 2004), although with less frequency.

The introduction of electronegative groups to produce Chl *b* and *c* causes a redistribution of electrons towards the periphery of the molecule along the Q_x axis. Consequently, as electrons are pulled away from the core of the molecule, the pK values of the central pyrrole nitrogens are reduced by about two pH units, an indication of less electron density on these atoms (Phillips, 1963; Smith, 1975). As suggested earlier from the data in Fig. 16.3a, the π electron system in Chl *b* is more circular than in Chl *a.* The Mg ion would then be less shielded, more strongly express its positive point charge, and react as a harder Lewis acid. As a result, the Mg interacts preferentially with ligands with a negative character, either the negative end of a dipole or even a ligand with a negative charge. Tamiaki et al. (1998) demonstrated experimentally that introduction of an oxygen atom on the periphery of a Zn-tetrapyrrole macrocycle, a reaction analogous to the conversion of Chl *a* to Chl *b*, increased the Lewis acid strength of the metal and consequently the equilibrium constant for a coordination complex with pyridine in benzene. Of particular importance is the stronger coordination bond of Chl *b* with water (Ballschmitter et al., 1969), which is probably the ligand present during the biosynthetic steps from Mg-protoporphyrin IX to Chl *a*. Water has a dipole moment of 2.70 Debye (D) in solution and 1.86 D in the gas phase (Gregory et al., 1997). The dipole moment of water within the nonpolar environment of a membrane is likely near the latter value. The imidazole group of histidine has a dipole moment of 3.66 D in the gas phase (Spackman, 1992) and is sufficiently strong to displace a water ligand from Chl *a.* During formation of the coordination bond, the electron-rich imidazole transfers a partial charge to the metal (Noy et al., 2000). The resulting positive charge on the ligand is apparently accommodated by the enclosing electron cloud.

The lack of imidazole as a ligand for the Mg in Chl *b* may result from repulsion of the induced positive charge on the imidazole by the more exposed positive charge on the metal in Chl *b*. The dipole strength of a charge-compensated carboxyl group or an uncharged carboxyl group (1.52 D) is insufficient to displace water from Chl *b* but provides an electron rich group for coordination with Chl *a* (Hoober et al., 2007). The backbone peptide bond in a protein has a dipole moment (4.2 D) (Gunner et al., 2000) that is sufficiently strong to displace a water ligand from Chl *b.* The electronegative oxygen of the peptide bond carbonyl retains its negative character, which allows strong electrostatic interaction. However, for a peptide bond carbonyl group to be available, the possibility for H-bonding to produce the alpha-helical protein structure must be eliminated by the occurrence of a proline residue one turn along the helix. As will be discussed in more detail later, the N-terminal region of the light-harvesting complex apoproteins (LHCPs), which bind Chl *b*, is unusually rich in proline (Jansson, 1999). Proline does not contribute to helical structures and the N-terminal region remains relatively unstructured (Liu et al., 2004). Displacement of the water ligand seems also to require an environment of low dielectric constant, which in a protein was estimated to be as low as 4 (Gunner et al., 2000). (For a further discussion of the potential role of dipole moments of ligands in coordination bonds with chlorophylls, see Hoober et al., 2007.)

VI. Experimental Demonstration of Ligand Preference

Differences in chemical activity of the chlorophylls were demonstrated by a simple experiment in which binding of the chlorophylls to a small peptide was studied (Eggink and Hoober, 2000). Detergent micelles substituted for a hydrophobic membrane environment, although the system was still largely aqueous. As shown in Fig. 16.4, Chls *a* and *d,* both of which exhibit a strong Q_y transition moment, bind to the peptide, which contains the imidazole group of histidine and a

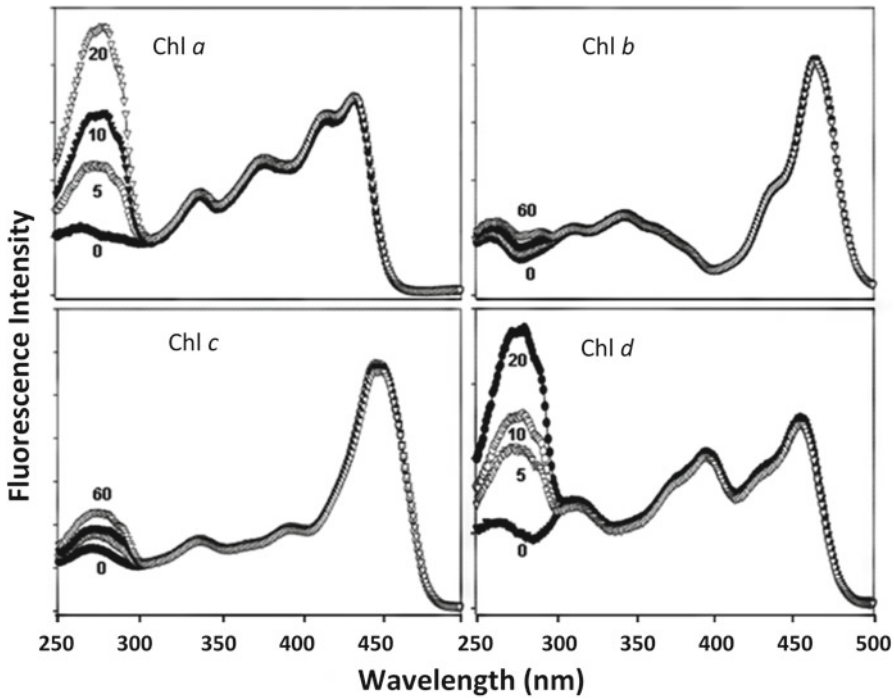

Fig. 16.4. Excitation spectra of the chlorophylls with various concentrations of a synthetic peptide containing the ligands in the chlorophyll-binding motif in membrane-spanning helix-1 of LHCP, as described in Eggink and Hoober (2000). Binding of chlorophyll to the peptide was detected by development of an excitation peak at 280 nm, the absorption maximum of the tryptophan residue next to the motif in the peptide. When bound to the peptide, chlorophyll was sufficiently close to the tryptophan residue for Förster resonance energy transfer to occur. Emission of fluorescence was measured at the long wavelength maximum for each type of chlorophyll. The *numbers* under the excitation peak at 280 nm indicate the concentration (micromolar) of peptide (Adapted from Chen et al., 2005).

glutamate-arginine ion-pair as ligands. In contrast, binding of Chls *b* and *c* was at least an order of magnitude lower (Chen et al., 2005). These results suggest that the dipolar water molecule is bound to Chl *b* and Chl *c* too strongly to be displaced by the amino acid side chain ligands in the peptide. Although non-hydrogen-bonded peptide bond carbonyl groups may have been present in the peptide, the high concentration of water in the environment (dielectric constant of solvent water = 80) apparently effectively competed with peptide ligands by mass action. Nevertheless, the absorbance characteristics of Chls *b* and *c* indicated that they indeed resided within the nonpolar environment of the micelles.

The above discussion is consistent with the ligands bound to chlorophylls in the major light-harvesting complex (Liu et al., 2004). Of the 8 Chl *a* molecules, 2 have imidazole groups (the LHCP has only 3 histidine residues), 2 have charge-compensated carboxyl groups of glutamate, 2 have amide groups of asparagine and glutamine, 1 is coordinated to a phosphodiester group of phosphatidyl glycerol, and 1 retains a water ligand. In contrast, 3 of the 6 Chl *b* molecules retain a water ligand, 2 are coordinated to backbone carbonyl groups and 1 to a carboxyl group of glutamate. Thus, all the Chl *b* molecules are coordinated to oxygen-containing ligands. Interestingly, one of the Chl *b* molecules binds to the backbone carbonyl of valine-119 but not to the adjacent imidazole group on histidine-120 (Liu et al., 2004).

Chl *a* shows a consistent pattern of donor-acceptor complexes, with a gradient of decreasing frequency of ligands from imidazole to side chain amide groups to water. A similar pattern is expected for Chl *b,* but instead, complexes formed in vivo are clustered at the extreme of this pattern, with only the peptide bond amide group, with the largest dipole moment, and water as ligands. This outcome is puzzling because the light-harvesting complex associated with PS II (LHCII) can be reconstituted in vitro with only

Chl *b* (Kleima et al., 1999). Although the total number of chlorophyll molecules per complex in this case is slightly less than those with normal Chl *a/b* ratios, the stability of complexes containing only Chl *b* is greater than those rich in Chl *a* (Reinsberg et al., 2001). Even more striking is the ability to reconstitute with Chl *b* the peridinin-Chl *a* protein from a dinoflagellate (Miller et al., 2005), and a light-harvesting complex (LHC) from a red alga (Grabowski et al., 2001), which are organisms that do not contain Chl *b*. Most of the binding sites normally occupied by Chl *a* can possibly contain Chl *b,* although direct evidence that Chl *b* binds to the same ligands is lacking. A study of the relative binding affinities of the chlorophylls to the apoprotein of the peridinin-chlorophyll-protein complex showed that Chl *b* is bound to the N-terminal domain approximately 2-times more strongly than Chl *a* (Brotosudarmo et al., 2008). A theoretical computational analysis of chlorophyll binding to a synthetic analogue of helix-1 of LHCP suggests that coordination bonds between Chl *b* and imidazole or the charge-compensated glutamate/arginine pair become thermodynamically favorable when bridged by a water molecule (Chen and Cai, 2007).

Nearly two-thirds of the Chl *a* in photosystems of the cyanobacterium *Synechocystis* sp. PCC 6803, which normally does not contain Chl *b*, was functionally replaced with Chl *b* when cells were transformed with the genes for Chlide *a* oxygenase and a LHCP (Xu et al., 2001). In the absence of Chl *a*, ligands normally bound to Chl *a*, such as side chain amide groups become available to Chl *b*. In systems that synthesize both Chls *a* and *b*, the two probably compete kinetically, and once a complex is formed with the kinetically more reactive Chl *a*, Chl *b* is excluded. Complexes then form with Chl *b* only when the ligand has a strong dipole capable of replacing a strongly held water molecule. The result is a strong coordination bond that is essential for enabling assembly of stable LHCs, an achievement that cannot be accomplished by Chl *a* alone.

The difference in the binding between Chl *a* and Chl *b* to the peptide (Fig. 16.4) suggests that the shift in the electronic distribution causes a dramatic change in coordination chemistry. Extrapolating this relationship to other chlorophylls suggests that the Lewis acid strength of the Mg should increase considerably in the Chl *c* series. We might expect, therefore, that Chl *c* will also retain water ligands, as suggested by the data in Fig. 16.4, or perhaps even coordinate with carboxyl groups. No structural information has emerged for LHCs isolated from organisms that contain Chl *c* rather than Chl *b* other than homology of the apoproteins (Durnford et al., 1999; De Martino et al., 2000). These issues are an open field for further investigations.

VII. Protein and Pigment Constituents of the Light-Harvesting Complexes

It is not certain whether the ancestral cyanobacterium that evolved into chloroplasts was capable of making Chl *b*, although species such as *Prochloron, Prochlorothrix* and *Prochlorococcus*, which are classified within the cyanobacterial family, have this capability (Tomitani et al., 1999; Hess et al., 2001). Modern cyanobacteria lack this ability. The green family of plants, including *Euglena*, the chlorophytic algae and plants, synthesize Chl *b* and incorporate it almost entirely into LHCs. The widely diverse group of algae that are products of secondary and tertiary endosymbiosis, such as the chromophytic algae that include the dinoflagellates, heterokonts, haptophytes and cryptophytes (see Chapter 1) (McFadden, 2001; Stoebe and Maier, 2002), make Chl *c* instead of Chl *b* but use this form of chlorophyll in the same way, i.e., in LHCs (Durnford et al., 1999; De Martino et al., 2000; Goss et al., 2000). These LHCs surround the core complexes of the photosynthetic reaction centers as peripheral antennae, in contrast to the core LHCs composed of CP43 and CP47 that contain only Chl *a* and are connected directly to the PS II reaction centers (Barber, 2003). It may be of particular importance that the apoproteins of the Chl *a/b*- and Chl *a/c*-complexes are synthesized in the cytosol of all these organisms, whereas proteins in the core antenna and reaction centers that bind only Chl *a* are synthesized in the plastid on ribosomes attached to thylakoid membranes (Harris et al., 1994; Zerges, 2000).

The green chlorophyte organisms contain chloroplasts that are surrounded by an envelope composed of two membranes. The single plastid per cell in the chromophytic algae is surrounded by four membranes, two of the typical envelope and two of the periplastid reticulum (reviewed in Hoober, 1984). The alga *Euglena gracilis* has

multiple plastids that are surrounded by three membranes. The differences in the number of membranes around the plastid require different pathways for import of these proteins after synthesis in the cytosol (Van Dooren et al., 2001), which leads to the question of whether Chl *b* (or Chl *c* in the case of the chromophytic algae) have a function in the import of LHCPs into the chloroplast and integration into membranes. LHCPs must be imported into the plastid along with several thousand other plastid proteins made outside of the organelle (Soll and Schleiff, 2004). In *Euglena* the LHCPs are synthesized as a polyprotein on ribosomes bound to the endoplasmic reticulum. After partial transfer into the lumen of the endoplasmic reticulum, the precursor polyproteins travel with the membrane through the Golgi apparatus and are transported by vesicle traffic to the plastids, where they are processed to the mature forms (Sulli et al., 1999; Van Dooren et al., 2001). Fusion of these vesicles with the outer (third) membrane surrounding the plastid would present the LHCPs to the typical plastid envelope import apparatus (Vothknecht and Soll, 2006), such that subsequent events possibly are similar to those in the chlorophyte organisms.

The LHCPs occur as a large family of proteins. In the alga *Chlamydomonas reinhardtii,* a well-studied organism, those associated with LHCs of PS II, referred to as the Lhcb family, are encoded by 9 genes for the major LHCPs (*Lhcbm* genes) and 2 genes for the minor complexes designated CP26 and CP29. Nine genes encode LHCPs for LHCI associated with PS I (the *Lhca* family) (Elrad and Grossman, 2004). In *Arabidopsis thaliana,* 26 genes for LHCPs were identified, 18 for Lhcb and 8 for Lhca (*Arabidopsis thaliana* Genome Initiative, 2000). These proteins of the Chl *a/b* complexes, along with those in Chl *a/c* complexes, are highly homologous products of an evolutionary lineage that reaches back to small peptides in cyanobacteria that have only a single membrane-spanning domain (see Chapter 14) (Dolganov et al., 1995; Funk and Vermaas, 1999). The proteins in algae and plants include three membrane-spanning regions, with a predominant Chl *a*-binding motif – ExxHxR – in the first and – ExxNxR – in the third. The glutamate (E) in helix-1 forms an ion-pair with the arginine (R) in helix-3, and vice versa with the other R and E to form a second ion-pair. These electrostatic bonds in the interior of the protein, an environment of low dielectric constant, contribute substantially to the stability of the LHC (Bassi et al., 1999; Remelli et al., 1999). However, the LHC must be completely assembled for these ion-pairs to form.

Two carotenoids, lutein and neoxanthin, are also required for the assembly and the stability of LHCs (Park et al., 2002). Two lutein molecules span the core of the complex, while neoxanthin is associated with the more peripheral helix-2 (Croce et al., 1999, 2002). The carotenoids play a minor role in light-harvesting, although lutein transfers absorbed energy primarily to Chl *a* while neoxanthin is more closely associated with Chl *b*. The carotenoids play a major role in protection of the complex from photooxidation by quenching triplet excited states of chlorophyll molecules (Croce et al., 1999; Cuttriss et al., 2006).

VIII. Functional Role of Chlorophyll *b* (and Chlorophyll *c*?)

Chl *b* has traditionally been thought to provide stability to LHCs, because in its absence most of the LHCPs do not accumulate. This scenario suggests that the LHPCs are imported into the plastid but subsequently degraded when stable LHCs do not assemble. The proteins indeed are synthesized in Chl *b-less* mutants, and Chl *b-less* plants are severely deficient in LHCs (reviewed in Hoober and Eggink, 1999; Hoober and Arygroudi-Akoyunoglou, 2004). However, chloroplasts isolated from Chl *b-less A. thaliana* plants were incapable of successfully importing LHCP precursors (Reinbothe et al., 2006). Thus a primary role for Chl *b* in *accumulation* of LHCPs may occur at the level of import into the plastid, rather than *stabilization* of LHCs within the plastid (Hoober and Eggink, 1999, 2001; Hoober et al., 2007). The first structure determined for LHCII (Kühlbrandt et al., 1994) described binding of Chl *a* to the helix-1 motif – ExxHxR – in the membrane-spanning helix-1 of LHCP. During import of LHCPs into the chloroplast, this conserved motif, which was the known chlorophyll binding site nearest the N-terminus, was suggested to bind chlorophyll in the chloroplast envelope inner membrane and retain the N-terminal domain until the remainder of the protein can be transferred through the outer membrane (Hoober and Eggink, 2001). It is now clear that this motif binds only Chl *a* (Bassi et al., 1999;

Remelli et al., 1999; Pascal et al., 2002; Chen et al., 2005), which is not sufficient for accumulation of LHCPs. Publication of a higher resolution structure of LHCII (Liu et al., 2004) revealed a Chl *b* bound to the backbone carbonyl of tyrosine-24, even closer to the N-terminus than the motif in membrane-spanning helix-1. Tyrosine-24 lies within a proline-rich N-terminal domain (Jansson, 1999). Because proline residues in a protein lack an amide hydrogen atom, formation of a helical structure by hydrogen-bonding cannot occur. Consequently, carbonyl groups of nearby peptide bonds are available as ligands for Chl *b.* Interaction of Chl *b* with the protein at this site fulfilled the predictions outlined earlier in this chapter for ligand preference and raised the possibility that binding of Chl *b* to this site is a critical event in retaining LHCPs in the chloroplast envelope.

The accumulation of LHCPs in the chloroplast is then possibly regulated by interaction of Chl *b* with the N-terminal domain of the protein. Kohorn (1990) showed that replacement of the histidine (H) in the helix-1 motif with alanine strongly attenuated import of the mutated LHCP into isolated chloroplasts. Therefore, binding of Chl *a* to this first membrane-spanning domain seems to be necessary for the retention of LHCP in the chloroplast envelope, although binding of Chl *a* alone is not sufficient. Concerted binding of Chls *a* and *b* to these N-terminal sites seems to hold LHCPs in the envelope membrane sufficiently long to allow further assembly of LHCs (Fig. 16.5). The chlorophylls are provided for this interaction by

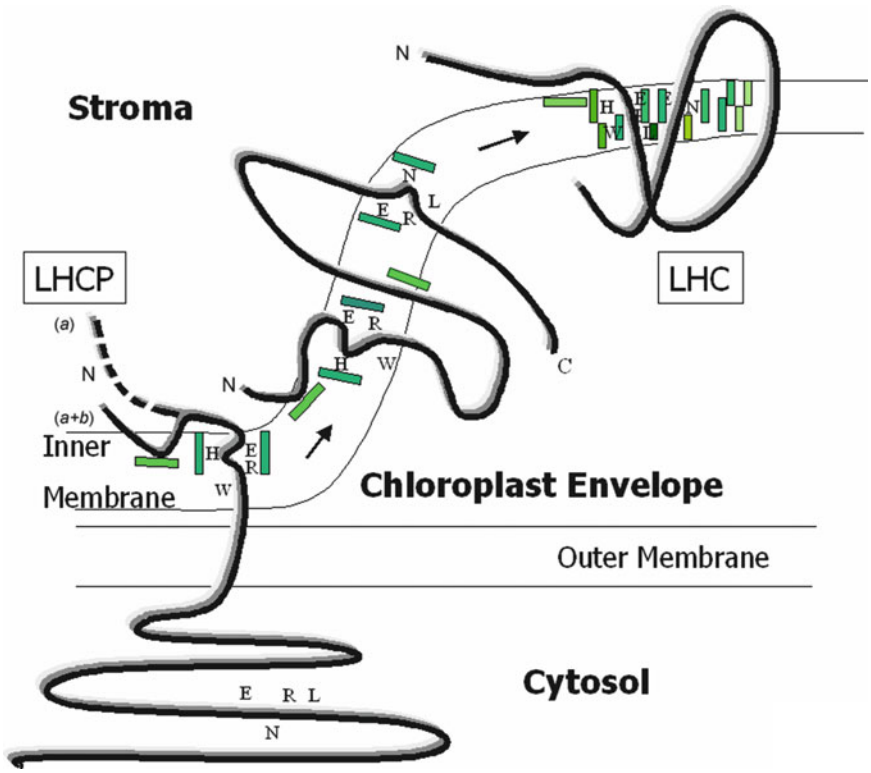

Fig. 16.5. Model of LHCII assembly in the chloroplast envelope and the proposed role of Chl *b*. Several proposed intermediates are shown in the sequence, *left* to *right*. After synthesis in the cytosol, an LHCP precursor is imported sufficiently into the stroma for removal of the transit sequence from the N-terminus and for the first membrane-spanning region to engage the inner membrane. Chl *a* (*dark rectangles*) binds to ligands in the motif provided by the ion-pair of the sidechains of glutamate and arginine and the imidazole group of histidine (*dotted line*, *a*). However, binding to these sites is not sufficient to retain the protein in the envelope, and without Chl *b* the protein slips back into the cytosol for transfer to the vacuole and degradation. Chl *b* (*light rectangles*) forms a strong coordination bond with the peptide bond carbonyl of tyrosine-24, closer to the N-terminus of the protein, and provides an additional hold on the protein (*solid line*, *a* + *b)*. These chlorophylls anchor the protein in the membrane sufficiently long for the remainder of the protein, including the conserved motif in membrane-spanning helix-3, to enter the membrane, bind additional chlorophyll and xanthophyll molecules, and complete assembly. Other proteins in the membrane and stroma apparently assist assembly of the complete complex (see text) (Adapted from Hoober et al., 2007).

the biosynthetic enzymes located in the envelope membranes (Joyard et al., 1998; Block et al., 2007). Chlide *a* oxygenase, the enzyme that coverts Chlide *a* to Chlide *b*, was detected on the chloroplast envelope inner membrane (Eggink et al., 2004; Reinbothe et al., 2006). Localization of the catalytic site of Chlide *a* oxygenase on the outer (cytosolic) surface of the inner membrane suggests that the N-terminus of LHCPs interacts with Chl *b* as it enters the membrane. The chlorophylls then likely serve as anchors to hold the N-terminal domain in place. Other factors are also involved in the assembly of LHCs such as the chloroplast signal recognition particle (Hutin et al., 2002; Schünemann, 2003), the Albino3 protein (Bellafiore et al., 2002) and other associated proteins (Eichacker and Henry, 2001).

IX. Assembly of Light-Harvesting Complexes and the Photosynthetic System

A. Observations with the Model Organism, Chlamydomonas reinhardtii

Our research group has extensively studied chloroplast development with "yellow-in-the-dark (*y*)" strains of the alga *C. reinhardtii*, with specific emphasis on identifying the initial site of incorporation of LHCPs into membranes. (For further information on *Chlamydomonas*, see Rochaix et al., 1998.) These mutant strains do not synthesize Chl in the dark and, moreover, strains are available that do not synthesize Chl *b*. Growth of the cells in the dark on acetate as a carbon source leads to formation of yellow cells that lack thylakoid membranes (Hoober et al., 1991). Experimental conditions were established that allowed these cells to initiate Chl synthesis immediately upon exposure to light at a nearly linear rate (Hoober and Stegeman, 1976; Maloney et al., 1989). Consequently, the fate of LHCPs could be analyzed biochemically and the cells examined ultrastructurally within minutes after initiation of membrane biogenesis.

Information on the post-translational modifications of LHCPs is quite complex. In vitro translation of poly(A)+-mRNA from *C. reinhardtii y1* generated two sharply defined protein bands for the precursor forms of the LHCPs (Hoober et al., 1982). When a soluble fraction of the cells was added to the translated products, the LHCP precursors were cleaved to proteins with the size of the mature LHCPs, 29.0 and 26.5 kDa (Marks et al., 1985). However, the in vitro processing was incomplete, achieving about 60% conversion to the mature form. When processing was attempted with LHCP precursors bound to antibodies, the larger precursor (31 kDa) was not cleaved whereas cleavage of the 29 kDa precursor was not affected. These experiments generated proteins with the same electrophoretic mobility on polyacrylamide gels as the mature proteins obtained from thylakoid membranes (Hoober et al., 1982). The surprising result that emerged from a proteomic analysis of thylakoid membrane proteins by Hippler et al. (2001) was that both of the two major 29 and 26.5 kDa fractions obtained from the membrane contained a mixture of the two major proteins after resolution by 2-dimensional electrophoresis. One source of variation was processing of the N-terminal transit sequence at two cleavage sites (Stauber et al., 2003; Stauber and Hippler, 2004), which led to different sizes of the same gene product. Electrophoresis also revealed that proteins migrating with essentially the same molecular mass were distributed among a series of species with different charge. This remarkable complexity, in terms of size and charge, of the distribution of the same gene products requires considerably more study to understand the processing of these proteins.

Nevertheless, we can still ask the question of how these proteins enter the chloroplast and become integrated into membranes. Measurements of the increase in Chl *a* and *b* during greening of *C. reinhardtii y1* showed that each increased immediately upon exposure of dark-grown cells to light and at approximately linear rates (Maloney et al., 1989). An important observation was made when the assembly of LHCs was monitored by the development of Förster resonance energy transfer from Chl *b* to Chl *a* within the complex. In this experiment, assembly of LHCs was detected by energy transfer as the two chlorophylls became sufficiently close for this process to occur. After a transient rise in chlorophyll fluorescence, the energy absorbed by the complexes was quenched by association of the LHCs with core complexes (Fig. 16.6). Addition of chloramphenicol, an inhibitor of synthesis of core complex

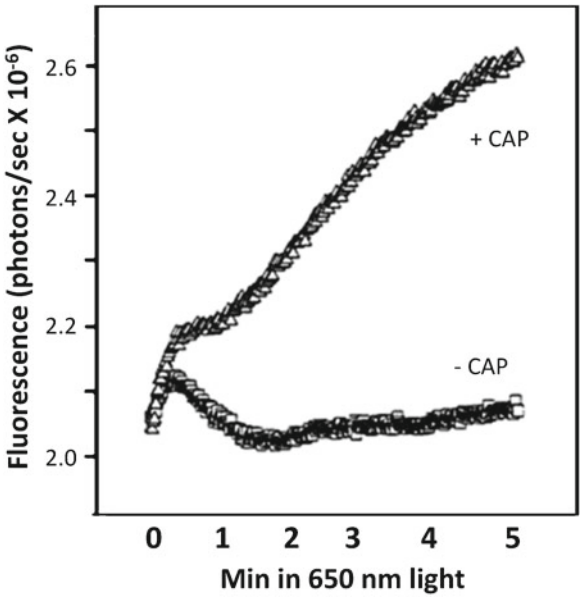

Fig. 16.6. Analysis by Förster resonance energy transfer of the increase in LHCII during greening of *dark-grown*, *yellow* cells of *C. reinhardtii cw15*. Degreened cells were incubated 1.5 h at 38°C and then exposed to 650 nm light in a spectrofluorimeter. Under these conditions, chlorophyll synthesis occurs linearly upon exposure of cells to light. The 650 nm light initiated light-dependent chlorophyll synthesis by photoreduction of Pchlide, and was also absorbed primarily by Chl *b*. Fluorescence emission from Chl *a* was measured at 679 nm (– CAP). Chloramphenicol (CAP) was added to 150 μM to a second suspension, which 15 min later was exposed to 650 nm light (+ CAP) and fluorescence emission was monitored. Fluorescence of the control sample (– CAP) was strongly quenched after the first minute of light exposure, which indicated that the complexes had become connected to an energy transduction pathway (Adapted from White et al., 1996).

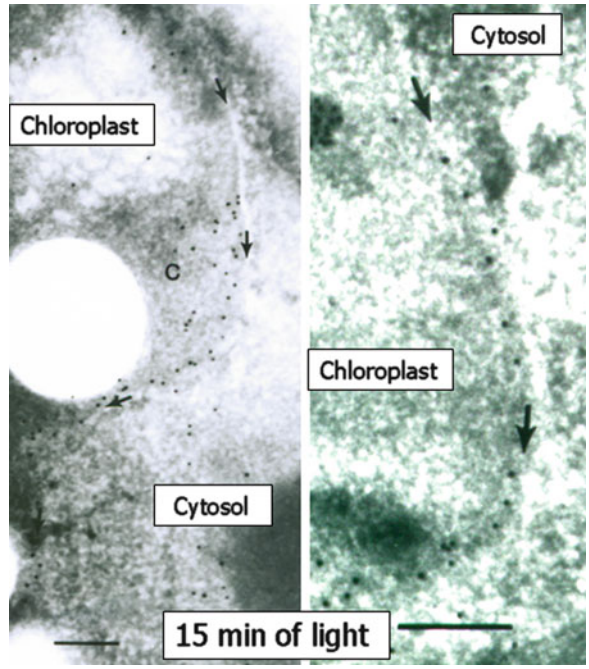

Fig. 16.7. Immunocytochemical localization of LHCP in sections of *dark-grown C. reinhardtii y1* after exposure to light for 15 min. Sections of cells from two experiments were treated with antibodies against LHCP and then with 10-nm gold particles conjugated with protein A, which binds to antibodies. The gold particles were found predominantly over the inner membrane of the chloroplast envelope (marked with *arrows*), which marked the initial site of integration of the proteins into membrane. Bar, 250 nm (Adapted from White et al., 1996).

proteins on chloroplast ribosomes, or of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to block electron flow out of PS II, was required to reveal the rate of assembly of the complexes (White and Hoober, 1994; White et al., 1996). This experiment demonstrated that LHCs were assembled and connected to reaction centers within seconds after the start of illumination, in the time frame required for folding of a protein upon completion of synthesis (Snow et al., 2005).

The rapid and nearly linear initial increase in the accumulation of LHCPs (Hoober et al., 1982) allowed identification of the site of assembly within the developing plastid. Sections of cells that were fixed within the first few minutes of greening were probed with antibodies raised against LHCPs (White et al., 1996; Eggink et al., 2001). These immunolabeling experiments provided the unequivocal result that the proteins were detected in situ predominantly along the chloroplast envelope (Fig. 16.7). To achieve this result, the cells were fixed as rapidly as possible with osmium tetroxide to prevent further migration of the proteins. These experiments revealed the initial accumulation of LHCPs in regions of extensive production of invaginations and vesiculation of the envelope (Fig. 16.8) (Hoober et al., 1991). The conclusion emerged that assembly of LHCs occurred initially in the envelope inner membrane, which expanded and then released vesicles into the interior of the chloroplast as membrane assembly proceeded (Eggink et al., 2001). (Further discussions of the structure and function of plastids are in Volume 26 of this series, Wise and Hoober, 2006.)

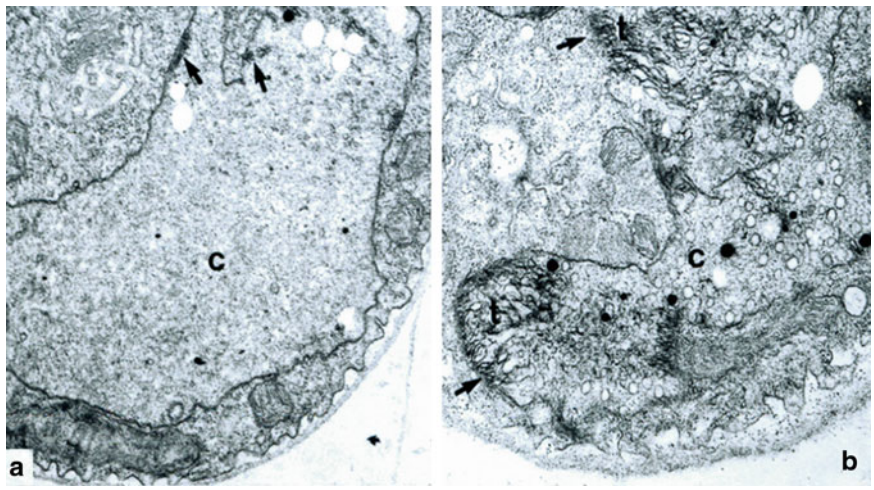

Fig. 16.8. Electron micrographs of sections of the chloroplast from (**a**) a *dark-grown* cell of *C. reinhardtii y1* and (**b**) after exposure to light for 15 min. In (**b**), extensive regions of the chloroplast envelope were associated with newly formed membranes (*arrows*). Numerous vesicles in the chloroplast stroma (**c**) were similar in morphology to membranes emanating from the envelope. Comparison of the two micrographs reveals extensive, light-induced production of thylakoid membrane (t) from the chloroplast envelope (Adapted from Hoober et al., 1991).

B. Biochemical Evidence for Site of Initial Assembly of Photosystems

Within the first few minutes of exposure of dark-grown cells of *C. reinhardtii y1* to light, fluorescence of newly assembled LHCs was quenched (Fig. 16.6). The increase in chlorophyll fluorescence, when cells were treated with DCMU or chloramphenicol, indicated that the LHCIIs were connected to functional reaction centers essentially immediately after assembly. Because fluorescence did not increase concomitantly with the increase in chlorophyll content during continuous illumination of untreated cells, these experiments indicated that a complete electron transport system was also assembled within minutes after the start of greening. These early membranes were examined by fluorescence induction kinetics to determine the status of the system and whether PS II and PS I were segregated as occurs in mature membranes in *State 1* or were intermingled in a *State 2* condition. In State 1, energy absorbed by LHCII is transferred to PS II and the electron acceptor pool of plastoquinone becomes reduced, which causes a temporary backup in excitons in the light-harvesting complexes and results in a rise in fluorescence as the excess energy is released. In State 2, LHCII is phosphorylated (Depège et al., 2003) and some complexes dissociate from PS II and move over to PS I, which allows more of the energy absorbed by LHCII to be transferred to PS I. Consequently, the maximal level of fluorescence is reduced, because PS I is weakly fluorescent. To provide the background for interpretation of the experimental results, the following discussion first describes the typical observation with mature chloroplasts.

C. Fluorescence Induction Curves During Greening of Chlamydomonas reinhardtii y1

In organic solvents, an environment that minimizes quenching of chlorophyll fluorescence by the solvent, the fluorescence quantum yield of Chl *a* (the number of photons emitted per number of photons absorbed) is in the range of 0.31–0.35 (Karukstis, 1991). Chlorophyll is relatively insoluble in water and functions within a non-polar environment provided by proteins and lipids in thylakoid membranes. The interior of the membrane is rich in the hydrocarbon portions of the membrane lipids and carotenoids, and thus provides a non-aqueous phase within the chloroplast. Although free chlorophyll in a membrane can retain much of its inherent fluorescence, most if not all chlorophyll is attached to proteins that position the molecules in such a fashion that absorbed energy is readily transferred to other molecules and thus eventually trapped (quenched) by reaction centers. Nearly 100% of absorbed

light quanta are productively used for electron transfer in photosynthesis. Therefore, chlorophyll in thylakoid membranes in vivo exhibits a low level of fluorescence, with a maximal quantum yield of only 0.03–0.05 (Krause and Weis, 1991). This level is reached when the acceptor of electrons from PS II is saturated (fully reduced), a physiological state that does not allow further trapping of energy.

The chlorophyll fluorescence yield is related to the ability of the remainder of the photosynthetic system to use the absorbed energy. Changes in the low level of fluorescence of Chl *a* in chloroplasts thus reflect its functional state, and measurement of fluorescence under a variety of conditions is a sensitive means to monitor photosynthetic activity. (See Papageorgiou and Govindjee, 2004, for detailed discussions of chlorophyll fluorescence in chloroplasts.) Figure 16.9 illustrates measurements made on a short time scale that describe the status of the reaction centers of PS II. In this experiment, light-grown cells of *C. reinhardtii y1* were dark-adapted for several minutes and then exposed to a modulated red light source (wavelength of maximal intensity, 650 nm) at a low intensity of 2.5 μmol photons m^{-2} s^{-1}. (Light of this wavelength is absorbed primarily by Chl *b* in the light-harvesting antenna; because of the close association of Chl *b* with Chl *a* within light-harvesting complexes of the antenna, energy is rapidly transferred to Chl *a* on a time scale of several hundred femtoseconds to a few picoseconds.) A low intrinsic level of fluorescence, designated F_o, is released by the antenna when the reaction centers and electron acceptors are fully oxidized. Upon exposure to a higher intensity (80 μmol photons m^{-2} s^{-1}) actinic red light, a rapid increase in fluorescence is observed on the time scale of several hundred milliseconds (White and Hoober, 1994).

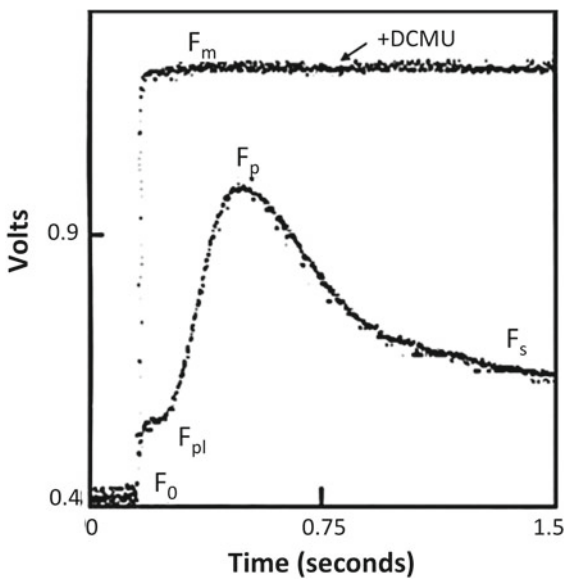

Fig. 16.9. Chlorophyll *a* fluorescence induction kinetics displayed by cells with a fully-developed, dark-adapted chloroplast, in a *State 1* condition. The rise to peak fluorescence (F_p) and decline to the steady-state level (F_s), within the maximal fluorescence (F_m) obtained in the presence of DCMU, are typical of results obtained with light-grown algae and leaves of higher plants. Addition of DCMU blocked electron flow out of PS II and consequently a maximal amount of the energy absorbed by the LHCs was emitted as fluorescence (Adapted from White and Hoober, 1994).

The first antenna complexes to become fully fluorescent were those not functionally connected to reaction centers, indicated by F_{pl}, a plateau level that varies with conditions; these unconnected complexes were relatively highly fluorescent. The time course of the rise in fluorescence to the peak value, F_p, is a measure of the net rate by which Q_A, the primary acceptor of PS II, is reduced. When Q_A is reduced, any additional energy absorbed by the antenna cannot be trapped and is then released by heat and/or fluorescence. The rate at which fluorescence rises from F_{pl} to F_p is a function of the intensity of actinic light as well as the size of the antenna that absorbs the light (Strasser et al., 1995, 2004). The subsequent fall from the peak level (F_p) of fluorescence occurs when the pathway for carbon fixation is activated and NADPH is oxidized. Electrons are then transported through PS I to $NADP^+$. Opening the gate at the end of the photochemical pathway results in re-oxidation of the plastoquinone pool and thus makes electron acceptors again available for PS II. The final steady-state level of fluorescence (F_s) is an indication of how well the two photosystems are balanced, or how readily electrons can be transferred through the complete system to the carbon fixing pathway.

Figure 16.9 shows a typical chlorophyll fluorescence transient for healthy chloroplasts. Deviation from this pattern is diagnostic of a change in physiological conditions. For example, herbicides such as DCMU act by blocking transfer of electrons from the reduced Q_A of PS II to

the next electron carrier, the plastoquinone Q_B. When such an inhibitor is added, fluorescence rapidly rises to a maximal value upon exposure to actinic light (curve labeled "+DCMU" in Fig. 16.9). The reduced form of electron acceptor Q_A can no longer donate electrons to Q_B , and the fluorescence rises. The maximal yield of fluorescence that can be generated in the assay is termed F_m. Maximal fluorescence of the system can also be induced by exposure to an intense burst of white light, which floods the reaction center with excitons at a higher rate than Q_A can release electrons (Schreiber et al., 1998). The maximal quantum yield of PS II is expressed by a simple relationship:

$$\Phi_F = F_m - F_o \left(\text{variable fluorescence}, F_v\right) / F_m$$

This value is between 0.75 and 0.85 for healthy plant and algal cells. When the reaction center is damaged, one result is an increase in F_o, which reduces the value of F_v. The decrease in this ratio indicates a reduced yield of photosynthesis.

D. Fluorescence Signature of Developing Thylakoid Membranes

LHCII, the major light-harvesting antenna, transfers energy primarily to PS II in State 1, the condition in which the photosynthetic units are segregated with PS II in grana and PS I units predominantly in stromal lamellae. When the intensity of incident light is high and Q_A is in a relatively reduced state, LHCII is phosphorylated (Depège et al., 2003) and functionally more energy is transferred into PS I. This arrangement is designated State 2, as described earlier. Because PS II is located within the granal stacks of thylakoid membranes, whereas PS I is more peripherally distributed, the state transition implies a reorganization of membrane components (Albertsson, 1995). PS I is a kinetically faster complex than PS II (Trissl and Wilhelm, 1993) and energy absorbed by LHCII is trapped more efficiently in State 2. Thus, the maximal level of fluorescence of the antenna that can be achieved with an intense flash of light is reduced, a term defined as F_m'. The value of F_p also decreases as fewer LHCIIs are connected to PS II. State 1 is restored when LHCII is dephosphorylated by the action of a protein phosphatase (Allen and Mullineaux, 2004).

An example of the use of the above information is shown in Fig. 16.10. Changes in the pattern of the fluorescence transient provide important information on the status of photosystems during chloroplast development. During the early phase, some of the newly formed thylakoid membranes are in the form of small vesicles, which are released from biogenic domains within the envelope inner membrane (Fig. 16.8). These vesicles apparently eventually fuse into lamellar structures that expand and adhere to form grana. Segregation of PS I and PS II does not occur until granal stacks are formed. Thus, in the separate, initially formed vesicles, and in the less organized biogenic domains in the envelope, reaction centers and antenna complexes should be distributed randomly within the membrane, with the light-harvesting complexes feeding exciton energy to PS I as well as PS II. Electrons produced by PS II are then rapidly pulled through the electron transport chain connecting the photosystems, and Q_A remains oxidized. This arrangement is characteristic of a State 2 condition. As shown in Fig. 16.10a, at the beginning of chloroplast development in dark-grown mutants of *C. reinhardtii y1*, the small number of antenna complexes were not connected to reaction centers. Fluorescence increased to the F_{pl} level upon exposure to actinic light, and addition of DCMU had essentially no effect. After an hour of chloroplast development, actinic light caused only a rise to the same plateau level (F_{pl}) of fluorescence (Fig. 16.10b). The subsequent rise in fluorescence to F_p, typical of mature thylakoid membranes, was not observed. However, a large increase to F_m upon addition of DCMU demonstrated that functional photosystems had indeed formed and some of the energy absorbed by LHCII was efficiently trapped by PS II. In these experiments, the typical fluorescence transient shown in Fig. 16.9 emerged gradually over several hours as the growing thylakoid membranes adhered to form grana (Hoober et al., 1991; White and Hoober, 1994). Similar conclusions were reached from studies of greening of an etiolated higher plant (Srivastava et al., 1999).

When utilization of the final forms of chemical energy produced by the photochemical reactions in thylakoid membranes is blocked, the entire

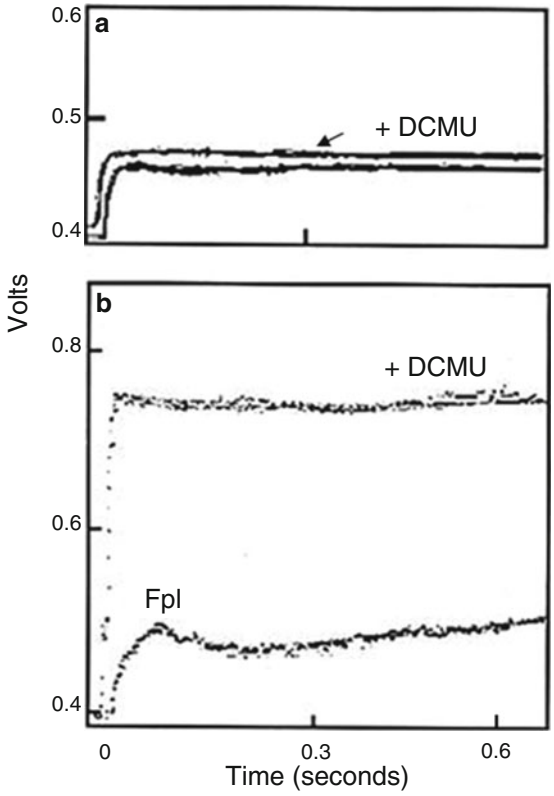

Fig. 16.10. (**a**) Fluorescence induction kinetics typical of *dark-grown*, *yellow* cells shows that fluorescence of residual chlorophyll rises rapidly to the F_{pl} level. Addition of DCMU did not significantly increase fluorescence, an indication that the LHCII were unconnected to reaction centers. (**b**) After *dark-grown* cells were exposed to white light for 1 h, kinetics typical of the State 2 condition were displayed. The curve for untreated cells was essentially unchanged from that obtained at the beginning of greening (**a**). The large increase in fluorescence when DCMU was added indicated that functional PS II centers were present but did not become sufficiently reduced in the absence of DCMU to cause re-emission of light from LHCs (Adapted from White and Hoober, 1994).

electron transport system gradually becomes fully reduced. Under these conditions, fluorescence does not fall to a lower F_s value. An illustration of this phenomenon is the fluorescence transient observed with purified thylakoid membranes (Fig. 16.11). The ultimate source of electrons, water, was still abundant in this assay, but $NADP^+$ and ADP were not present. Without acceptors of chemical energy, fluorescence of the antenna gradually increased as the entire system became reduced when exposed to light (White and Hoober, 1994). The slow rise to F_m in the absence of DCMU again indicated that a

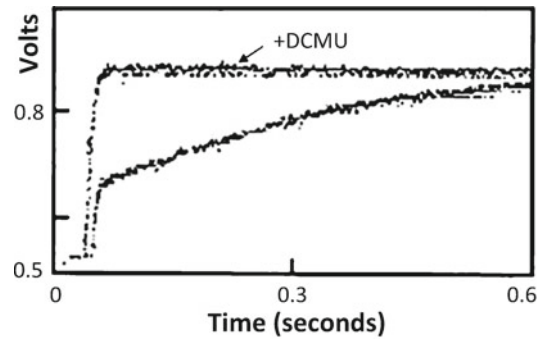

Fig. 16.11. Dark-grown cells of *C. reinhardtii y1* were exposed to light for 1 h, broken by passage through a French pressure cell, and then fractionated to obtain a thylakoid membrane fraction. The membranes were suspended in buffer and the fluorescence induction curves were obtained with the same conditions shown in Fig. 16.10. Because no terminal electron acceptors were added, fluorescence increased gradually as the complete energy transduction pathway became reduced, eventually reaching the level of fluorescence in samples treated with DCMU.

complete energy transduction pathway was assembled and that more time was required for all the components to become reduced than when the measurement included only PS II in the presence of DCMU.

These fluorescence data support the proposal that small vesicles are released from the chloroplast envelope when biogenesis of thylakoid membrane is initiated, as revealed by electron microscopy. These vesicles contained co-mingled LHCs, PS I, PS II as well as the intermediate electron carriers. In addition, invaginations of the inner envelope membrane may contain unsegregated photosystems still attached to the larger membrane structure. Sedimentation analysis of membranes from gently broken, greening cells of the cell-wall mutant *C. reinhardtii cw15* showed that about half of the chlorophyll sedimented as large particles, expected for newly formed thylakoid domains within large fragments of the envelope membrane (Fig. 16.12a). Approximately a third of the chlorophyll sedimented at a rate expected for vesicles. Fluorescence data on these fractions indicated that the chlorophylls were photosynthetically active and that the results shown in Figs. 16.6 and 16.10 reflected the presence of chlorophylls predominantly associated with the envelope (Hoober et al., 1991). These observations are consistent with the images obtained by electron microscopy for invaginations

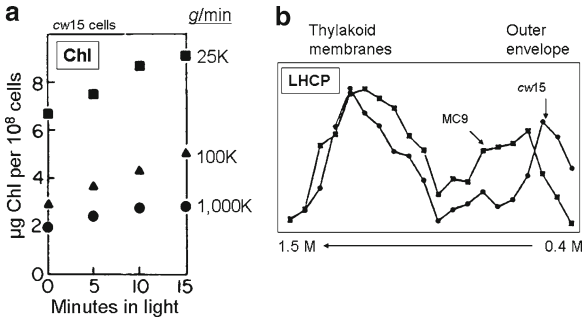

Fig. 16.12. (**a**) Distribution of chlorophyll in cell fractions. Degreened cells of *C. reinhardtii cw15* were exposed to light for 0, 5, 10 or 15 min at 38°C, broken and centrifuged at (■) 5,000 *g* for 5 min (25 kg/min) to collect large fragments, (▲) 10,000 *g* for 10 min (100 kg/min) to collect small fragments and vesicles and (●) 30,000 *g* for 35 min (1,000 kg/min) to collect small vesicles. At these times, most of the newly synthesized chlorophyll was associated with large fragments, most likely the envelope membrane, in addition to smaller particles (Adapted from Hoober et al., 1991). (**b**) Degreened cells of *C. reinhardtii cw15* and an insertional mutant strain, MC9, were exposed to light at 38°C in the presence of [^{14}C] arginine for 5 min. Cells were then broken and placed on sucrose gradients for equilibrium density sedimentation. After electrophoretic resolution of the proteins in separate fractions of the gradient, the radioactivity in the LHCP bands was quantified. The results were normalized to the maximal level in the higher density fractions typical of thylakoid membranes (about 1.3 M sucrose). In both strains, a significant amount of labeled LHCPs were recovered in low-density membranes characteristic of the outer membrane of the chloroplast envelope (From Eggink et al., 1999).

of the inner envelope membrane and appearance of a population of small vesicles (Fig. 16.8). When cells were pulse-labeled with [^{14}C]arginine during the first 5 min of greening, most of the labeled LHCPs were found in sucrose gradients at the position expected for the density of thylakoid membranes (Eggink et al., 1999). However, a substantial amount was also recovered with the much lower density of the outer membrane of the chloroplast envelope that may represent proteins in the process of import (Fig. 16.12b).

X. Conclusions

From experiments, discussed in this chapter, we conclude that the photosystems are initially assembled in small domains within the envelope inner membrane, in which LHCs, PS I, PS II and all components of the electron transport system are assembled with remarkable speed. The data support a model in which the LHCPs originating in the cytosol are assembled into LHCs and connect to the core complexes with kinetics that correspond with the rates of folding of individual proteins. Further evidence for the interaction of LHCPs with chlorophyll and their integration into the envelope membrane were the observations that, when the proteins are made in excess, the extra proteins are recovered in vacuoles in the cytoplasm rather than in the plastid (White et al., 1996). In a Chl *b-less* mutant, LHCPs that were synthesized in the dark did not accumulate in the plastid but were detected in the cytosol and vacuoles. Furthermore, most of the LHCPs synthesized during initial greening of this mutant were recovered in vacuoles (Park and Hoober, 1997). An assembly-deficient mutant shunted nearly all LHCPs to vacuoles after processing to the mature size (Eggink et al., 1999). The mechanism of the diversion of LHCPs to the vacuole is not known. However, an important conclusion from these experiments is that the excess LHCPs were not recovered within the chloroplast. Thus, import and accumulation of LHCPs in the organelle appear to require – either regulated or facilitated by – association with chlorophyll, and particularly Chl *b*, within the chloroplast envelope (Reinbothe et al., 2006).

Data obtained with the *Chlamydomonas reinhardtii* system are supported by observations of chloroplast development in plants. Vesicle formation at the level of the chloroplast envelope is observed when plants are treated with cool temperatures. The vesicles disappear when the normal growth temperature is restored (Morré et al., 1991). Formation of vesicles from the envelope inner membrane has also been observed by other investigators (Kroll et al., 2001; Westphal et al., 2003). Vesicle formation and subsequent thylakoid membrane biogenesis are essentially absent in the *VIPP1* mutant strain of *Arabidopsis thaliana* (Kroll et al., 2001), which is deficient in a vesicle-inducing plastid protein. The VIPP1 protein forms large ring structures that may be involved in the initiation of vesicle formation (Aseeva et al., 2004). Application of inhibitors of vesicle traffic to isolated chloroplasts also provides evidence for vesicle traffic from the chloroplast envelope (Westphal et al., 2001), which is supported by a bioinformatics analysis (Andersson

and Stina, 2004). These data support the concepts of thylakoid formation proposed many years ago by von Wettstein (1959, 2001).

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References

Adra AN and Rebeiz CA (1998) Chloroplast biogenesis 81. Transient formation of divinyl chlorophyll *a* following a 2.5 millisecond light flash treatment of etiolated cucumber cotyledons. Photochem Photobiol 68: 852–856

Albertsson P-Å (1995) The structure and function of the chloroplast photosynthetic membrane—a model for the domain organization. Photosynth Res 46: 141–149

Allen JF and Mullineaux CW (2004) Probing the mechanism of state transitions in oxygenic photosynthesis by chlorophyll fluorescence spectroscopy, kinetics and imaging. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 447–461. Springer, Dordrecht

Andersson MX and Stina A (2004) A chloroplast-localized vesicular transport system: a bio-informatics approach. BMC Genomics 5:40

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–813

Aseeva E, Ossenbühl F, Eichacker LA, Wanner G, Soll J and Vothknecht U (2004) Complex formation of Vipp1 depends on its α-helical PspA-like domain. J Biol Chem 279: 35535–35541

Balaban TS (2005) Relevance of the diastereotopic ligation of magnesium atoms of chlorophylls in the major light-harvesting complex II (LHC II) of green plants. Photosynth Res 86: 215–262

Balaban TS, Fromme P, Holzwarth AR, Krauss N and Prokhorenko VI (2002) Relevance of the diasterotopic ligation of magnesium atoms of chlorophylls in photosystem I. Biochim Biophys Acta 1556: 197–207

Ballschmitter K, Cotton TM and Katz JJ (1969) Chlorophyll-water interactions. Hydration, dehydration and hydrates of chlorophyll. Biochim Biophys Acta 180: 347–359

Barber J (2003) Photosystem II: the engine of life. Quart Rev Biophys 36: 71–89

Bassi R, Croce R, Cugini D and Sandonà D (1999) Mutational analysis of a higher plant antenna protein provides identification of chromophores bound into multiple sites. Proc Natl Acad Sci USA 96: 10056–10061

Belanger FC and Rebeiz CA (1984) Chlorophyll biogenesis. Spectroscopic study of net spectral shifts induced by axial coordination in metalated tetrapyrrole. Spectrochim Acta 40A: 807–827

Bellafiore S, Ferris P, Naver H, Göhre V and Rochaix JD (2002) Loss of Albino3 leads to the specific depletion of the light-harvesting system. Plant Cell 14: 2303–2314

Blankenship RE (2002) Molecular Mechanisms of Photosynthesis. Blackwell Scientific, Oxford

Block MA, Douce R, Joyard J and Rolland N (2007) Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. Photosynth Res 92: 225–244

Brotosudarmo THP, Mackowski S, Hofmann E, Hiller RG and Scheer H (2008) Relative binding affinities of chlorophylls in peridinin-chlorophyll-protein reconstituted with heterochlorophyllous mixtures. Photosynth Res 95: 247–252

Chen M and Cai ZL (2007) Theoretical study on the thermodynamic properties of chlorophyll *d*-peptides coordinating ligand. Biochim Biophys Acta 1767: 603–609

Chen M, Eggink LL, Hoober JK and Larkin AWD (2005) Influence of structure on binding of chlorophylls to peptide ligands. J Am Chem Soc 127: 2052–2053

Chow HC, Serlin R and Strouse CE (1975) The crystal and molecular structure and absolute configuration of ethyl chlorophyllide *a* dehydrate. A model for the different spectral forms of chlorophyll *a.* J Am Chem Soc 97: 7230–7237

Clayton RK (1973) Primary processes in bacterial photosynthesis. Annu Rev Biophys Bioengin 2: 131–156

Clegg RM (2004) Nuts and bolts of excitation energy migration and energy transfer. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 83–105. Springer, Dordrecht

Cox PA (2000) Inorganic Chemistry, BIOS Scientific Publ, Oxford

Croce R, Weiss S and Bassi R (1999) Carotenoid-binding sites of the major light-harvesting complex II of higher plants. J Biol Chem 274: 29613–29623

Croce R, Canino G, Ros F and Bassi R (2002) Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry 41: 7334–7343

Cuttriss AJ, Mimica JL, Howitt CA and Pogson BJ (2006) Carotenoids. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 315–334. Springer, Dordrecht

De Martino A, Douady D, Quinet-Szely M, Rousseau B, Crépineau F, Apt K and Caron L (2000) The light-harvesting antenna of brown algae. Highly homologous proteins encoded by a multigene family. Eur J Biochem 267: 5540–5549

Depège N, Bellafiore S and Rochaix JD (2003) Role of chloroplast protein kinase Stt7 in LHCII phosphorylation

and state transition in *Chlamydomonas.* Science 299: 1572–1575

Dolganov NAM, Bhaya D and Grossman AR (1995) Cyanobacterial protein with similarity to the chlorophyll *a/b* binding proteins of higher plants: evolution and regulation. Proc Natl Acad Sci USA 92: 636–640

Dougherty RC, Strain HH, Svec WA, Uphaus RA and Katz JJ (1970) The structure, properties, and distribution of chlorophyll c. J Am Chem Soc 92: 2826–2833

Durnford DG, Deane JA, Tan S, McFadden GI, Gantt E and Green BR (1999) A phylogenetic assessment of the eukaryotic light-harvesting proteins, with implications for plastid evolution. J Mol Evol 48: 59–68

Eggink LL and Hoober JK (2000) Chlorophyll binding to peptide maquettes containing a retention motif. J Biol Chem 275: 9087–9090

Eggink LL, Park HS and Hoober JK (1999) The role of the envelope in assembly of light-harvesting complexes in the chloroplast: distribution of LHCP between chloroplast and vacuoles during chloroplast development in *Chlamydomonas reinhardtii.* In: Argyroudi-Akoyunoglou JH and Senger H (eds) The Chloroplast: From Molecular Biology to Biotechnology, pp 161–166. Kluwer Academic Publishers, Dordrecht

Eggink LL, Park HS and Hoober JK (2001) The role of chlorophyll *b* in photosynthesis: hypothesis. BMC Plant Biol 1: 2

Eggink LL, LoBrutto R, Brune DC, Brusslan J, Yamasato A, Tanaka A and Hoober JK (2004) Synthesis of chlorophyll *b*: localization of chlorophyllide *a* oxygenase and discovery of a stable radical in the catalytic subunit. BMC Plant Biol 4: 5

Eichacker LA and Henry A (2001) Function of a chloroplast SRP in thylakoid protein export. Biochim Biophys Acta 1541: 120–134

Elrad D and Grossman AR (2004) A genome's-eye view of the light-harvesting polypeptides of *Chlamydomonas reinhardtii*. Curr Genet 45: 61–75

Ferris T (1988) Coming of Age in the Milky Way, Doubleday, New York

Frackowiak D, Bauman D, Manikowski H, Browett WR and Stillman MJ (1987) Circular dichroism and magnetic circular dichroism spectra of chlorophylls *a* and *b* in nematic liquid crystals. Biophys Chem 28: 101–114

Fragata M, Nordén B and Kurucsev T (1988) Linear dichroism (250-700 nm) of chlorophyll *a* oriented in a lamellar phase of glycerylmonooctanoate/H_2O. Characterization of electronic transitions. Photochem Photobiol 47: 133–143

Funk C and Vermaas W (1999) A cyanobacterial gene family coding for single-helix proteins resembling part of the light-harvesting proteins from higher plants. Biochemistry 38: 9397–9404

Gadella TWJ Jr, Van der Krogt GNM and Bisseling T (1999) GFP-based FRET microscopy in living plant cells. Trends Plant Sci 4: 287–291

Goss R, Wilhelm C and Garab G (2000) Organization of the pigment molecules in the chlorophyll *a/b/c* containing alga *Mantoniella squamata* (Prasinophyceae) studied by means of absorption, circular and linear dichroism spectroscopy. Biochim Biophys Acta 1457: 190–199

Grabowski B, Cunningham FX and Gantt E (2001) Chlorophyll and carotenoid binding in a simple red algal light-harvesting complex crosses phylogenetic lines. Proc Natl Acad Sci USA 98: 2911–2916

Gregory JK, Clary DC, Liu K, Brown MG and Saykally RJ (1997) The water dipole moments in water clusters Science 275: 814–817

Grimm B, Porra RJ, Rüdiger W and Scheer H (eds) (2006) Chlorophylls and Bacteriochlorophylls. Biochemistry, Biophysics, Functions and Applications, Advances in Photosynthesis and Respiration, Vol 25. Springer, Dordrecht

Gunner MR, Saleh MA, Cross E, ud-Doula A and Wise M (2000) Backbone dipoles generate positive potentials in all proteins: origins and implications of the effect. Biophys J 78: 1126–1144

Hanson LK (1991) Molecular orbital theory of monomer pigments. In: Scheer H (ed) Chlorophylls, pp 993–1014. CRC Press, Boca Raton

Harris EH, Boynton JE and Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev 58: 700–754

Helfrich M, Bommer B, Oster U, Klement H, Mayer K, Larkum AWD and Rüdiger W (2003) Chlorophylls of the *c* family: absolute configuration and inhibition of NADPH:protochlorophyllide oxidoreductase. Biochim Biophys Acta 1605: 97–103

Hess WR, Rocap G, Ting CS, Larimer F, Stilwagen S, Lamerdin J and Chisholm SW (2001) The photosynthetic apparatus of *Prochlorococcus*: insights through comparative genomics. Photosynth Res 70**:** 53–71

Hippler M, Klein J, Fink A, Allinger T and Hoerth P (2001) Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*. Plant J 28: 595–606

Hoff AJ and Amesz J (1991) Visible absorption spectroscopy of chlorophylls. In: Scheer H (ed) Chlorophylls, pp 723–738. CRC Press, Boca Raton

Hoober JK (1984) Chloroplasts. Plenum Press, New York

Hoober JK (2006) Chloroplast development: whence and wither. In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 27–51. Springer, Dordrecht

Hoober JK and Arygroudi-Akoyunoglou JH (2004) Assembly of light-harvesting complexes of photosystem II and the role of chlorophyll *b*. In: Papageorgiou G and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 679–712. Springer, Dordrecht

Hoober JK and Eggink LL (1999) Assembly of light-harvesting complex II and biogenesis of thylakoid membranes in chloroplasts. Photosynth Res 61: 197–215

Hoober JK and Eggink LL (2001) A potential role of chlorophylls *b* and *c* in assembly of light-harvesting complexes. FEBS Lett 489: 1–3

Hoober JK and Stegeman WJ (1976) Kinetics and regulation of synthesis of the major polypeptides of thylakoid membranes in *Chlamydomonas reinhardtii y-1* at elevated temperatures. J Cell Biol 70: 326–337

Hoober JK, Marks DB, Keller BJ and Margulies MM (1982) Regulation of accumulation of the major thylakoid polypeptides in *Chlamydomonas reinhardtii y*-1 at 25°C and 38°C. J Cell Biol 95: 552–558

Hoober JK, Boyd CO and Paavola LG (1991) Origin of thylakoid membranes in *Chlamydomonas reinhardtii y*-1 at 38°C. Plant Physiol 96: 1321–1328

Hoober JK, Eggink LL and Chen M (2007) Chlorophylls, ligands and assembly of light-harvesting complexes in chloroplasts. Photosynth Res 94: 387–400

Houssier C and Sauer K (1970) Circular dichroism and magnetic circular dichroism of the chlorophyll and protochlorophyll pigments. J Am Chem Soc 92: 779–791

Hutin C, Havaux M, Carde JP, Kloppstech K, Meiherhoff K, Hoffman N and Nussaume L (2002) Double mutation cpSRP43^{-}/cpSRP54^{-} is necessary to abolish the cpSRP pathway required for thylakoid targeting of the light-harvesting chlorophyll proteins. Plant J 29: 531–543

IUPAC Compendium of Chemical Technology, 2nd Edition (1997) http://www.iupac.org/goldbook/O04339.pdf (August 15, 2005)

Jansson S (1999) A guide to the *Lhc* genes and their relatives in *Arabidopsis*. Trends Plant Sci 4: 236–240

Jeffrey SW (1969) Properties of two spectrally different components in chlorophyll *c* preparations. Biochim Biophys Acta 177: 456–467

Jeffrey SW and Wright SW (1987) A new spectrally distinct component in preparations of chlorophyll *c* from the micro-alga *Emiliania huxleyi* (Prymnesiophyceae). Biochim Biophys Acta 894: 189–188

Joyard J, Teyssier E, Miège C, Berny-Seigneurin D, Maréchal E, Block MA, Dorne A-J, Rolland N, Ajlani G and Douce R (1998) The biochemical machinery of plastid envelope membranes. Plant Physiol 118: 715–723

Karukstis KK (1991) Chlorophyll fluorescence as a physiological probe of the photosynthetic apparatus. In: Scheer H (ed) Chlorophylls, pp 769–795. CRC Press, Boca Raton

Kleima FJ, Hobe S, Calkoen F, Urbanus ML, Peterman EJG, Van Grondelle R, Paulsen H and Van Amerongen H (1999) Decreasing the chlorophyll *a/b* ratio in reconstituted LHCII: structural and functional consequences. Biochemistry 38: 6587–6596

Knox RS and Spring BQ (2003) Dipole strengths in the chlorophylls. Photochem Photobiol 77: 497–501

Kohorn BD (1990) Replacement of histidines of light harvesting chlorophyll *a/b* binding protein II disrupts chlorophyll-protein complex assembly. Plant Physiol 93: 339–342

Kolossov VL and Rebeiz CA (2001) Chloroplast biogenesis 84: solubilization and partial purification of membrane-bound [4-vinyl] chlorophyllide *a* reductase from etiolated barley leaves. Anal Biochem 295: 214–219

Krause GH and Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annu Rev Plant Physiol Plant Mol Biol 42: 313–349

Kroll D, Meierhoff K, Bechtold N, Kinoshita M, Westphal S, Vothknecht UC, Soll J and Westhoff P (2001) *VIPP1*, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. Proc Natl Acad Sci USA 98: 4238–4242

Kühlbrandt W, Wang DN and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Ledford HK and Niyogi KK (2005) Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell Environ 28: 1037–1045

Linnanto J and Korppi-Tommola J (2001) Spectroscopic properties of Mg-chlorin, Mg-bacteriochlorin, and bacteriochlorophylls *a, b, c, d, e, f, g,* and *h* studied by semiempirical and ab initio MO/CI methods. J Phys Chem A 105: 3855–3866

Linnanto J and Korppi-Tommola J (2004) Semiempirical PM5 molecular orbital study on chlorophylls and bacteriochlorophylls: comparison of semiempirical, ab initio, and density functional results. J Comput Chem 25: 123–137

Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X and Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. Nature 428: 287–292

Maloney MA, Hoober JK and Marks DB (1989) Kinetics of chlorophyll accumulation and formation of chlorophyll-protein complexes during greening of *Chlamydomonas reinhardtii y*-1 at 38°C. Plant Physiol 91: 1100–1106

Marks DB, Keller BJ and Hoober JK (1985) *In vitro* processing of precursors of thylakoid membrane proteins of *Chlamydomonas reinhardtii* y-1. Plant Physiol 79: 108–113

McFadden GI (2001) Primary and secondary endosymbiosis and the origin of plastids. J Phycol 37: 951–959

Miller DJ, Catmull J, Puskeiler R, Tweedale H, Sharples FP and Hiller RG (2005) Reconstitution of the peridinin-chlorophyll *a* protein (PCP): evidence for functional flexibility in chlorophyll binding. Photosynth Res 86: 229–240

Miyashita H, Adachi K, Kurano N, Ikemoto H, Chihara M and Miyachi S (1997) Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll *d* as the major chlorophyll. Plant Cell Physiol 38: 274–281

Morré DJ, Selldén G, Sundqvist C and Sandelius AS (1991) Stromal low temperature compartment derived from the inner membrane of the chloroplast envelope. Plant Physiol 97: 1558–1564

Nagata N, Tanaka R, Satoh S and Tanaka A (2005) Identification of a vinyl reductase gene for chlorophyll synthesis in *Arabidopsis thaliana* and implications for the evolution of Prochlorococcus species. Plant Cell 17: 233–240

Nishigaki A, Ohshima S, Nakayama K, Okada M and Nagashima U (2001) Application of molecular orbital calculations to interpret the chlorophyll spectral forms of pea photosystem II. Photochem Photobiol 73: 245–248

Noy D, Yerushalmi R, Brumfeld V, Ashur I, Scheer H, Baldridge KK and Scherz A (2000) Optical absorption and computational studies of [Ni]-bacteriochlorophyll-*a.* New insight into charge distribution between metal and ligands. J Am Chem Soc 122: 3937–3944

Oba T and Tamiaki H (2002) Which side of the π-macrocycle plane of (bacterio)chlorophylls is favored for binding of the fifth ligand? Photosynth Res 74: 1–10

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll a Fluorescence. A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19. Springer, Dordrecht

Park H and Hoober JK (1997) Chlorophyll synthesis modulates retention of apoproteins of light-harvesting complex II by the chloroplast in *Chlamydomonas reinhardtii.* Physiol Plant 101: 135–142

Park H, Kreunen SS, Cuttriss AJ, DellaPenna D and Pogson BJ (2002) Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. Plant Cell 14: 321–332

Pascal A, Caffarri S, Croce R, Sandonà D, Bassi R and Robert B (2002) A structural investigation of the central chlorophyll *a* binding sites in the minor photosystem II antenna protein, Lhcb4. Biochemistry 41: 2305–2310

Phillips JN (1963) Physico-chemical properties of porphyrins. In: Florkin M and Stotz EH (eds) Comprehensive Biochemistry, Vol 9, pp 34–72. Elsevier, Amsterdam

Porra RJ (1991) Recent advances and re-assessments in chlorophyll extraction and assay procedures for terrestrial, aquatic, and marine organisms, including recalcitrant algae. In: Scheer H (ed) Chlorophylls, pp 31–57. CRC Press, Boca Raton

Porra RJ (1997) Recent progress in porphyrin and chlorophyll biosynthesis. Photochem Photobiol 65: 492–516

Rabinowitch E and Govindjee (1969) Photosynthesis. John Wiley & Sons, New York

Reinbothe C, Bartsch S, Eggink LL, Hoober JK, Brusslan J, Andrade-Paz R, Monnet J and Reinbothe S (2006) A role for chlorophyllide *a* oxygenase in the regulated import and stabilization of light-harvesting chlorophyll *a/b* proteins. Proc Natl Acad Sci USA 103: 4777–4782

Reinsberg D, Ottmann K, Booth PJ and Paulsen H (2001) Effects of chlorophyll *a*, chlorophyll *b,* and xanthophylls on the *in vitro* assembly kinetics of the major light-harvesting chlorophyll *a/b* complex, LHCIIb. J Mol Biol 308: 59–67

Remelli R, Varotto C, Sandonà D. Croce R and Bassi R (1999) Chlorophyll binding to monomeric light-harvesting complex: a mutational analysis of chromophore-binding residues. J Biol Chem 274: 33510–33521

Rochaix JD, Goldschmidt-Clermont M and Merchant S (eds) (1998) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7. Kluwer Academic Publishers, Dordrecht

Sauer K (1975) Primary events and the trapping of energy. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 115–181. Academic Press, New York

Sauer K, Smith JRL and Schultz AJ (1966) The dimerization of chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll in solution. J Am Chem Soc 88: 2681–2688

Scheer H (1991) Structure and occurrence of chlorophylls. In: Scheer H (ed) Chlorophylls, pp 3–30. CRC Press, Boca Raton

Schreiber U, Bilger W, Hormann H and Neubauer C (1998) Chlorophyll fluorescence as a diagnostic tool: basics and some aspects of practical relevance. In: Raghavendra AS (ed) Photosynthesis: A Comprehensive Treatise, pp 320–336. Cambridge University Press, Cambridge

Schünemann D (2003) Structure and function of the chloroplast signal recognition particle. Curr Genet 44: 295–304

Serlin R, Chow HC and Strouse CE (1975) The crystal and molecular structure of ethyl chlorophyllide *b* dihydrate at -153. J Am Chem Soc 97: 7237–7242

Simonetto R, Crimi M, Sandonà, Croce R, Cinque G, Breton J and Bassi R (1999) Orientation of chlorophyll transition moments in the higher-plant light-harvesting complex CP29. Biochemistry 38: 12974–12983

Smith KM (1975) General features of the structure and chemistry of porphyrin compounds. In: Smith KM (ed) Porphyrins and Metalloporphyrins, pp 1–28. Elsevier Scientific, Amsterdam

Snow CD, Sorin EJ, Rhee YM and Pande VS (2005) How well can simulation predict protein folding kinetics and thermodynamics? Annu Rev Biophys Biomol Struct 34: 43–69

Soll J and Schleiff E (2004) Protein import into chloroplasts. Nature Rev Mol Cell Biol 5: 198–208

Spackman MA (1992) Molecular electric moments from X-ray diffraction data. Chem Rev 92: 1769–1797

Srivastava A, Strasser RJ and Govindjee (1999) Greening of peas: Parallel measurements of 77K emission spectra, OJIP chlorophyll *a* fluorescence transient, period four oscillation of the initial fluorescence level, delayed light emission, and P700. Photosynthetica 37: 365–392

Stauber EJ and Hippler M (2004) *Chlamydomonas reinhardtii* proteomics. Plant Physiol Biochem 42: 989–1001

Stauber EJ, Fink A, Markert C, Kruse O, Johanningmeier U and Hippler M (2003) Proteomics of *Chlamydomonas reinhardtii* light-harvesting proteins. Eukaryot Cell 2: 978–994

Stoebe B and Maier UG (2002) One, two, three: nature's tool box for building plastids. Protoplasma 219: 123–130

Strasser RJ, Srivastava A and Govindjee (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochem Photobiol 61: 32–42

Strasser RJ, Tsimilli-Michael M and Srivastava A (2004) Analysis of the chlorophyll *a* fluorescence transient. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 321–362. Springer, Dordrecht

Sulli C, Fang ZW, Muchhal U and Schwartzbach SD (1999) Topology of *Euglena* chloroplast protein precursors within endoplasmic reticulum to Golgi to chloroplast transport vesicles. J Biol Chem 274: 457–463

Sundholm D (2003) A density-functional-theory study of bacteriochlorophyll *b*. Phys Chem Chem Phys 5: 4265–4271

Tamiaki H, Yagai S and Miyatake T (1998) Synthetic zinc tetrapyrroles complex with pyridine as a single axial ligand. Bioorg Med Chem 6: 2171–2178

Thiel C (1999) http://www.physics.montana.edu/students/thiel/docs/Osc.pdf (August 16, 2011)

Tomitani A, Okada K, Miyashita H, Matthijs HCP, Ohno T and Tanaka A (1999) Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400: 159–162

Trissl HW and Wilhelm C (1993) Why do thylakoid membranes from higher plants form grana stacks? Trends Biochem Sci 18: 415–419

Umetsu M, Wang ZY, Kobayashi M and Nozawa T (1999) Interaction of photosynthetic pigments with various organic solvents. Magnetic circular dichroism approach and application to chlorosomes. Biochim Biophys Acta 1410: 19–31

Van Dooren GG, Schwartzbach SD, Osafune T and McFadden GI (2001) Translocation of proteins across the multiple membranes of complex plastids. Biochim Biophys Acta 1541: 34–53

Van Grondelle R and Gobets B (2004) Transfer and trapping of excitation in plant photosystems. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 107–132. Springer, Dordrecht

Van Zandvoort MAM, Wróbel D, Lettinga P, Van Ginkel G and Levine YK (1995) The orientation of the transition dipole moments of chlorophyll *a* and pheophytin *a* in their molecular frame. Photochem Photobiol 62: 299–308

von Wettstein D (1959) The effect of genetic factors on the submicroscopic structures of the chloroplast. J Ultrastruct Res 3: 235–237

von Wettstein D (2001) Discovery of a protein required for photosynthetic membrane assembly. Proc Natl Acad Sci USA 98: 3633–3635

Vothknecht U and Soll J (2006) Protein import into chloroplasts: who, when, and how? In: Wise RR and Hoober JK (eds) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23, pp 53–74. Springer, Dordrecht

Westphal S, Soll J and Vothknecht UC (2001) A vesicle transport system inside of chloroplasts. FEBS Lett 506: 257–261

Westphal S, Soll J and Vothknecht UC (2003) Evolution of chloroplast vesicle transport. Plant Cell Physiol 44: 217–222

White RA and Hoober JK (1994) Biogenesis of thylakoid membranes in *Chlamydomonas reinhardtii y*1: a kinetic study of initial greening. Plant Physiol 106: 583–590

White RA, Wolfe GR, Komine Y and Hoober JK (1996) Localization of light-harvesting complex apoproteins in the chloroplast and cytoplasm during greening of *Chlamydomonas reinhardtii* at 38°C. Photosynth Res 47: 267–280

Wise RR and Hoober JK (eds) (2006) The Structure and Function of Plastids, Advances in Photosynthesis and Respiration, Vol 23. Springer, Dordrecht

Wu P and Brand L (1994) Resonance energy transfer: methods and applications. Anal Biochem 218: 1–13

Xu H, Vavilin D and Vermaas W (2001) Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria. Proc Natl Acad Sci USA 98: 14168–14173

Zerges W (2000) Translation in chloroplasts. Biochimie 82: 583–601

Chapter 17

Photosynthetic Water Splitting: Apparatus and Mechanism

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Summary

This chapter reviews our current knowledge on the reactions leading to light-induced water splitting into molecular oxygen and hydrogen, bound in the form of plastoquinol. This process takes place in the multimeric protein complex of Photosystem II (PS II). After a brief description of the basic principles of biological solar energy exploitation through photosynthesis, general features of the kinetics, energetics and the structural array and nature of the cofactors of PS II are presented. The overall reaction pattern of the water:plastoquinone oxidoreductase comprises three types of sequences: (a) light-induced charge separation leading to the "stabilized" radical pair $P680^{+\bullet}Q_A^{-\bullet}$; (b) oxidative water splitting into molecular oxygen and four protons released into the lumen, driven by $P680^{+\bullet}$ as oxidant and with tyrosine Y_Z involved as intermediate, and (c) two-step reduction of plastoquinone to quinol under uptake of two protons from the cytoplasm/stroma by the use of $Q_A^{-\bullet}$ as reductant. Evidence is presented that within the

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(Chl a)$_4$ Pheo$_x$ (x = 0, 1 or 2) unit, which constitutes the photoactive pigment P680, the lowest singlet exciton state predominantly located on Chl a_{D1} acts as electron donor for the primary charge separation and that the exceptionally high reduction potential of P680 mainly originates from a specific hydrophobic protein microenvironment. The key step of oxidative water splitting is the O-O bond formation which is shown to occur most likely at the level of a binuclearly complexed peroxide. It is emphasized that the protein matrix plays a key role in tuning the energetics and kinetics. Examples are presented for effects of protein relaxation and flexibility on the reaction pattern of PS II.

I. Introduction

Biological organisms are open systems far away from the thermodynamic equilibrium by an estimated gap of 20–25 kJ mol^{-1} (Morovitz 1978). Therefore Gibbs free energy fluxes are an indispensable prerequisite for the development and sustenance of living matter. The unique Gibbs free energy source for life on Earth is the electromagnetic radiation in the near UV, visible and near infrared regions, which is emitted from the Sun as a huge extraterrestrial power station (for a description of this fundamental Gibbs free energy transformation process within the framework of irreversible thermodynamics, see Bell and Gudkov 1992; Walz 1997). The annual energy input form the Sun on our planet is estimated to be about 5×10^{21} kJ mol^{-1} (Zamaraev and Parmon, 1980). Almost half of the solar radiation is either reflected by the atmosphere or absorbed by it, thus giving rise to storms and water movement in the oceans. The other half irradiates the surfaces on Earth (oceans and land at a ratio of 2–2.5). Only a very small fraction of less than 2.5×10^{18} kJ mol^{-1}, i.e., <1% of this solar radiation is eventually transformed via photosynthesis into chemical Gibbs free energy. In the overall balance the average temperature on Earth remains virtually constant due to the energy release as black body radiation with a maximum of the wavelength distribution in the far infrared (at about 10 mm). The Gibbs free energy content of the total reserves of fossil fuels is estimated to be about 4×10^{19} kJ (Zamaraev and Parmon, 1980), which corresponds to less than the energy of the solar radiation reaching the Earth's surface every year. This comparison nicely illustrates that sufficient radiation energy is available to satisfy the increasing populations and demands of mankind's civilization provided that suitable devices become available to perform efficient solar energy exploitation. With this challenge in mind it is important to analyse the structure and mechanism of the photosynthetic apparatus not only as a fascinating scientific problem but also to unravel nature's masterpiece for exploitation of solar radiation as a unique Gibbs free energy source in order to use it as a blue print for constructing efficient biomimetic systems of practical application.

Abbreviations: Ant – antenna complexes of PS II; BChl, Bphe – bacteriochlorophyll, bacteriopheophytin; Chl – chlorophyll; Chl$_{D1}$, Chl$_{D2}$ – "monomeric chlorophylls" of the D1- and D2-branches, respectively of the RC; CP – chlorophyll-binding proteins; CP43, CP47 – core antenna subunits of PS II; Cyt b_{559} – cytochrome b_{559}; Cyt b_6f – cytochrome b_6f complex; DFT – density functional theory; EET – excitation energy transfer; E$_m$ – midpoint redox potential; ENDOR – electron nuclear double resonance; ERPE – exciton-radical pair equilibrium; EPR – electron paramagnetic resonance; ESSEM – Electron spin echo envelope modulation; ETC – electron transfer chain; EXAFS – extended X-ray absorption fine structure; FTIR – Fourier Transform Infrared; LHCII – light-harvesting complex II; $M_jL_kW_l$ – detailed symbol for redox states S_i of the WOC; MLS – EPR multiline signal for the S_2 of the WOC; NET – nonadiabatic electron transfer; NHFe – non-heme iron center; PS II – Photosystem II; PS II CC – Photosystem II core complex; PBRC – RC of purple bacteria; PG – phosphatidylglycerol; $P680^{+\bullet}$ – oxidized state of the RC pigments; P_{D1}, P_{D2} – "special pair" chlorophylls of the D1- and D2-branches, respectively, of the RC; Pheo$_{D1}$, Pheo$_{D2}$ – pheophytins of the D1- and D2-branches, respectively, of the RC; PQ – plastoquinone; Q_A, Q_B – plastoquinones of the RC; QENS – quasielastic neutron scattering; RC – reaction center; RC-PC – reaction center pigment complex; ^{1}(RC - PC)*, ^{1}P680* – excited singlet state of RC pigments; WOC – water oxidizing complex; Y_Z, Y_D – redox active tyrosines of the D1- and D2-branches, respectively; XANES – X-ray absorption near edge spectroscopy

II. Photosynthetic Solar Energy Exploitation by Light-Induced Water Cleavage

The key point of large-scale light transformation into Gibbs free energy is an apparatus able to use substrates that are both available in global abundance and suitable for light-induced synthesis of stable products with a higher Gibbs free energy content. According to Van Niel's fundamental concept (Van Niel, 1941) the photosynthetic process can be described by the overall reaction

$$X\mathbf{H_2} + T \xrightarrow{h\nu} T\mathbf{H_2} + X \qquad (17.1)$$

where XH_2 is an available hydrogen donor and T a suitable hydrogen acceptor with $G^{\circ}(TH_2) > G^{\circ}(XH_2)$. Early photosynthetic organisms used hydrogen (electron) donors XH_2 with a comparatively low Gibbs energy gap $\Delta G^{\circ}(H_2X/X)$ and of limited abundance. During evolution the specific capacity $\Delta G^{\circ}(H_2X/X)$ increased up to the highest possible value of systems existing in an aqueous environment $\Delta G^{\circ}(H_2O/O_2)$. The cornerstone of this development was the "invention" of a biomolecular device, which enables light-induced water splitting into molecular oxygen and metabolically bound hydrogen. This event occurred about three billion years ago at the level of cyanobacteria (Buick, 1992; de Marais, 2000; Xiong and Bauer, 2002) and had two consequences of paramount importance: (a) the huge water pool on the Earth's surface became available as a hydrogen source for the biosphere and (b) the release of oxygen as a "waste" product of photosynthetic water splitting led to the present day aerobic atmosphere (Kasting and Seifert, 2002; Lane, 2003) thus opening the road for a much more efficient (exceeding a factor of 10) exploitation of the Gibbs free energy content of food through the aerobic respiration of heterotrophic organisms (for thermodynamic considerations, see Renger, 1983). Accordingly, the "invention" of a machinery for light-induced water splitting that resulted in the formation of an aerobic atmosphere simultaneously established the bioenergetic prerequisite for the development of all higher forms of life on Earth. Furthermore, the molecular oxygen led to generation of the stratospheric ozone layer, which is the indispensable protective "umbrella" against deleterious UV-B irradiation. For a review, see Vass and Aro (2008).

The hierarchy of structural and functional organization of photosynthesis in higher plants is illustrated in Fig. 17.1. Within the photosynthesizing cells of a leaf the apparatus is localized in special organelles, the chloroplasts which are the evolutionary result of symbiotic uptake of cyanobacteria by ancestor eukaryotic cells (Gray, 1992; McFadden, 1999; Larkum, 2008). Chloroplasts contain a membrane system forming vesicular structures, termed thylakoids, where the key processes take place that lead to light-induced transformation into electrochemical Gibbs free energy. This reaction sequence, which is characteristic for all oxygen-evolving organisms, and often referred to as the "primary processes of photosynthesis", can be summarized by the overall reaction:

$$2NADP^+ + 2\mathbf{H_2O} + 3ADP + P_i \xrightarrow{8h\nu} 2NADP\mathbf{H} + \mathbf{O_2} + 3ATP + 2H^+ \qquad (17.2)$$

where P_i is inorganic phosphate (for the sake of clarity the protonation states of $P_i = HPO_4^{2-}$, $ADP = ADP^{3-}$ and $ATP = ATP^{4-}$ at physiological pH and the consumption of protons and formation of H_2O in ATP synthesis are omitted). The products of this process, NADPH and ATP, subsequently lead via a sequence of enzyme catalysed "dark" reactions in the stroma, referred to as the Calvin-Benson cycle (see Calvin 1989; Benson, 2002 and Chapter 26), to CO_2 fixation (see left side of the lower part of Fig. 17.1).

The right side of the lower part of Fig. 17.1 schematically illustrates the general shape of the pigment/cofactor complexes that perform the process of Eq. 17.2 and the array of these units in the thylakoid membrane. The overall sequence is energetically driven by the light-induced charge separation within Photosystem I (PS I) and Photosystem II (PS II) that act in series, functionally connected via the cytochrome b_6f complex (Cyt b_6f complex) and the mobile carriers PQ/PQH_2 and plastocyanin (PC) (in cyanobacteria

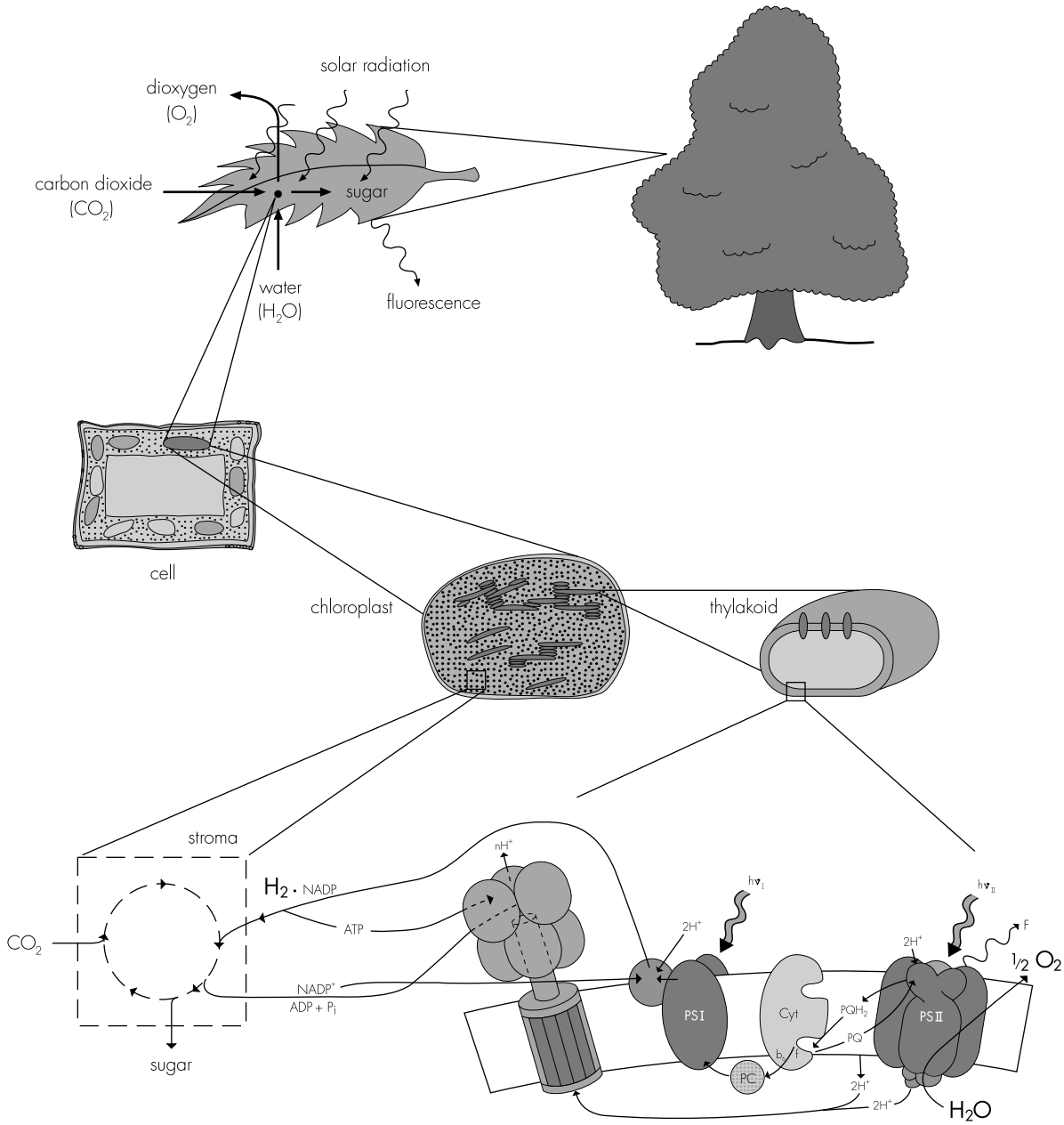

Fig. 17.1. Hierachy of the structural and functional organization of photosynthesis in higher plants (for details, see text). PS I, Photosystem I; PS II, Photosystem II; Cyt b_6f, cytochrome b_6f complex, and PQ, plastoquinone. The ATP synthase on the left side of the membrane section is not explicitly labeled.

PC is replaced by cytochrome c_6). The pigment-protein complexes PS I and PS II and the Cyt b_6f complex are anisotropically incorporated into the thylakoid membrane so that the light-induced electron transfer in these units is vectorial with respect to the normal of the membrane and the reductive and oxidative pathways are spatially separated thus giving rise to formation of a transmembrane electrochemical potential difference of protons[1]. According to the theory of Peter Mitchell (Mitchell, 1961), recognized by the Nobel prize in 1978, the Gibbs free energy transiently stored in the form of a p.m.f (proton motive force or proton electrochemical potential) provides the driving force for ATP synthesis via vectorial proton transfer through the ATP synthase complex (for reviews, see Witt, 1971; Junge and Jackson, 1982; Junge, 2008) (see Chapter 22).

It must be emphasized that the overall reaction described by Eq.17.2 represents the linear electron transport chain (ETC), which gives rise to a NADPH/ATP stoichiometry of 1:1.5. This ratio can be increased by "switching on" cyclic electron transport pathways that are of physiological relevance (Munekage et al., 2004; Havaux et al., 2005; Joliot and Joliot, 2005).

This brief description reveals that the key steps of photosynthetic water splitting take place in PS II and therefore the following description will be entirely restricted to the structure and functional mechanism of this complex.

III. Water Splitting in Photosystem II

PS II is a multimeric pigment-protein complex, which acts as a water:plastoquinone-oxidoreductase:

$$2\mathrm{PQ} + 2\mathbf{H_2O} \xrightarrow{4h\nu} 2\mathrm{PQ}\mathbf{H_2} + \mathbf{O_2} \qquad (17.3)$$

The overall process of Eq.17.3 comprises three types of reaction sequences (for a review see Renger (1999)):

(a) light-induced "stable" charge separation

$$\mathrm{Ant} \bullet \mathrm{P680PheoQ_A} \xrightarrow{h\nu} {}^1[\mathrm{Ant} \bullet \mathrm{P680}]^* \mathrm{PheoQ_A} \rightarrow \mathrm{Ant} \bullet \mathrm{P680^{+\bullet}Pheo^{-\bullet}Q_A} \rightarrow \mathrm{Ant} \bullet \mathrm{P680^{+\bullet}PheoQ_A^{-\bullet}} \qquad (17.4)$$

(b) oxidative water splitting

$$4 \oplus + 2\mathrm{H_2O} \rightarrow \mathrm{O_2} + 4\mathrm{H^+_{Lumen}} \qquad (17.5)$$

(c) plastoquinol formation

$$4 \ominus + 4\mathrm{H^+_{Stroma}} + 2\mathrm{PQ} \rightarrow 2\mathrm{PQH_2} \qquad (17.6)$$

where Ant denotes the antenna pigments which transfer their excitation energy to the photoactive pigment P680 where an electron is ejected from its lowest excited electronic singlet state $^1\mathrm{P680}^*$ and accepted by pheophytin *a* (Pheo). This crucial step of light transformation into electrochemical Gibbs free energy leads to the formation of the primary cation-anion radical pair $\mathrm{P680^{+\bullet}Pheo^{-\bullet}}$. A subsequent rapid electron transfer from $\mathrm{Pheo^{-\bullet}}$ to the secondary acceptor $\mathrm{Q_A}$ is required for a sufficient stabilization of the light-induced charge separation. The symbols $\oplus$ and $\ominus$ represent redox equivalents (including the cofactors $\mathrm{P680^{+\bullet}}$ and $\mathrm{Q_A}$ involved, see Sections III.C and III.D) that give rise to oxidative water splitting (sequence (b)) and plastoquinol formation (sequence (c)), respectively; $\mathrm{H^+_{Lumen}}$ and $\mathrm{H^+_{Stroma}}$ symbolize proton release into the thylakoid lumen and uptake from the stroma, respectively.

In the following sections the structure of the PS II complex and the mechanisms of the three reaction sequences (17.4)–(17.6) will be discussed.

A. Structure of PS II and Cofactor Arrangement

The whole PS II is the composite of a core complex (PS II CC) and associated pigment-protein units, which comprise the peripheral/proximal antennas. Compared to the reaction centers of

[1]The term "proton gradient" is often used in an incorrect manner. A gradient of protons, by definition, is $\nabla[\mathrm{H^+}(\vec{r})]$ where ∇ is a vector operator (derivative to space coordinates) and $[\mathrm{H^+}(\vec{r})]$ the scalar field of the local proton concentration at position $\vec{r}$. In fact the vector $\nabla[\mathrm{H^+}(\vec{r})]$ is the driving force for a vectorial process; however, the actual value of this space dependent quantity is very difficult to determine in biological membrane systems and requires detailed calculations. Therefore only the difference of the trans-membrane electrochemical potential of protons $\Delta\bar{\mu}[\mathrm{H^+}]$ is known and this parameter should not be misleadingly referred to as "proton gradient."

Table 17.1. Cofactor and lipid stoichiometry of purified dimeric PS II core complexes (PS II CC) from *T. elongatus* and redissolved crystals

	Dimeric PS II CC before crystallization	Redissolved PS II CC crystals
Chl*a*[a]	34 ± 2.1 (8)	34 ± 2.2 (3)
Car[b]	9.1 ± 1 (10)	9.1 ± 0.8 (5)
PQ9[a]	2.9 ± 0.8 (6)	2.2 ± 0.2 (3)
Mn[b]	3.8 ± 0.5 (4)	3.6 ± 0.5 (4)
Lipids[b]	10 ± 4 (6)	nd
βDM[b]	110 ± 20 (6)	nd

The numbers of independent experiments are given in parentheses

[a,b] Values are normalized per 2 Pheo *a* and 36 Chl *a*, respectively; βDM: n-dodecyl β-D-maltoside

nd: not determined due to the high amount of protein required for measurement (data from Kern et al., 2005)

anoxygenic bacteria and PS I, the PS II core is characterized by a unique and surprisingly complex polypeptide pattern, which consists of at least 20 subunits (Shi and Schröder, 2004). This feature remained nearly invariant to evolutionary development of all oxygen-evolving organisms (Raymond and Blankenship, 2004). In marked contrast to the highly conserved PS II CC the associated proximal/peripheral antenna systems drastically changed during evolution from cyanobacteria to higher plants (for reviews, see Larkum, 2008; Mimuro et al., 2008; Van Amerongen and Croce, 2008). In vivo, PS II exists predominantly as dimers in the thylakoid membrane of cyanobacteria, algae and higher plants (Peter and Thornber, 1991; Boekema et al., 1995; Bibby et al., 2003). The reactions of Eq. 17.3 take place in PS II CC and therefore only this part of PS II will be briefly described.

PS II CC retaining full water-cleavage activity can be isolated in purified form from both cyanobacteria (Sugiura and Inoue, 1999; Kern et al., 2005) and higher plants (Franzen et al., 1986; Haag et al., 1990). The cofactor and lipid composition of highly purified dimeric PS II CC from the thermophilic cyanobacterium *Thermosynechococcus elongatus* is summarized in Table 17.1 (Kern et al., 2005). Similar values were obtained for PS II CC from higher plants after correction for the "contamination" by the antenna protein CP 29 (Irrgang et al., 1998). High resolution X-ray diffraction crystallography data revealed a higher content of 20 and 25 lipids per PS II core in *T. vulcanus* (Umena et al., 2011) and *T. elongatus* (Guskov et al., 2010), respectively.

The pigments and cofactors of PS II CC are bound to six subunits: D1, D2, CP43, CP47 and PsbE and PsbF of cytochrome b_{559} (Cyt b_{559}). In all photosynthetic reaction centers the cofactors that perform the light-induced stable charge separation are incorporated into a dimeric protein matrix consisting of polypeptides D1 and D2 that not only binds P680, Pheo and Q_A but also houses the sites for oxidative water splitting (see Eq. 17.5 and Section III.C) and PQH_2 formation (see Eq. 17.6 and Section III.D). CP43 and CP47 contain chlorophyll (Chl) *a* and carotenoids and act both as core antenna and provide an indispensable structural element (loop E) in the assembly of the water-oxidizing complex (WOC) (Bricker and Frankel, 2002; Eaton-Rye and Putnam-Evans, 2005). Additionally, the two subunits PsbE and PsbF bind the heme group of Cyt b_{559} which exhibits an unusual high potential form among the b-type cytochromes (Stewart and Brudvig, 1998; Kaminskaya et al., 1999, 2005). A deeper mechanistic understanding of the reaction sequences (17.4)–(17.6) requires detailed knowledge on the spatial arrangement of the cofactors and their environment.

During the last decade enormous progress has been achieved in unravelling the structure of PS II by X-ray diffraction crystallography. Structure models at resolution of 2.9–3.8 Å were presented for PS II CC from the thermophilic cyanobacteria *T. elongatus* (Zouni et al., 2001; Ferreira et al., 2004; Biesiadka et al., 2004; Loll et al., 2005; Zouni, 2008; Guskov et al. 2010) and *T. vulcanus* (Kamiya and Shen, 2003). A key step towards a structure with significantly higher resolution of PS II was achieved by Jian-Ren Shen and coworkers due to minimizing the radiation damage effects and increasing the X-ray diffraction crystallography data resolution to 1.9 Å (Umena et al., 2011). Less detailed information is available for the structure of PS II from higher plants (for a review, see Rhee, 2001). Figure 17.2 shows the array of the cofactors within the D1/D2 heterodimer. In addition to the Chl *a* and Pheo molecules and Q_A that were identified as the cofactors of stable charge separation (see Eq. 17.4.), the tetranuclear manganese cluster of the WOC (see Section III.C.2), the redox active tyrosines (Y_Z and Y_D) and the non-heme iron center (NHFe) are also seen. Furthermore, the D1/D2 matrix contains two β-carotenes (Kobayashi et al., 1990; Tomo et al., 1997; Telfer 2002), which are probably involved in

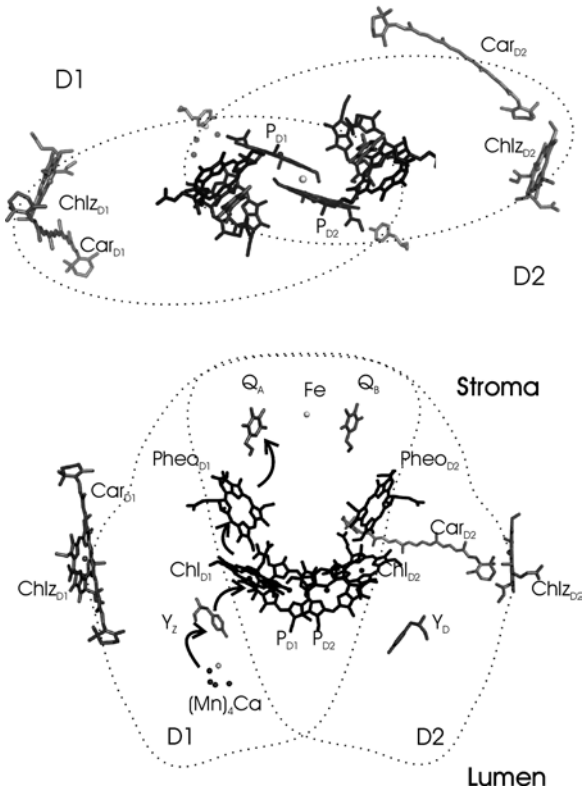

Fig. 17.2. Structural array of the cofactors within the heterodimer of polypeptides D1 and D2. The upper panel presents a top view, the lower panel the side view of the complex. Chl_{D1}, Chl_{D2}, P_{D1} and P_{D2} are the four Chl *a* and $Pheo_{D1}$ and $Pheo_{D2}$ the two Pheo *a* molecules of the reaction center pigments at the core of the photosystem. $Chlz_{D1}$ and $Chlz_{D2}$, the distal Chl *a* molecules; Q_A and Q_B, the primary and secondary plastoquinone acceptors, respectively; $(Mn)_4$, the tetranuclear manganese cluster of the water-oxidizing complex; and Fe, the non heme iron center. The electron transfer steps are symbolized by bent arrows. The dimensions of D1 and D2 are symbolized by dotted contour lines. See text for details.

side reactions of PS II with Cyt b_{559} and Chl *a* (see Faller et al., 2005; Pogson et al., 2005).

An inspection of the arrangement of the eight chlorins reveals a clear structural separation into two peripheral Chls, $Chlz_{D1}$ and $Chlz_{D2}$, and a reaction center pigment complex (RC-PC) consisting of Chl_{D1}, P_{D1}, P_{D2}, Chl_{D2} and the pheophytin *a* molecules $Pheo_{D1}$ and $Pheo_{D2}$. This striking structural feature has important implications for the function of these Chl *a* molecules (see Section III.B.1). The Chl molecules $Chlz_{D1}$ and $Chlz_{D2}$ are separated from RC-PC and do not contribute to formation of P680. Interestingly, no corresponding counterparts of these pigments exist in purple bacterial reaction centers (PBRCs), in contrast to the other six chlorins whose arrangement is strikingly similar to that of the corresponding four bacteriochlorophylls (BChl) and two bacteriopheophytins (BPheo) in the PBRCs (see Lancaster, 2008). Therefore, it seems likely that $Chlz_{D1}$ and $Chlz_{D2}$ are of special functional relevance for PS II. This idea is confirmed by studies on mutants of *Chlamydomanas reinhardtii* in which the axial histidine ligands of $Chlz_{D1}$ (H118 of polypeptide D1) and $Chlz_{D2}$ (H117 of polypeptide D2) were replaced by other amino acid residues (Ruffle et al., 2001). The mutants exhibit significant differences in various properties, inter alia in light-harvesting efficiency and sensitivity to photoinhibition (Ruffle et al., 2001). It was suggested that these two pigments could be constituents of the excitation energy transfer (EET) pathway from the core antenna pigment-protein complexes CP43 and CP47 to P680 (Johnston et al., 2000). However, model calculations based on the PS II CC structure revealed that $Chlz_{D1}$ and $Chlz_{D2}$ probably play only a minor role compared with the direct EET from Chls of CP43 and CP47 to the RC-core (Vasil'ev et al., 2002, 2004; Raszewski and Renger, 2008). Therefore, a role in protection to light stress (Schweitzer et al., 1998; Lince and Vermaas, 1998) appears to be the most likely function of $Chlz_{D1}$ and $Chlz_{D2}$.

B. Light-Induced Charge Separation $P680^{+\bullet}Q_A^{-\bullet}$ Formation

The stepwise light-induced formation of a stable charge separation is the general principle of the RC in all photosynthetic organisms (for a discussion, see Renger and Holzwarth, 2005). The unique property of PS II, however, is the nature of the photoactive pigment P680 and in particular its very high reduction potential. The cation radical $P680^{+\bullet}$ which is one of the strongest oxidants in biological systems is the indispensable prerequisite for oxidative water splitting (see Eq. 17.5). Accordingly, the nature of P680 has been a focus of considerable scientific attention and will be discussed in more detail.

1. Nature and Properties of Cofactors P680, Pheo and Q_A

The four Chl *a* molecules of the RC-PC are potential candidates as constituents of P680. Theoretical

calculations based on the PS II structure at 3.2 Å resolution (Biesiadka et al., 2004) revealed that the four Chl *a* molecules and two Pheos are excitonically coupled with values ranging from about 30 up to 160 cm^{-1} and the strongest coupling was found between the two Chl *a* molecules P_{D1} and P_{D2} (Raszewski et al., 2005). The structural array of P_{D1} and P_{D2} is similar to that of the special pair (see Lancaster, 2008 and references therein) that acts as the photochemically active species for the primary charge separation in PBRCs. Therefore at a first glance it might be attractive to assign P680 to a special pair consisting of P_{D1} and P_{D2}. However, closer inspection reveals that the center-to-center distances between P_{D1} and P_{D2} (8.3 Å) is larger than the distance of 7.6 Å between the two BChl molecules in the special pair of PBRCs. Furthermore, the π-π stacking interaction between the two rings of moieties P_1 and P_2 on the special pair of BPRCs is interrupted between P_{D1} and P_{D2} by an in-plane tilt of one pigment (Raszewski et al., 2008; Renger and Renger, 2008). As a consequence of the larger distance and smaller Q_Y transition dipole moments of Chl compared to BChl, the value of about 160 cm^{-1} (Raszewski et al., 2005) for excitonic coupling between P_{D1} and P_{D2} is lower than values reported for the special pair of PBRCs (see Warshel and Parson, 1987) and the spectral differences between the chlorins of RC-PC in PS II are much less pronounced than the marked difference between the special pair and the four "monomeric" molecules $BChl_A$, $BChl_B$, $BPheo_A$ and $BPheo_B$ in PBRCs (for details see Lancaster, 2008; Parson, 2008). These considerations reveal that for PS II an analogous separation between a "special pair" consisting of P_{D1}/P_{D2} and the remaining four chlorins of the RC-PC is not justified.

Based on a review of the currently available experimental data and theoretical analyses it seems necessary to assign P680 to the pigment cluster of the RC-PC unit $(Chl\ a)_4$ $Pheo_x$ where index x denotes the number of coupled Pheo molecules. The mode of excitonic coupling of $Pheo_{D1}$ and $Pheo_{D2}$ is a matter of controversy, with values of x = 0, 1 or 2 having been reported (for review, see Renger and Holzwarth, 2005). A clear illustration of the close interrelationship among the six pigments of the RC-PC is provided by results on a *Chlamydomonas reinhardtii* mutant where $Pheo_{D2}$ was transformed into a Chl *a* by replacement of Leu 210 in polypeptide D1 by a His residue that acts as a ligand for Mg^{2+} in the center of the chlorine ring. Xiong et al. (2004) found that PS II CC isolated from the D1 – L210H mutant were virtually unable to evolve oxygen (the rate under saturating light was <4% of the value of the wild type) and to photoaccumulate $Q_A^{-\bullet}$. On the other hand, the kinetics of primary charge separation were not substantially altered in isolated D1/D2/Cyt b_{559} preparations[2] from this mutant. These results were interpreted by the assumption that the distribution of the exited singlet state energy in ^{1}(RC-PC)* of the mutant differs from that in wild type, thus giving rise to a dissipative decay via fluorescence at the expense of charge separations (Xiong et al., 2004). Interestingly, a replacement of the strongly conserved Glu 129 of polypeptide D2 in the nearest neighbourhood of $Pheo_{D2}$ affects the energetics of Q_B (Perrine and Sayre, 2011).

The assignment of P680 to [Chl_{D1}, P_{D1}, P_{D2}, Chl_{D2}, $Pheo_{D1}$, $Pheo_{D2}$] allows that each component of RC-PC contributes to a quite different extent to various states of P680, i.e., the lowest electronically excited singlet state $^1P680^*$, the triplet state $^3P680$ and the cation radical $P680^{+\bullet}$. The molecular orbital (MO) schemes of $^1(RC\text{-}PC)^*$, $^3(RC\text{-}PC)$ and $(RC\text{-}PC)^{+\bullet}$ depend not only on the chemical structures of the pigments themselves, but also on both the pigment-pigment and pigment-protein interactions. Detailed quantum chemical calculations are required to unravel the electronic configuration of these states.

Apart from restrictions by the capacity of quantum chemical and molecular mechanics methods due to the size of the system, these studies were also limited so far by the resolution of the X-ray structure analysis and problems arising from spectral overlap. In spite of these obstacles important information has been obtained which is

[2] D1/D2/Cyt b_{559} preparations were first isolated by Nanba and Satoh (1987) and shown to enable light-induced $P680^{+\bullet}$ $Pheo^{-\bullet}$ formation. This reaction takes place with a high quantum yield (Kurreck et al., 1997a). However, these preparations which are completely deprived of Q_A, Q_B and the NHFe (Kurreck et al., 1997b) cannot stabilize the primary charge separation. Therefore these samples are not an analogue of the isolated reaction centers from anoxygenic purple bacteria (PBRC) (for reviews, see Lancaster, 2008, Parson, 2008) and should not be termed "PS II reaction centers" although this terminology is widely used in the literature.

summarized below (for a review, see also Renger and Renger, 2008).

a. The Electronic State of $^1P680^*$

A serious problem for straightforward spectral analyses of experimental data is the strong overlap of the bands in the Q_Y region (spectral congestions). Numerous attempts have been made to unravel the spectral properties of $^1P680^*$. Most of the studies were performed with D1/D2/Cyt b_{559} preparations (Konermann and Holzwarth, 1996; Germano et al., 2001; Jankowiak et al., 2002; Vacha et al., 2002). In order to cope with the overlapping spectral properties of the eight chlorins in these preparations two different approaches were used for selective modification of individual pigments: (i) site-directed mutagenesis of binding sites and (ii) replacement by other chlorine derivatives.

Based on Fourier Transform Infrared (FTIR) measurements Noguchi et al. (1998) showed that the central Mg^{2+} of P_{D1} and P_{D2} is pentacoordinated, with histidines (D1-His 198 and D2-His 197, respectively, in *Synechocystis* sp. PCC 6803) as axial ligands.

The nature of the electronic state of $^1(RC - PC)^*$ (Raszewski et al., 2005) has been theoretically analysed on the basis of experimental spectral data and the structure of PS II gathered from X-ray diffraction crystallographic analysis at 3.2 Å resolution (Biesiadka et al., 2004) and further refined (Raszewski et al., 2008) by using the structure data of 3.0 Å resolution (Loll et al., 2005). The most striking result of this study is the finding that the lowest exciton state (n = 1) is dominated by Chl_{D1} and not by a "special pair" $P_{D1} - P_{D2}$. This phenomenon clearly contrasts with the situation in PBRCs where the "red-most" Q_Y transition is associated with the lower exciton state of the special pair. In marked contrast the exciton states n = 3 and n = 6 are ascribed to the pair $P_{D1} - P_{D2}$ in PS II and characterized by peaks at 675 and 660 nm, respectively. The second lowest exciton state (n = 2) is dominated by $Pheo_{D2}$, while states n = 4 and n = 5 are mainly due to absorption by $Pheo_{D1}$ and Chl_{D2}, respectively. A complementary theoretical study revealed that mixed exciton states have the tendency to be localized over an average of three chlorins (Barter and Klug, 2005).

An inspection of the calculated data reveals that the strongest coupling exists between P_{D1} and P_{D2} so that these two molecules might resemble a kind of "special pair" within the RC-PC unit although its functional role differs drastically from that of the "special pair" in PBRCs: the "special pair" $P_{D1} - P_{D2}$ in PS II does not act as either the trap of excitation energy or as the primary electron donor (Section III.B.2). Therefore the term "special pair" is highly misleading for P680 and should not be used in this connection.

b. Triplet State $^3P680$

A markedly different situation arises for the electronic configuration of the triplet state $^3P680$. In the case of triplet states the excitonic coupling is vanishingly small due to a spin forbidden transition (Schmidt and Reineker, 1985) and can be ignored. First information on the triplet localization within the state $^3(RC - PC)$ was obtained from orientation studies. The plane of the chlorin ring where the triplet resides was shown to deviate drastically from the normal to the thylakoid membrane (Van Mieghem and Rutherford, 1993) in marked contrast to the parallel orientation of triplet state of the PBRC special pair to the chromatophore membrane (Tiede and Dutton, 1981). An experimental study on *Synechocystis* sp. PCC 6803 mutants (Diner et al., 2001) and theoretical analyses (Raszewski et al., 2005, 2008) led to the conclusion that at low temperatures the triplet state is localized at Chl_{D1}, thus explaining the former results of Van Mieghem and Rutherford (1993). At higher temperatures the triplet state becomes delocalized with an energy gap of the order of 10 meV (Noguchi et al., 1998).

c. Cation Radical $P680^{+\bullet}$

The cation radical $P680^{+\bullet}$ is one of the strongest oxidants in biology and the energetic prerequisite for oxidative water splitting into molecular oxygen and four protons. With an estimated reduction potential of +1.1 to +1.26 V for P680 (Jursinic and Govindjee, 1977; Klimov et al., 1979; Rappaport et al., 2002) the cation radical $P680^{+\bullet}$ exceeds the oxidizing power of the corresponding cation radical of the special pair in all natural PBRCs and of $P700^{+\bullet}$ in PS I by at least 0.5 V.

A comparison with P700 readily shows that the chemical nature of Chl *a* per se is not nature's "clou" for achieving the unique redox properties of P680. It is therefore reasonable to assume that the embedding protein matrix of the RC core plays the key role in establishing the exceptionally high E_m values. Several factors have been discussed as possibly being responsible for this effect: (i) hydrogen bonding; (ii) distance between the Chls P_{D1} and P_{D2}; (iii) planarity of the chlorin ring, and (iv) dielectric environment (for a review, see Renger and Holzwarth, 2005).

An essential step forward in our understanding was achieved on the basis of theoretical analyses. Hasegawa and Noguchi (2005) showed by density functional theory (DFT) calculations that the reduction potential of Chl *a* reaches values of +1.3 V within a hydrophobic environment with a dielectric constant of about 2. On the basis of the structures that were gathered from X-ray diffraction crystallography (see Section III.A) the protein environment of P680 was inferred to be more hydrophobic than that of the "special pair" in PBRCs and of P700 in PS I. These findings provide clear evidence for a low dielectric environment around P680 as a major factor in furnishing Chl *a* species with unusually high reduction potentials (Hasegawa and Noguchi, 2005). Calculations based on an electrostatic continuum model and the PS II structures at 3.5 and 3.2 Å resolution resulted in values of +1.1 to +1.3 V for the four Chls of (RC-PC) while those of $Chlz_{D1}$ and $Chlz_{D2}$ were found to be much lower (about 0.9 V) (Ishikita et al., 2005) and not far from the value of monomeric Chl *a* in solution (Watanabe and Kobayashi, 1991). The calculations also reveal that the essential contributions to the drastic upshift of E_m in RC-PC originates form the D1/D2 protein matrix (about 80% of the total effect) and only to a minor extent from the other subunits.

Regardless of some unresolved details (Renger and Holzwarth, 2005; Renger and Renger, 2008) it is clear that the nature of the dielectric properties of the protein matrix is the key factor for the unusual redox properties of P680. Furthermore, the spin distribution of the electron within RC-PC of the cation radical state $P680^{+\bullet}$ also depends on the details of the microenvironment as is clearly illustrated by comparative FTIR studies on D1/D2/Cyt b_{559} preparations and PS II CCs (Takahashi et al., 2008). For functional reasons the spin density should be high at P_{D1} in order to achieve a directed hole transfer to Y_Z. Theoretical calculations (Raszewski et al., 2008) and experimental data (Takahashi et al., 2008) show this to be the case for PS II CCs. Likewise the distance from the Chls of RC-PC to other redox active groups including the peripheral Chl_{Z1} and Chl_{Z2} must be large enough in order to avoid undesired dissipative and destructive side reactions.

For many years, Chl *a* seemed to be unique among the Chl species in constituting the photochemically active species P680 of PS II and P700 of PS I, whereas the special pair in the RCs of anoxygenic bacteria was shown to contain a variety of BChl derivatives ranging from BChl *a* in *Rhodobacter spheroides* to BChl *b* in *Blastochloris viridis* and BChl *g* in heliobacteria (for reviews on the structure of BChls and the types of anoxygenic bacteria, see Scheer, 2008; and Parson, 2008, respectively). With the discovery of the Chl *d*-containing oxygen-evolving cyanobacterium *Acaryochloris marina* (Miyashita et al., 1997) this unique role of Chl *a* for P680 became a matter of debate. It was shown that the Chl *a*–Chl *a*' heterodimer of P700 (Fromme et al., 2008) is replaced by Chl *d*–Chl *d*' in PS I (P740) of this organism (Akiyama et al., 2002). Questions arise on the nature of P680 in *A. marina*. An unambiguous answer is complicated by the finding that all thylakoid preparations from *A. marina* contain a small amount of Chl *a* (Akiyama et al., 2002). At present different models are discussed for the nature of the four Chl molecules in the (RC-PC) complex (for review, see Renger, 2008) while the primary acceptor seems to be a Pheo *a* (Razeghifard et al., 2005).

With respect to the nature of P680 interesting findings were obtained by constructing mutants through genetic engineering of *Synechocystis* sp. PCC 6803 where Chl *a* and Pheo *a* were partly replaced by Chl *b*/Pheo *b* in the PS II core (Vavilin et al., 2002). These findings reveal that Chl *a* and Pheo *a* are not necessarily the only chlorins that can constitute a functionally competent PS II with intact WOC activity.

d. Properties of Pheo

Figure 17.2 shows that RC-PC contains two Pheo *a* molecules ($Pheo_{D1}$ and $Pheo_{D2}$) that are structurally homologous to the two BPheo molecules

in PBRCs. The most striking feature of the charge separation process of PBRCs is the "unidirectionality" although the array of both BPheo's is virtually symmetric (Bylina et al., 1988; Michel-Beyerle et al., 1988; Zinth and Kaiser, 1993). Only the BPheo in the "active" branch ($BPheo_A$) is involved in the photochemical reaction; the other one ($BPheo_B$) located in the "inactive" branch participates in a charge separation only under special conditions (e.g., in PBRCs from specifically "engineered" mutants, see Laible et al., 2003; Wakeham et al., 2003). An analogous "sideness" of the light-induced electron transfer reactions is assumed to prevail in PS II. In this case the feature of "unidirectionality" is even more pronounced because it not only comprises the light-induced charge separation and PQH_2 formation (see, Section III.D) but also extends to the donor-side reactions involving Y_Z and the WOC (see, Section III.C).

In marked contrast to the drastic upshift of the reduction potential of P680 the modulation of the redox properties of $Pheo^{-\bullet}/Pheo$ is less pronounced. The E_m value of −610 mV determined for PS II (Klimov et al., 1977) is almost the same as that of Pheo *a* in solution (Watanabe and Kobayashi, 1991). However, when taking into account electrostatic effects due to the presence of $Q_A^{-\bullet}$ during the redox titration, the actual "working potential" of $Pheo_{D1}/Pheo_{D1}^{-\bullet}$ is expected to be shifted towards less negative values. An experimental study revealed that this effect is about 85 mV (Gibasiewicz et al., 2001). Therefore, a value of −525 mV seems to be more realistic for the reduction potential of $Pheo_{D1}^{-\bullet}$ in competent functional PS II complexes with oxidized Q_A. This number is in line with recently reported data of redox titrations on PS II CC from thermophilic cyanobacteria (Kato et al., 2009; Allakhverdiev et al., 2010) but it must be emphasized that in these studies the interaction between $Q_A^{-\bullet}$ and $Pheo_{D1}^{-\bullet}$ has also been ignored.

e. Properties of Q_A

In PBRCs, the component Q_A is either a ubiquinone (UQ) (e.g., *Rhodobacter spheroides*) or a menaquinone (MQ) (*Blastochloris viridis*) (see, Parson, 2008 and references therein), whereas Q_A is exclusively plastoquinone-9 (PQ-9) in all PS II complexes analyzed so far. The PQ-9 molecule of Q_A is noncovalently attached to the binding site in the D2 polypeptide.

One striking feature of Q_A in both PBRCs and PS II is its functioning as a one electron redox component under normal conditions, i.e., the UQ (MQ) and PQ-9 attain only the redox states of the quinone and semiquinone forms. A double reduction to the quinol form requires nonphysiological conditions such as continuous illumination in the presence of $Na_2S_2O_4$ (Van Mieghem et al., 1989). Numerous redox titration experiments have been performed over the last four decades to determine the E_m value of $Q_A^{-\bullet}/Q_A$. A collection of data gathered from these measurements shows a clustering of the E_m values around −300, −100, 0 and +50 mV (for illustration, see Fig. 1 of Krieger et al., 1995). For general considerations it must also be taken into account that probably two different E_m forms of $Q_A^{-\bullet}/Q_A$ exist (Bao et al., 2010) and that the energetics of this cofactor in addition depends on the nature of the species (mesophilic, thermophilic cyanobacteria, plants) (Fufezan et al., 2007; Shibamoto et al., 2010).

Values of -60 mV were found for PS II CC from *T. elongatus* (Ido et al., 2011) when the titration was performed in the absence of redox mediators but significantly lower (by about 100 mV) numbers were reported for the same type of sample material in the presence of these chemicals (Shibamoto et al., 2009, 2010). The effect of redox mediators remains to be clarified.

When taking together all available information from the literature a value of -50 to -100 mV seems to be a reasonable benchmark for the reduction potential of $Q_A^{-\bullet}$ in intact PS II complexes.

The redox properties of Q_A are modulated by the protein matrix as is illustrated by an E_m upshift of about 150 mV in samples from higher plants which lack an intact WOC (Krieger et al., 1995). An analogous shift of about 120 mV was observed in PS II complexes from *T. elongatus* (Shibamoto et al., 2009; Ido et al., 2011). This effect is probably of physiological relevance because it diminishes the yield of $^3P680$ formation by favoring the pathway of direct $P680^{+\bullet}Q_A^{-\bullet}$ recombination via tunnelling (Section III.C.3).

The structure of the Q_A pocket has been unravelled by X-ray diffraction crystallography analysis of PS II core complexes from *T. elongatus* at 3.0 Å resolution (Loll et al., 2005). It is formed by residues Ile213, His214, Thr217, Met246, Trp253,

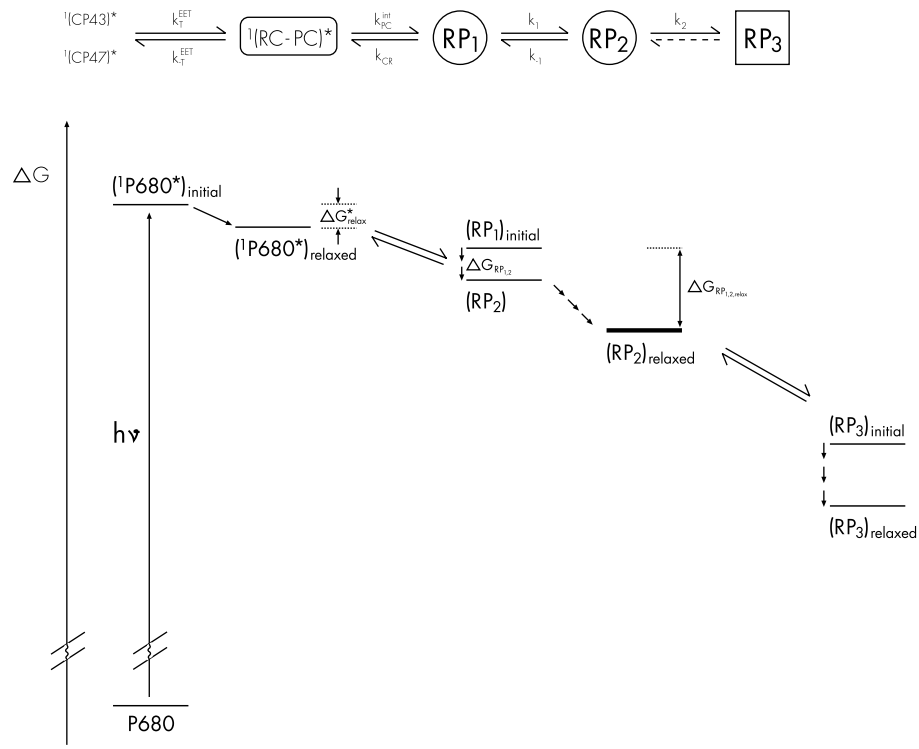

Fig. 17.3. Kinetics (*upper part*) and energetics (*lower part*) of light-induced charge separation in PS II. The Gibbs free energy scale is not linear and interrupted (in order to illustrate effects owing to protein relaxation: for details on the energetics, see text); the rate constants k_T^{EET} and k_{-T}^{EET} describe EET processes from the core antenna to the (RC-PC) unit in the forward and back direction, respectively, k_{PC}^{int} and k_{CR} the primary charge separation and its reversal, respectively, k_1, k_{-1} and k_2 electron transfer steps between the different radical pairs states RP_1, RP_2 and RP_3. Symbols: RP_1 is $P680_1^{+\bullet}Pheo_{D1}^{-\bullet}$; RP_2 is $P680_2^{+\bullet}Pheo_{D1}^{-\bullet}$, and RP_3 is $P680_2^{+\bullet}Pheo_{D1}Q_A^{-\bullet}$.

Ala260, Pheo261 and Leu267 of polypeptide D2 with the quinone ring of PQ-9 being sandwiched between Leu267 and Trp253. Electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopy studies on samples deprived of the paramagnetic high spin NHFe (located between Q_A and the Q_B binding pocket, see Fig. 17.2) by a special treatment (MacMillan et al., 1990; Kurreck et al., 1996) revealed that $Q_A^{-\bullet}$ forms asymmetric hydrogen bonds with the protein matrix (MacMillan et al., 1995a). FTIR measurements confirm that the mode of $Q_A^{-\bullet}$ interaction with the environment in these iron-depleted PS II membrane fragments closely resembles that of the native state (Noguchi et al., 1999). Electron spin echo envelope modulation (ESEEM) studies suggested hydrogen bonding to nitrogens of His and the backbone (MacMillan et al., 1995b). This conclusion is in perfect agreement with the currently available X-ray structure where His214Nε and the backbone NH of Phe261 of polypeptide D2 are shown to be hydrogen bond partners of Q_A (Kern et al., 2005; Kern and Renger, 2007). It must be emphasized that the X-ray diffraction crystallography data probably reflect the structural arrangement of $Q_A^{-\bullet}$ rather than of Q_A due to the effects of X-ray irradiation as is shown for PBRCs (Utschig et al., 2008). Recent ENDOR and Q-Band EPR spectroscopy data reveal that Q_A does not change its orientation upon reduction to $Q_A^{-\bullet}$ (Flores et al., 2010).

2. Mechanism of $P680^{+\bullet}Q_A^{-\bullet}$ Formation

The reaction sequence of light-induced charge separation in PS II is schematically summarized in Fig. 17.3. The process starts with the formation of an electronically excited singlet state by light absorption. Since P680 acts as a shallow trap for

electronic excitation energy from the antenna (Eckert et al., 1987; Schatz et al., 1987, 1988) and since the excitation energy of $^1P680^*$ is distributed over several different molecules (see Section III.B.1.a), the kinetics of $P680^{+\bullet}Pheo^{-\bullet}$ formation reflect a series of equilibration steps. Depending on the rate of the individual EET and electron transfer steps the overall reaction can be kinetically limited either by the EET from the antenna to the trap (a diffusion- or transfer-to-the-trap limited process) or by electron transfer at the trap (a trapping-limited process). It was found that the rate of trapping of the excited singlet state by $P680^{+\bullet}Pheo^{-\bullet}$ formation, gathered from fluorescence lifetime measurements, depends on the antenna size (Schatz et al., 1987, 1988; Roelofs and Holzwarth, 1990; Trissl and Lavergne, 1995; Renger et al., 1995; Barter et al., 2001; Engelmann et al., 2005). This finding could be consistently described within the framework of the "exciton-radical pair equilibrium" (ERPE) model (Schatz et al., 1987, 1988). In this model the measured rate constant for photochemical $P680^{+\bullet}Pheo^{-\bullet}$ formation, k_{PC}^{trap}, is expressed as the product of the intrinsic rate constant, k_{PC}^{inst}, and a constant K_{eq}^* that summarizes all equilibration steps of the excited singlet state between the antenna and P680. The "equilibrium" constant K_{eq}^* is the probability of an excited singlet state in PS II to populate the state $^1P680^*$ from where the primary charge separation takes place. In the ERPE model the rate constant k_{PC}^{trap} is assumed to be proportional to the "spectrally weighted" antenna size (Renger et al., 1995) provided that the EET processes are sufficiently fast to assure excitation energy equilibration. It is a matter of controversial debate as to what extent this equilibration is really achieved (Vasil'ev et al., 2001; Van Amerongen and Dekker, 2003; Engelmann et al., 2005; Renger and Holzwarth, 2005). Progress in PS II structure analysis (Loll et al., 2005) offered a more reliable basis for theoretical studies (Raszewski and Renger, 2008), which led to the conclusion that this condition is not satisfied and the overall reaction sequence be actually kinetically limited by EET from CP43 and CP47 to (RC-PC) where the excited singlet state population equilibration between the pigments is very fast (100–500 fs) leading to $^1(RC\,PC)^*_{equil}$. Interestingly, the EET from the peripheral to the core antenna was inferred to be much faster than from CP43/CP47 to PC-RC (Broess et al., 2008; for a review, see Renger and Renger, 2008). In Fig. 17.3, top, rate constants k_T, k_{-T} describe forward and back EET between the antenna (including CP43 and CP47), symbolized by Ant, and (RC-PC).

The primary charge separation step takes place within RC-PC. According to our current state of knowledge the formation of the radical pair $P680^{+\bullet}Pheo^{-\bullet}$ comprises at least two steps with $^1Chl^*_{D1}$ and $Pheo_{D1}$ (see Fig. 17.2) acting as primary electron donor and acceptor, respectively (for details see Renger and Renger, 2008). Alternative models with Chl_{D1} as primary acceptor and $^1P^*_{D1}$, as primary donor via charge transfer (CT) states have been also proposed (see Shelaev et al., 2011; Novoderezhkin et al., 2011). It cannot be excluded that both types of mechanisms take place, depending on excitation and environmental conditions.

Values of k_T^{EET}, k_{-T}^{EET}, the intrinsic rate constants k_{PC1}^{int} and k_{PC2}^{int} for ET in the forward direction and k_{-PC1}^{int}, k_{-PC2}^{int} for the back direction have been gathered from deconvolution of flash induced absorption changes and fluorescence decay kinetics at high time resolution. However, it must be emphasized that a straightforward and unambiguous interpretation is prevented due to spectral congestion. Therefore quite different sets of rate constants were gathered from very similar experimental data (see Szczepaniak et al., 2009; Van der Weij–de Wit et al., 2011). At present a definite conclusion on the mode of limitation ("transfer to the trap" or "trapping" limited) cannot be presented. Furthermore differences are likely to emerge among the variety of PS II complexes (for a discussion, see Croce and Van Amerongen, 2011).

Regardless of the uncertainties of the rate constants, a closer inspection of the reaction sequence reveals that the mechanisms of photochemical charge separation are strikingly different in PBRCs and PS II. In PBRCs the lower exciton state of the special pair acts both as the trap for excitation energy and as primary donor for electron transfer to the monomeric $BChl_A$, i.e., the reaction sequence is $^1P^*Bchl_ABPheo_A \rightarrow P^{+\bullet}BChl_A^{-\bullet}BPheo_A \rightarrow P^{+\bullet}BChl_ABPheo_A^{-\bullet}$ (where P stands for the special pair). In marked contrast, the Chl_{D1} at a similar spatial position in the D1/D2 heterodimer as the $BChl_A$ in the L/M protein matrix of PBRCs is the

dominant site of the lowest excited state in ^{1}(RC PC)* of PS II (see Section III.B.1.a) and the primary electron donor to $Pheo_{D1}$ (a role of Chl_{D1} as primary acceptor under special conditions, however, cannot be excluded, vide supra). Interestingly, also the monomeric Chl *a* of PS I has been inferred recently to be the primary donor rather than P700 (Holzwarth et al., 2006b; Giera et al., 2010). If this is really the case, then a remarkable difference exists in the mechanism of primary charge separation in the RCs of anoxygenic bacteria (type I and II) and PS I and II of oxygen-evolving photosynthetic organisms. This would lead to interesting ramifications on the functional relevance of this evolutionary change. In fact, the scheme of Fig. 17.3 and Eq. 17.4 are likely to be oversimplifications because an explicit consideration of mixing singlet excited and radical pair states is ignored. It has been predicted by theoretical calculations that 5–10% of charge transfer states are formed directly on photon absorption by (RC-PC) (Barter and Klug, 2005). An energetically low lying charge transfer state is probably responsible for an efficient charge separation in PS II occurring up to excitations in the wavelength region of 700–730 nm (Krausz et al., 2008 and references therein). The implications of this finding for the details of the primary photochemistry in PS II remain to be clarified.

The radical ion pair $P680^{+\bullet}Pheo^{-\bullet}$ has to be "stabilized" by rapid electron transfer from $Pheo_{D1}^{-\bullet}$ to Q_A in order to minimize the probability for loss reaction(s) via dissipative recombination of $P680^{+\bullet}Pheo^{-\bullet}$ (see, Renger and Holzwarth, 2005, and Section III.D). The kinetics of the "stabilization" reaction have been resolved by measurements of flash-induced absorption changes that are characteristic for transient populations of $Pheo^{-\bullet}$ and $Q_A^{-\bullet}$. A comparison of the data reveals that $Pheo^{-\bullet}$ reoxidation (Nuijs et al., 1986) and $Q_A^{-\bullet}$ formation (Eckert et al., 1988; Bernarding et al., 1994) exhibit virtually the same kinetics with lifetimes of (300 ± 100) ps. This time constant, which is confirmed by recent studies (Miloslavina et al., 2006; Holzwarth et al. 2006a; Broess et al., 2008), closely resembles the corresponding values of about 200 ps for the electron transfer from $BPheo_A^{-\bullet}$ to Q_A in PBRCs (Rockley et al., 1975; Kirmeier et al., 1985; Zinth and Kaiser, 1993).

The formation of the radical pair $P680^{+\bullet}Q_A^{-\bullet}$ is indispensable for a sufficiently stable charge separation that provides the driving force for the reaction sequences summarized by Eqs. 17.5 and 17.6. Therefore, only PS II complexes containing Q_A in their oxidized form are functionally competent for efficient trapping of electronically excited states in the form of a useful electrochemical potential difference. PS II complexes in state P680Pheo Q_A are referred to as "open" reaction centers, while PS II is "closed" when lacking Q_A or containing reduced PQ-9 in the form of the semiquinone $Q_A^{-\bullet}$ or the double-reduced quinone Q_AH_2. As a consequence, Q_A acts as a photochemical quencher of Chl *a* fluorescence (Duysens and Sweers, 1963). This property is the basis of widely used fluorometric methods for noninvasive monitoring of the functional state of the photosynthetic apparatus (for a review, see Papageorgiou and Govindjee, 2004).

3. Energetics of $P680^{+\bullet}Q_A^{-\bullet}$ Formation

The levels of the initial and the "final" states determine the overall energetics. The initial state of charge separation in PS II is the lowest electronically excited singlet state formed at P680 after photon absorption and subsequent rapid vibrational relaxation. A Q_Y transition of 680–684 nm corresponds to an energy of 1.83–1.82 eV. According to Lev Krishtalik, the configurational rather than the Gibbs free energy is the key parameter for the energetics of the reactions in PS II complexes (Krishtalik 1986) and entropy contributions owing to excited state equilibration among antenna pigments (Renger et al., 1995) have to be ignored.

The assessment of the energy level of the "final" state of stable charge separation, $P680^{+\bullet}Q_A^{-\bullet}$ is a difficult problem. In principle, redox and reduction potentials are thermodynamic parameters that are defined for equilibrium states. However, the transient radical pair $P680^{+\bullet}Q_A^{-\bullet}$ with its limited lifetime does not attain a true equilibrium. Furthermore, even the values of thermodynamically well-defined redox potentials are not precisely known for both $P680/P680^{+\bullet}$ and $Q_A^{-\bullet}/Q_A$. When using the reported midpoint potentials (see Sections III.B.1.c and III.B.1.e) the Gibbs free energy gap between $^1P680^*Q_A$ and $P680^{+\bullet}Q_A^{-\bullet}$ is estimated to be 0.5–0.6 eV and the efficiency of the "stable" charge separation in PS II is calculated to be about 65–70% (Renger and Holzwarth, 2005). A comparison with PBRCs

reveals that the energetic efficiency of charge separation in PS II is much higher than the reported value of about 35% for the radical pair $P870^{+\bullet}Q_A^{-\bullet}$ in *Rhodobacter sphaeroides* (Warncke and Dutton, 1993). This marked difference between the two types of organisms is almost entirely due to the necessity of developing the strongly oxidizing species $P680^{+\bullet}$ for photosynthetic water oxidation.

The cofactors involved in $P680^{+\bullet}Q_A^{-\bullet}$ formation are embedded into a tailored protein matrix. Any charge density redistribution within the cofactor(s) owing to population of a particular redox state necessarily leads to changes of the interaction with the atoms of other cofactors and the protein matrix. This response is time dependent and therefore a ladder of energetic states emerges (vide infra).

Reliable calculations of the energetics require detailed information on the electronic configuration of the different states (see Fig. 17.3) and their interaction with the environment. An indispensable – but not sufficient – prerequisite for a straightforward analysis is the knowledge of a high-resolution structure of the whole system. The new information on the PSII structure of 1.9 Å resolution (Umena et al., 2011) offers a suitable basis for further theoretical calculations. In the following our fragmentary knowledge will be briefly described.

Ultra fast excited singlet state equilibration within $^1(RC - PC)^*$ (Durrant et al., 1992; Müller et al., 1996; Raszewski et al., 2005) is most likely followed by a response of the protein matrix, but the energetics of this rapid environmental response is not yet resolved. Values of tens of meV are reported for PBRCs (Paschenko et al., 2003).

The radical ion pair $P680^{+\bullet}Pheo^{-\bullet}$ attains at least two different states: $P680_1^{+\bullet}Pheo_{D1}^{-\bullet}$ (RP1 in Fig. 17.3) and $P680_2^{+\bullet}Pheo_{D1}^{-\bullet}$ (RP2 in Fig. 17.3), where $P680_1^{+\bullet}=[Chl_{D1}^{+\bullet}P_{D1}P_{D2}Chl_{D2}]$ and $P680_2^{+\bullet}=[Chl_{D1}P_{D1}^{+\bullet}P_{D2}Chl_{D2}]$, that are distinguished by their mode of hole localization. An energetic gap of about 50 meV would be sufficient to assure that the electron density moves from $Chl_{D1}^{+\bullet}$ to P_{D1} so that the spin of the cation radical is predominantly located at P_{D1} (Renger and Holzwarth, 2005; Raszewski et al., 2008). The exact spin distribution within $(RC - PC)^{+\bullet}$ depends on the protein environment and markedly differs for different sample types. In PS II CCs with an intact WOC the electron hole is almost completely located on P_{D1} (Takahashi et al., 2008).

Based on the static X-ray diffraction crystallography structure of PBRCs, the energy gap between the excited singlet state of the special pair ($^1P^*$) and the initially formed radical pair $P^{+\bullet}BPheo_A^{-\bullet}$ was calculated to be about −90 meV and that of the subsequent relaxation to about −200 meV (Parson et al., 1990). These theoretical values have to be compared with estimates gathered from experimental results. Delayed fluorescence measurements lead to corresponding $\Delta G°$ values of −170 and −80 meV (Woodbury et al., 1986; Ogrodnik et al., 1988), while numbers of −55 and −135 meV, respectively, are obtained from an analysis of time-resolved decay kinetics of prompt fluorescence of an antenna-free mutant strain of *Rhodobacter capsulatus* (Müller et al., 1995). Apart from quantitative differences due to limitations of both theoretical and experimental approaches (for a discussion, see Renger and Holzwarth, 2005) the results clearly show the relevance of protein relaxation for the energetics of light-induced charge separation in PBRCs (for theoretical calculations on the role of nuclear motions on the primary electron transfer steps in PBRCs, see Parson and Warshel, 2004). The limitations of the primary charge separation by protein dynamics has been experimentally shown for PBRCs by measurements of time resolved Trp absorption changes and comparison with electron transfer reactions (Wang et al., 2007).

The overall relaxation comprises a ladder of states with progressively increasing free energy gaps. As a consequence, the energetics of charge separation become a time-dependent parameter. A clear illustration of this principle is presented in a study on time-resolved fluorescence from PBRCs. Data gathered over a wide range of temperatures (10–295 K) and from mutants where the redox potential of the special pair couple $P/P^{+\bullet}$ is shifted by up to 350 mV are well described by a sequence of relaxations of the radical pair $P^{+\bullet}BPheo_A^{-\bullet}$, and include three different states (Katiliene et al., 2003).

Comparative detailed theoretical calculations on the protein dynamics are not yet reported for PS II. Experimental values of about −150 meV for the total $\Delta G°$ between $^1P680^*$ and $P680^{+\bullet}Pheo_{D1}^{-\bullet}$ in "open" PS II complexes were gathered from analyses of fluorescence decay

kinetics (Vasil'ev et al., 1996). Time-resolved photovoltage measurements with unstacked PS II membrane fragments and data evaluation within the framework of the exciton radical pair equilibrium model (Schatz et al., 1987, 1988) lead to a very similar value of about −160 meV (Gibasiewicz et al., 2001). In PS II centers "closed" by keeping $Q_A^{-\bullet}$ reduced, the negative charge on $Q_A^{-\bullet}$ diminished the $\Delta G°$ gap by about 85 meV whereas formation of Q_AH_2 leads to marginal effects at most (Gibasiewicz et al., 2001). The influence of the negative charge of $Q_A^{-\bullet}$ on the properties of $Pheo^{-\bullet}$ is clearly reflected by a pronounced electrochromic effect on the Q_x band of $Pheo_{D1}$ that gives rises to the "C550 signal" (Van Gorkom, 1974).

Information on the energetics of relaxation processes was gathered from an analysis performed at room temperature by using His-tagged PS II CCs from *Synechocystis* sp. PCC 6803 with high O_2-evolving capacity. The results were interpreted by the existence of a sequence of relaxation steps which take place within a ladder of four radical pair states with a total $\Delta G°$ of −167 meV (Vasil'ev et al., 2002). The latter value nicely fits with the –150 meV gathered from earlier data (Vasil'ev et al., 1996).

The above mentioned studies on both PBRCs and PS II reveal that the radical pair energetics are characterized by a sequence of relaxation steps via a series of radical pairs of the reaction centers as is shown schematically in the bottom part of Fig. 17.3. This behavior is fundamentally different from static distributions of free energy differences that are due to sample heterogeneity and characterized by inherently different free energy gaps. It reflects the essential time-dependent role of protein conformational relaxation in stabilizing intermediate redox states. As a consequence, the protein plays an active functional role not only in tuning the redox properties of cofactors but also in providing rapid and efficient relaxation channels to increase the free energy gap once the radical pair is formed which has the effect of preventing back reactions. It is attractive to hypothesize that probably the protein environment around the RC has been optimized during evolution to fulfill this task.

In summary, proteins play an active role in supporting charge separation by providing efficient relaxation channels that span a wide time domain from picoseconds up to nanoseconds and perhaps extending into the microsecond range. The modulation of the energetics via protein relaxations is not a peculiarity of light-induced charge separation leading to $P680^{+\bullet}Pheo^{-\bullet}$ formation but appears to be a general feature which is also relevant for subsequent reactions like $P680^{+\bullet}$ reduction by Y_Z (see Section III.C.1).

C. Oxidative Water splitting: The Kok Cycle

The key step leading to a deeper understanding of the underlying reaction pattern of oxidative water splitting was the discovery of the period four oscillation of oxygen yield per flash when dark-adapted algae cells or chloroplasts from higher plants are excited with a train of single turnover flashes (Joliot et al., 1969). Kok and coworkers (Kok et al., 1970) interpreted this characteristic feature by a basic scheme that is referred to as the Kok-cycle.

Figure 17.4 presents an extended version of this scheme. An inspection readily shows that the overall reaction sequence comprises two different types of reactions: (i) reduction of $P680^{+\bullet}$ by a component Y_Z and (ii) stepwise oxidation of the WOC by Y_Z^{OX} until, after the accumulation of four oxidizing redox equivalents, molecular oxygen is formed and released via an exchange reaction with substrate water.

1. $P680^{+\bullet}$ Reduction by Y_Z

The cofactors P680 and Y_Z are bound to the D1/D2 heterodimeric protein matrix in a well-defined manner (Ferreira et al., 2004; Loll et al., 2005; Zouni, 2008; Umena et al., 2011). At first glance, it is therefore surprising that the $P680^{+\bullet}$ reduction by Y_Z exhibits rather complex kinetics. In a reasonable approximation, the overall time course of this process can be approximated satisfactorily by three-exponential processes with "fast" ns, "slow" ns and μs components. This general feature was observed in thylakoids (Renger et al., 1983), PS II membrane fragments (Brettel et al., 1984; Eckert and Renger, 1988; Schilstra et al., 1998) and PS II CCs from both spinach and thermophilic cyanobacteria (Kühn et al., 2004; Sugiura et al., 2004). Indirect lines of evidence suggest that it also pertains to intact leaves (*Arabidopsis thaliana*) (Steffen et al., 2005a). These kinetics depend on the redox state of the WOC and change

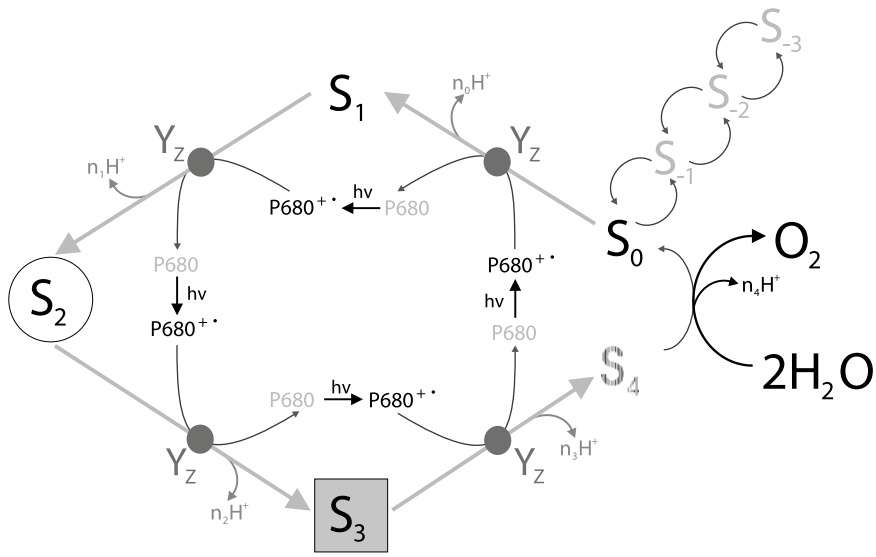

Fig. 17.4. Kok cycle. Extended Kok-cycle of oxidative water splitting. The photo oxidation of P680 is marked by arrows (the input of each photon is indicated by hv, the intermediary redox component Y_Z is symbolized by a dark grey dot, the S_i states are symbolized in the following way: the dark stable redox state S_1 by a bold capital, the metastable redox state S_2 and S_3 by capitals encircled and grey square background (pointing to the possibility of a multiple S_3 state, see Fig. 17.9), respectively, and the transient "elusive" state S_4 by a dashed symbol, super reduced states are marked in grey. For the sake of simplicity the slow dark relaxation reactions of S_1, S_2 and S_3 are omitted (for review, see Renger, 1999; Messinger and Renger, 2008).

drastically in systems lacking the WOC (Gläser et al., 1976; Brettel et al., 1984; Eckert and Renger, 1988). Two alternative explanations can be considered for the origin of the multiphasic kinetics in systems with an intact WOC: (i) "static" sample heterogeneity (an ensemble of PS II complexes with different structural features and/or energetic parameters, e.g., distribution of distances between Y_Z and P680 and/or Gibbs free energy gap/reorganization energy, respectively) or (ii) transition through a sequence of redox equilibria of the type $[P680^{+\bullet}Y_Z \Leftrightarrow P680\ Y_Z^{OX}]_j$ due to relaxation processes analogous to those discussed for the radical pairs of charge separation (for details see, Renger and Holzwarth, 2005), where index j symbolizes one of several stages of relaxation. A critical survey of the experimental data favours the idea of a sequence of relaxation steps as outlined by Kühn et al. (2004). Figure 17.5 shows a scheme, which has been developed for the reaction sequence and the energetics of $P680^{+\bullet}$ reduction by Y_Z. In this sequence the initial step is a rapid equilibration that comprises the transfer of an electron from Y_Z to $P680^{+\bullet}$ concomitant with a proton shift within a hydrogen bond between the OH group of Y_Z and a nearby base X (Eckert and Renger, 1988). Evidence gathered from site-directed mutagenesis experiments (see Hayes et al., 1998 and references therein) and X-ray diffraction crystallography data (Ferreira et al., 2004; Loll et al., 2005) revealed that base X is His190 of polypeptide D1. The rate of the equilibration within the initial state I can be described consistently by the Marcus theory of nonadiabatic electron transfer (NET) (Marcus and Sutin, 1985) with a reorganization energy of about 0.5 eV (Renger et al., 1998; Tommos and Babcock, 2000). Therefore this reaction is kinetically limited by the electron transfer step which is characterized by rate constants of $(50\ ns)^{-1}$–$(20\ ns)^{-1}$ (Renger et al., 1983; Brettel et al., 1984; Eckert and Renger, 1988; Schilstra et al., 1998; Sugiura et al., 2004; Kühn et al., 2004; Steffen et al., 2005a), activation energies of 10–20 kJ/mol (Eckert and Renger, 1988; Jeans et al., 2002; Kühn et al., 2004) and a vanishingly small kinetic H/D isotope effect (Karge et al., 1996). The normalized extent of the "fast" ns kinetics to the overall $P680^{+\bullet}$ reduction by Y_Z is large in redox states S_0 and S_i of the WOC and significantly smaller in S_2 and S_3 (Brettel et al., 1984; Eckert and Renger, 1988; Schilstra et al.,

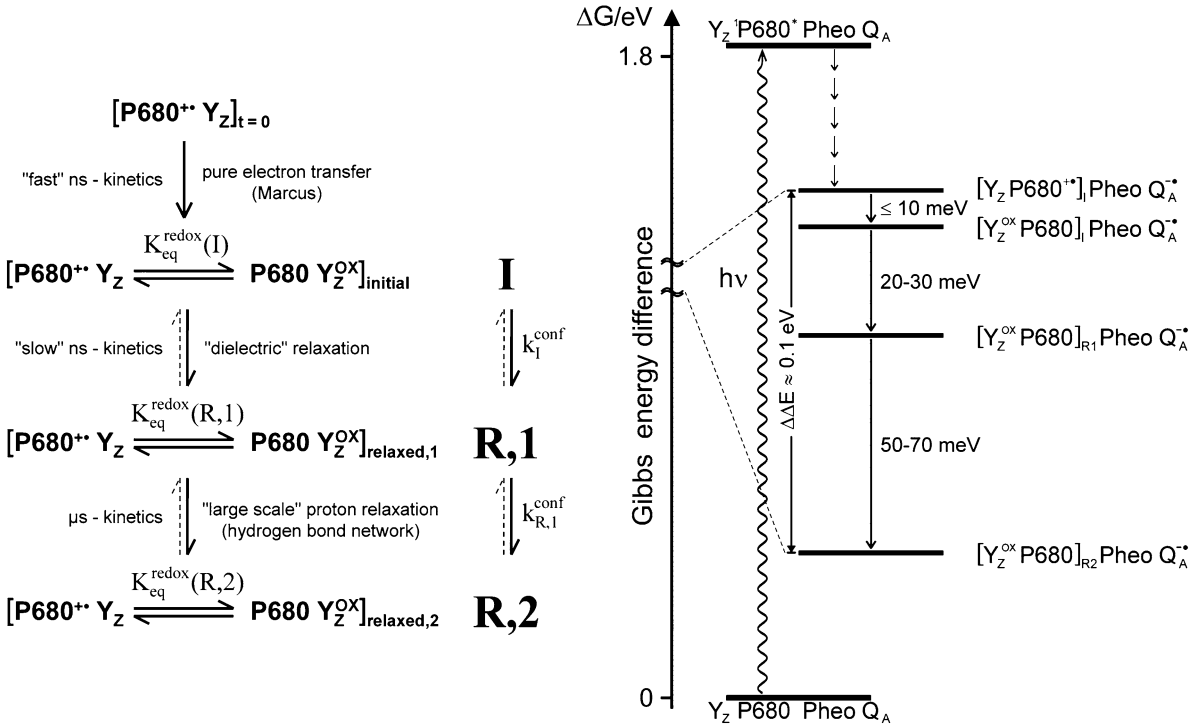

Fig. 17.5. Reaction scheme (*left side*) and energetics (*right side*) of $P680^{+\bullet}$ reduction by Y_Z in dark-adapted PS II complexes. A value of about 100 meV for the total Gibbs free energy gap between states Y_Z $P680^{+\bullet}$ Pheo $Q_A^{-\bullet}$ and [Y_Z^{OX} P680 Pheo $Q_A^-]_{relaxed}$ was taken from Metz et al. (1989). The differences between the states I, R,1 and R,2 were gathered from an analysis of the multiphasic kinetics as outlined in Kühn et al. (2004). These values are typical for PS II where the WOC attains oxidation states S_0 and S_1 (for further discussion, see Kühn et al., 2004).

1998). This phenomenon is assumed to reflect mainly the dependence on the S_i -states of the equilibrium constant in the initial state $[P680^{+\bullet}Y_Z \Leftrightarrow P680\ Y_Z^{OX}]_I$. The "slow" ns components with rate constants of $(300–600\ ns)^{-1}$ are assumed to originate from a local response of the nearest protein environment that does not comprise "large scale" hydrogen bond rearrangements (Kühn et al., 2004). These kinetics are invariant to replacement of exchangeable protons by deuterons (Karge et al., 1996) and the activation energy is somewhat larger (a factor of 1.5–2) than that for the "fast" ns kinetics (Jeans et al., 2002; Kühn et al., 2004). The normalized amplitudes exhibit a dependence on the redox state S_i of the WOC which is opposite to that of the "fast" ns component, i.e., higher values in S_2 and S_3 and lower values in S_0 and S_1 (Brettel et al., 1984; Eckert and Renger, 1988; Schilstra et al., 1998).

In marked contrast to the "fast" and "slow" ns kinetics, the component with 30–35 µs kinetics exhibits a pronounced H/D exchange effect (Schilstra et al., 1998; Christen et al., 1998, 1999). This phenomenon is ascribed to the rearrangement of a hydrogen bond network in the environment of Y_Z (Christen et al., 1998; Schilstra et al., 1998).

The energetics of the different relaxation steps are sensitive to environmental changes and also slightly dependent on the sample type (P. Kühn and G. Renger, unpublished results). Regardless of these subtleties it is clear that the protein matrix plays an active role in $P680^{+\bullet}$ reduction by Y_Z through the relaxation processes, which give rise to the sequence of energetic states shown in Fig. 17.5 (see also Kühn et al., 2004). The hydrogen bond between Y_Z and His190 is critical for the kinetics of Y_Z oxidation by $P680^{+\bullet}$. The possibility of a low barrier hydrogen bond configuration has been discussed (Christen et al., 1999). The distance between the oxygen atom of Y_Z and the imino nitrogen atom of His 190 has been shown to be

2.5 Å. Furthermore, two additional hydrogen bonds are formed between Y_Z and two water molecules (Umena et al., 2011). A protonation of His190 is expected to disrupt this bond. Indeed the normalized extent of the fast ns kinetics was shown to decrease with a pK of 4.6 upon lowering the pH of suspension of the PS II CC from *T. elongatus* (Kühn et al., 2005). This value is markedly lower than the pK of His in solution of about 6.0 (see *Textbooks of Biochemistry*). However, marked shifts of pK values of amino acid residues are not unusual in protein, in particular when hydrogen bonds are involved. DFT calculations reveal that the pK of His also depends on the conformation (angle of the imidazole ring plane) and can vary by about two pH units (Hudaky and Perczel 2004).

The nature of the relaxation processes and of the hydrogen bond network shift leading to states R1 and R2, respectively (see Fig. 17.5), remain to be unravelled. The reaction pattern of $P680^{+\bullet}$ reduction by Y_Z is markedly different in PS II complexes which are lacking a functionally competent WOC: the ns kinetics disappear in the physiological pH range and the reaction is dominated by μs kinetics. In thylakoids or PS II membrane fragments deprived of the WOC, the kinetics with typical lifetimes of about 10 μs at pH = 7.0 become retarded when the pH decreases (Conjeaud et al., 1979; Renger et al., 1984), the activation energy is significantly larger (30–40 kJ/mol) than in samples with an intact WOC (Reinman and Mathis, 1981; Ahlbrink et al., 1998; Renger et al., 1998) and the kinetics are characterized by a pronounced H/D exchange effect of about 3 (Christen et al., 1997; Ahlbrink et al., 1998; Diner et al., 1998). The oxidation of Y_Z in these samples is coupled to a stoichiometric proton release into the lumen (Renger and Völker, 1982) and to a markedly higher reorganization energy of about 1.6 eV (Renger et al., 1998; Tommos and Babcock, 2000). These findings indicate that the reaction coordinate is significantly altered in PS II complexes without a functional WOC, and they imply changes of the environment of Y_Z, probably in its hydrogen bonding (for a review, see Berthomieu and Hienerwadel, 2005) whereas the orientation of Y_Z remains virtually unaffected (Matsuoka et al., 2011). Of physiological relevance is the high susceptibility of the reaction to photoinhibition (Callahan et al., 1986; Theg et al., 1986; Eckert et al., 1991). The quantum yield of this deleterious effect exceeds that of PS II complexes with an intact WOC by a factor of about 1,000 (Eckert et al., 1991).

Newly synthesized PS II complexes lack an intact WOC (Strasser and Sironval, 1972; Cheniae and Martin, 1973). Likewise, several stress factors such as cold or heating (Thompson et al., 1989; Enami et al., 1994), drought (Noguchi and Sugiura, 2002), low or high pH (Briantais et al., 1977; Renger et al., 1977) and UV-B-irradiation (Renger et al., 1989; Vass et al., 2002) lead to elimination or deterioration of the WOC function. Accordingly, these PS II complexes are especially prone to photoinhibition and need to be protected. It is assumed that Cyt b_{559} is an essential component of a protective mechanism that most likely involves a cyclic electron flow (Poulson et al., 1995; Stewart and Brudvig, 1998; Kaminskaya et al., 2003). This idea seems to be supported by the different properties of Cyt b_{559} in the two sample types, i.e., PS II samples with and without an intact WOC (Gadjeva et al., 1999). A complementary protection of systems lacking the WOC emerges from the drastic increase of $E_m(Q_A^{-\bullet}/Q_A)$ (see sections I.B.1.c and III.C.3)

2. Water-Oxidizing Complex (WOC): Structure, Reaction Pattern and Mechanism

The Kok cycle per se provides only a formal description of the four-step reaction sequence energetically driven by the strongly oxidizing cation radical $P680^{+\bullet}$ with Y_Z acting as redox carrier. For a deeper understanding of oxidative water splitting, information is required on: (i) the structure of the WOC; (ii) the electronic configuration and nuclear geometry of the catalytic site in each redox state S_i, and (iii) the reaction coordinates of the individual redox steps. In the following our knowledge on these points will be summarized and an attempt presented to cast this information into a proposed model.

a. Structure of the WOC

Different lines of experimental evidence indicate that the catalytic site of the WOC is a Mn_4O_XCa cluster, where x denotes the number

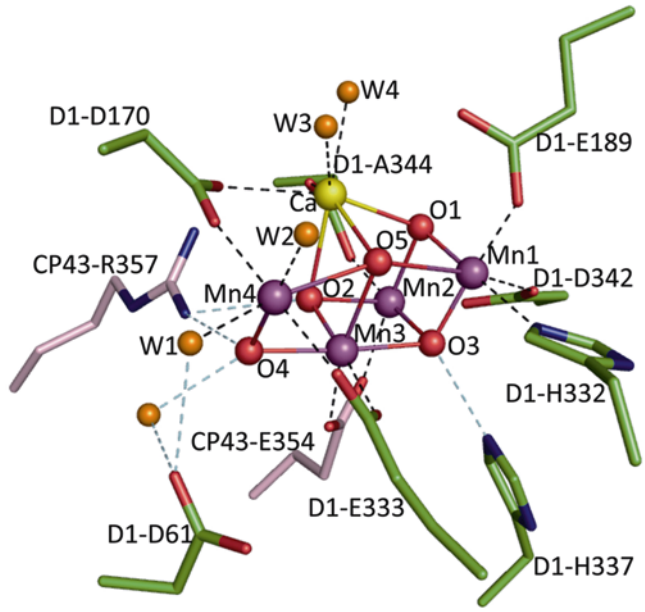

Fig. 17.6. Structural arrangement of the Mn_4O_xCa cluster according to the X-ray diffraction crystallography data of Umena et al. (2011). For an assignment of amino acid residues and water molecules as ligands, see text. Mn1, Mn2, Mn3 and Mn4 denote the different manganese ions of the WOC (for details, see text).

of μ-oxo-bridges (for recent reviews, see Yachandra, 2005; Messinger and Renger, 2008). Therefore the structure of the WOC is defined by: (a) the spatial arrangement of the four manganese centers; the bridging oxygens and the Ca^{2+} ion; (b) the coordination sphere of the Mn_4O_XCa cluster, and (c) the surrounding protein matrix.

X-ray diffraction crystallography and extended X-ray absorption fine structure (EXAFS) analyses are the currently most widely used methods to unravel the structure of the Mn_4O_XCa cluster. Several X-ray diffraction crystallography structures of PS II from thermophilic cyanobacteria have been published since 2001 (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Biesiadka et al., 2004; Loll et al., 2005).

X-ray diffraction crystallography analyses are generally faced with problems that originate from the use of the required high doses of X-ray radiation. This leads to "radiation damage" effects owing to the generation of photoelectrons which interact with several amino acid side chains and in particular with reducible transition metal centers (Carugo and Carugo-Djinovic, 2005). The latter effect is of special relevance for the Mn_4O_XCa cluster of the WOC. EXAFS studies performed at much weaker X-ray radiation (factor of about 1,000) and at 10 K unambiguously show that the manganese is reduced to Mn(II) at the doses used for X-ray diffraction crystallography analyses and that all Mn-Mn distances and Mn-O-Mn bridges are lost (Yano et al., 2005a).

A breakthrough in unravelling a high resolution structure of the WOC was achieved by Jian-Ren Shen and coworkers. The new 1.9 Å structure offered for the first time a complete picture of the geometry including the oxygen atoms of the μ-oxo-bridges and of the water molecules that were not seen at the lower resolution of former studies (for a review, see Guskov et al., 2010). Fig. 17.6 shows that the cluster in its resting state (a population of super-reduced S_{-i} states due to electrons generated by the X rays cannot be excluded so that the structure might reflect the geometry of the S_{-3} state which is rather stable, see Messinger et al., 1997) is identified as a Mn_4O_5Ca core. However the symbol Mn_4O_xCa is more appropriate to characterize the cluster because it comprises the possibility that the number of oxo-bridges can change during the Kok cycle. Therefore Mn_4O_xCa will be used throughout this chapter.

Precise data on Mn-Mn and Mn-O distances and on the number of these structural elements within the Mn_4O_XCa cluster are obtained by using X-ray

spectroscopy. The seminal work of Mel Klein, Ken Sauer, Vittal Yachandra and coworkers revealed that the Mn_4O_XCa cluster is characterized by two or three Mn-Mn distances of about 2.7 Å and one Mn-Mn distance of about 3.3 Å (for a review, see Yachandra et al., 1996). Progress in the detection technique led to a significant improvement of the distance resolution down to 0.09 Å with an accuracy of ±0.02 Å (Yano et al., 2005b). The results obtained with this method unambiguously showed that the Mn_4O_XCa cluster in the dark stable S_1 state comprises three short Mn-Mn distances (two of about 2.7 Å and one of about 2.8 Å) and one long Mn-Mn distance of 3.3 Å. Likewise, two similar Ca^{2+}-Mn distance (3.3–3.4 Å) were discovered (Yano et al., 2005b).

In addition to the μ-oxo bridges, the Mn and Ca^{2+} ions are coordinated by other ligands, in particular by amino acid residues of polypeptide D1 and – most important – by the substrate molecules. Based on site-directed mutagenesis studies Asp170, His332, Glu333, His337, Asp342 of polypeptide D1 and its carboxyl terminus of Ala344 were inferred to be possible ligands of the Mn_4O_XCa cluster (see Debus, 2005 and references therein). Figure 17.6 shows that the Mn_4O_xCa cluster is coordinated by six carboxylate ligands (Asp170, Glu189, Glu333, Asp 342 and the C-terminus of Ala344 of polypeptide D1 and Glu354 of CP43) and one His (D1-H332) from the protein matrix and in addition by four water molecules. The ligation by amino acid residues confirms former conclusions gathered from FTIR data on mutants of mesophilic cyanobacteria, except of Glu189 and His332 (both are ligands to Mn1) and His337 which coordinates to O3 rather than to Mn. One striking feature of the first coordination sphere is the bidentate ligation of the metal centers by carboxylate groups (D1-Asp170,D1-Glu333,D1-Asp342,D1-Ala344 and CP43-Glu354). This mode of bridging is inferred to stabilize the Mn_4O_XCa cluster and – even more important – to provide the indispensable scaffold for fixing and/or tuning functionally important mutual distances between the metal ions when the internal bridging mode is changed during the catalytic cycle, e.g., by formation or a change of μ-oxo bridges between two metal ions (Kern and Renger, 2007). According to Fig. 17.6 all manganese centers are hexa-coordinated. This finding is at variance with results of theoretical modelling by DFT where Mn1 (coordinated with His332) is inferred to be penta-coordinated (Siegbahn, 2009, 2011). Analogous conclusions were derived for redox state S_2 of the WOC on the basis of analyses of data from EPR/ENDOR spectroscopy at low temperatures (Cox et al., 2011). The mode of coordination of Mn1 is of mechanistic relevance and needs to be clarified by taking into account possible changes during the S_i state transitions. The ligand-metal interaction can be probed by using FTIR spectroscopy. It was shown that D1-Asp170 is ligating a Mn, which does not change its redox state from S_0 up to S_3 (Debus et al., 2005), An analogous feature was found for D1-Ala189 (Strickler et al., 2006). D1- Ala344 is the ligand of a Mn that is the site of oxidation in the $S_1 \rightarrow S_2$ transition (Chu et al., 2004; Kimura et al., 2005a) and D1-His332 coordinates a Mn which is not oxidized in the conventional Kok-cycle up to S_3 but is sensitive to structural changes of the Mn_4O_XCa cluster coupled with the S_i-state transitions of the WOC (Kimura et al., 2005b; Sugiura et al., 2009). The key role of D1-His332 has been illustrated by X-ray spectroscopic studies on a D1-H332E mutant (Yano et al., 2011).

The conclusion drawn from the above mentioned FTIR data would imply that only two manganese of the Mn_4O_xCa cluster are redox active, in marked contrast to most of the published models (ses section III.C.2.d). The unravelling of the origin of this serious discrepancy is of utmost relevance because it raises general questions on the interpretation of experimental results obtained by using different approaches under different conditions (low versus room temperature, hydration level, sample material etc.).

In addition inorganic ligands like Cl^- and HCO_3^- where often discussed as possible members of the first coordination sphere (for reviews, see Van Gorkom and Yocum, 2005; Van Rensen and Klimov, 2005) (Chapter 20). HCO_3^- was modelled into the X-ray diffraction crystallography structure proposed by Ferreira et al. (2004) but more refined data are not in favor with this suggestion (Loll et al., 2005). Recent gas chromatography-mass spectrometric studies reveal that HCO_3^- is not part of an intact WOC (Ulas et al., 2008; Shevela et al., 2008). This finding is in line with FTIR data indicating that HCO_3^- is not a ligand of the Mn_4O_XCa cluster (Aoyama et al., 2008). The new X-ray diffraction

crystallography data (Umena et al., 2011; Kawakami et al., 2011) definitely rule out HCO_3^- as a ligand to the Mn_4O_XCa cluster. With respect to Cl^-, a possible signature has been reported for binding to Mn based on EXAFS studies on oriented samples with the WOC in redox state S_3 (Yachandra, 2005). On the other hand FTIR data led to the conclusion that Cl^- is not a direct ligand to Mn (Hasegawa et al., 2004), which was supported by X-ray spectroscopy measurements on bromide reconstituted PS II membrane fragments (Haumann et al., 2006). Replacement Cl^- by Br^- retards the kinetics of the WOC oxidation by Y_Z^{OX} to virtually the same extent in redox states S_1 and S_3 (Ishida et al., 2008). Accordingly, Cl^- appears to be part of a hydrogen bond network close to the Mn_4O_XCa cluster as originally proposed by Olesen and Andréasson (2003). This network could facilitate H^+ release from the catalytic site and might be involved in substrate water interaction.

Direct information on chloride binding sites near the Mn_4O_XCa cluster was obtained from X-ray diffraction crystallography data. Two Cl^- are anchored to the protein – mainly via hydrogen bonds – at distances of 6.7 Å and 7.4 Å to Mn4 and Mn2, respectively (for a review, see Kawakami et al., 2011).

The functionally most relevant ligands are the substrate molecules, their protonation in the different S_i-states and the mode of hydrogen bonding with the protein environment. Binding of water molecules to the Mn_4O_XCa cluster was identified by the X-ray diffraction crystallography structure of 1.9 Å resolution (see Fig. 17.6) which reveals that Mn4 and Ca^{2+} each coordinates two H_2O and at least three further molecules are located in the close environment thus forming a hydrogen bond network that probably exerts a functional role. This finding of several H_2O molecules in the WOC confirms earlier indirect lines of evidence for the existence of a cluster of 6–12 water molecules gathered from EPR and mass spectrometry data (Hansson et al., 1986; Bader et al., 1993; Burda et al., 2001). Measurements of the exchange kinetics in the different S_i-states revealed that two substrate water molecules are bound in a different manner (Hillier and Wydrzynski, 2000). It must be emphasized that in addition to the two molecules that are eventually linked together to form the O-O bond, further water molecules probably play an essential role in mediating proton transfer and affecting reaction pathways (see also Noguchi, 2008). Recent progress in the application of theoretical methodology (combined quantum mechanical (QM) and molecular mechanics (MM) approaches) stimulated several attempts to model the structure of the Mn_4O_xCa cluster on the basis of XRDC, EXAFS and EPR/ENDOR spectroscopic data (Kusunoki, 2011; Siegbahn, 2009, 2011; Sproviero, 2008; Zein et al., 2008). The XRDC structure model of 1.9 Å resolution will provide a new promising starting point for refined theoretical analyses.

b. Electronic Configuration and Nuclear Geometry of S_i States

The electronic structure of each individual S_i-state is determined by the valence of manganese and its first coordination sphere. For a more precise description it appears useful to separate conceptionally contributions due to manganese (M), ligands (L) except the substrate and the substrate (W) itself. Therefore the S_i-states will be symbolized by $S_i = M_jL_kW_l$ with $i = j + k + l$, where $j = k = l = 0$ for S_0. This nomenclature permits a straightforward semantic distinction between metal- and ligand/substrate-centered oxidation steps in the WOC. In some cases it is of advantage to summarize the contributions of either the oxidation states of M and L, i.e., $S_i = (ML)_mW_l$ with $m = j + k$, or the ligands and substrate, i.e., $S_i = M_j(LW)_n$ with $n = k + 1$ (for further details, see Messinger and Renger, 2008).

Integers are used for the indices j, k, l, m and n (e.g., $l = 0$, 1 and 2 correspond with substrate, oxo-radical and peroxide like species, respectively). It must be emphasized that these indices represent only formal redox states – without giving any information on the protonation state – and even more importantly, they do not reflect the real charge at each atom in the Mn_4O_XCa cluster and its ligands including the substrate molecules. In fact, DFT calculations and a charge population analysis reveal that actually Ca^{2+} carries the highest positive charge within the Mn_4O_XCa cluster although manganese attains the redox states III and IV (vide infra) (Sproviero et al., 2006, 2008; Zein et al., 2008). Detailed information on the electron density distribution within the WOC attaining the different S_i states are expected from future analyses on the basis of further spectroscopic studies in combination with advanced QM/MM calculations that include the protein environment

of the more refined 1.9 Å structure, in particular the position of water molecules.

The electronic configuration of the Mn_4O_XCa cluster can be best probed by X-ray absorption near edge (XANES), EPR and ^{55}Mn ENDOR spectroscopy (for recent reviews, see Messinger and Renger, 2008; Sauer et al., 2008). A cornerstone in analyzing this complex was the discovery of a g = 2 EPR multiline signal (MLS) for S_2 at low temperatures of about 10 K (Dismukes and Siderer, 1981). Later also the S_0 state was shown to exhibit a multiline signal but of different shape (Messinger et al., 1997a, b; Åhrling et al., 1997). These signals originate from the magnetic coupling of the electron spin of an unpaired electron with the nuclear spin I = 5/2 of the manganese. An analysis of the number of lines and the spectral widths of these MLSs indicates that S_0 and S_2 are mixed valence states of four magnetically coupled manganese centers (Peloquin et al., 2000; Kulik et al., 2005a, b). The S_1 and S_3 states have been shown to be characterized by integer spin signals that can be detected by parallel mode EPR (Dexheimer and Klein, 1992; Yamauchi et al., 1997; Matsukawa et al., 1999).

Interpretation of ^{55}Mn ENDOR data within the framework of a structural model of the Mn_4O_XCa cluster gathered from EXAFS studies on single crystals of *T. elongatus* (Yano et al., 2006) leads to the following conclusions on the electronic structure of the redox states (Kulik et al., 2007): $S_0 = Mn_A(III)Mn_B(III)Mn_C(IV)Mn_D(III)$, $S_1 = Mn_A(III)Mn_B(IV)Mn_C(IV)Mn_D(III)$ and $S_2 = Mn_A(III)Mn_B(IV)Mn_C(IV)Mn_D(IV)$ where indices A,B,C and D correspond with numbers 4, 3, 1 and 2, respectively, shown in Fig. 17.6. This assignment based on 10 K experimental data obtained on samples containing 3% methanol implies that the manganese centers can be distinguished with respect to their valence states. On the other hand X-ray resonant Raman scattering experiments suggest that the electron removed from the manganese during the $S_1 \rightarrow S_2$ transition probably originates from a delocalized orbital (Glatzel et al., 2004). At present, extent and mode of delocalization and its possible variation with temperature are not known.

A more complex picture emerges for S_3. In spite of full agreement on the experimental XANES data in the pure S_i-states (Messinger et al., 2001; Haumann et al., 2005a) controversial interpretation still exists on the nature of the $S_2 \rightarrow S_3$ transition. Basically three types of models are currently under discussion: (i) the oxidation step is metal-centered ("Mn-only" model, see Ono et al., 1992; Haumann et al., 2005a); (ii) the reaction is ligand-centered, probably leading to formation of a bridged oxo-radical ("oxo-radical" model, see Messinger et al., 2001; Pushkar et al., 2008), and (iii) the redox level S_3 of the WOC is not a single redox state but the ensemble of three different configurations ($M_3L_0W_0$, $M_2(LW)_1$ and $M_1L_0W_2$), which rapidly interchange via redox isomerism and proton tautomerism equilibria ("multiple S_3 state" model, see Renger, 1993, 2001, 2004). In this case the essential O-O bond is already preformed at the level of a binuclearly complexed peroxidic state $M_1L_0W_2$ as originally proposed in Renger (1978).

The nature of the kinetically elusive transient state S_4 is also a matter of controversial discussions and speculation (see Section III.C.2.d).

A word of caution should be added. It is not yet known as to what extent data from low temperature experiments where the WOC is fully blocked in its function really reflect the situation of the system at work under physiological conditions (Renger, 1987). The limited signal/noise ratio of room temperature X-ray spectroscopic data (Haumann et al., 2005a) does not permit a straightforward answer to this most important question.

Furthermore, even the most important characteristics of the electronic configuration of the WOC are not unambiguously clarified and a matter of controversial discussion, i.e., the overall redox level of the manganese within the Mn_4O_xCa cluster remains uncertain. In spite of an enormous set of experimental data and numerous theoretical analyses leading to the widely accepted $Mn(III)Mn_3(IV)$ configuration for S_2 (vide supra), the "low overall valence" configuration $Mn_3(III)Mn(IV)$ cannot be definitely ruled out for S_2 with corresponding ramifications to the other S_i states (for thorough discussions, see Gatt et al., 2011 and references therein). The "water proof" identification of the overall redox level of the manganese is an indispensable prerequisite for any reliable considerations on the molecular mechanism of oxidative water splitting in photosynthesis.

The nuclear geometry of the Mn_4O_XCa cluster in the different S_i-states can be best analyzed by EXAFS (vide supra) performed on samples where – apart from the S_1 state populated in dark-

adapted samples – the redox state of the WOC is trapped in S_0, S_2 or S_3. The results obtained reveal a significant structural difference between S_0 and S_1 in that one short Mn-Mn distance is larger (2.85 Å) in S_0 than in S_1 (about 2.7 Å) while only minor structural changes occur in the Mn_4O_XCa -cluster during the $S_1 \rightarrow S_2$ transition. In marked contrast, the $S_2 \rightarrow S_3$ transition is coupled with a marked rearrangement of the nuclei as reflected by an elongation of the short (~2.7 Å) Mn-Mn distances to 2.82 and 2.95 Å (Liang et al., 2000). Likewise, measurements on Sr^{2+}-containing PS II CCs show that also the Mn-Ca vectors (3.3–3.4 Å) decrease (Pushkar et al., 2008; Yachandra and Yano, 2011). The conclusion of significant structural changes coupled with this redox transition is supported by other indirect lines of evidence: (i) the striking difference in the reorganization energy compared to the other oxidation steps (Renger, 1997); (ii) the drastically different reactivity of S_2 and S_3 towards NH_2OH and NH_2NH_2 (Messinger and Renger, 1990; Messinger et al., 1991), and (iii) the binding of Ca^{2+} (Boussac and Rutherford, 1988). Changes of the ligand sphere during the Kok cycle can be monitored by attenuated total reflection FTIR. It has been found that the orientation of the carboxylate group of CP43-Glu354 changes by 8° during the $S_1 \rightarrow S_2$ step due to transition from a bridging ligand of two metals to a bidentate coordination of one Mn (Iizawa et al., 2010).

c. Reaction Coordinate of Oxidative Water Splitting

The reaction coordinate of oxidative water splitting in the WOC is determined by the energetics of the individual S_i-states and the activation energy barriers for the stepwise transitions between these states in the Kok-cycle (see Fig. 17.5). Absolute values of the S_i-state energy levels are not known. Likewise, the standard Gibbs free energy differences between redox states S_i and S_{i+1}, $\Delta G^{\circ}(S_{i+1}/S_i)$ cannot be directly experimentally determined. Estimations on these values are obtained on the basis of the relation:

$$\Delta G^{\circ}(S_{i+1}/S_i) = \Delta G(P680^{+\bullet}/P680) + \Delta G^{\circ}(P680Y_Z^{OX}S_i/P680^{+\bullet}Y_ZS_i) + \Delta G^{\circ}(Y_ZS_{i+1}/Y_Z^{OX}S_i) \quad (17.7)$$

where $\Delta G^{\circ}(P680^{+\bullet}/P680)$, $\Delta G^{\circ}(P680Y_Z^{OX}S_i/P680^{+\bullet}Y_ZS_i)$ and $\Delta G^{\circ}(Y_ZS_{i+1}/Y_Z^{OX}S_i)$ are the Gibbs free energy differences for the formation of the cation radical $P680^{+\bullet}$, oxidation of Y_Z by $P680^{+\bullet}$ and S_i-state transitions, respectively. The formulation of Eq. 17.7 tacitly implies that $\Delta G^{\circ}(P680^{+\bullet}/P680)$ is virtually independent of the redox states of Y_Z and the WOC (S_i). This assumption is justified by the finding that the shift by the redox state of the nearly equidistant Y_Z^{OX} is ≤10 meV (Diner et al., 2004). Since the Mn_4O_XCa cluster is further apart than Y_Z from $P680^{+*}$ the electrostatic effect is expected to be even significantly smaller than 10 meV.

The reduction potential of P680 has been recently estimated to be about +1.25 V (for details see Renger and Holzwarth, 2005; Rappaport and Diner, 2008), corresponding to a $\Delta G^{\circ}(P680^{+\bullet}/P680)$ value of 1.25 eV. Values of $\Delta G^{\circ}(P680Y_Z^{OX}S_i/P680^{+\bullet}Y_ZS_i)$ were reported to be –90 meV for S_0 and S_1 and −20 meV for S_2 and S_3 (Brettel et al., 1984) and data on $\Delta G^{\circ}(Y_ZS_{i+1}/Y_Z^{OX}S_i)$ are presented in Vass and Styring (1991) and Vos et al. (1991). On the basis of these numbers, the values of $\Delta G^{\circ}(S_{i+1}/S_i)$ are calculated to be about 0.85, 1.10, 1.15 and 1.0 eV for i=0, 1, 2 and 3 which exceed former estimates by about 0.15 eV. The difference originates from the revised value of $\Delta G^{\circ}(P680^{+\bullet}/P680)$, which is higher by 0.1–0.15 V than former estimates (Jursinic and Govindjee, 1977; Klimov et al., 1979).

It is important to note that a value of +0.85 eV for $\Delta G^{\circ}(S_1/S_0)$ is in contradiction to the experimental value +0.76 V reported for the reduction potential of Y_D (Boussac and Etienne, 1984) because Y_Z^{OX} is known to slowly oxidize S_0 to S_1 (vide infra). This discrepancy indicates that at present precise values of the energetics are still lacking.

Regardless of the uncertainties on the exact numbers, the obtained $\Delta G^{\circ}(S_{i+1}/S_i)$ values show that – except for the S_0 oxidation – all S_i-state transitions in the WOC are characterized by very similar Gibbs free energy differences of 1.0–1.15 eV. This characteristic of the WOC entirely differs from the energetics of a four-step oxidation for water splitting in aqueous solutions (Anderson and Albu, 1999) as is illustrated in Fig. 17.7.

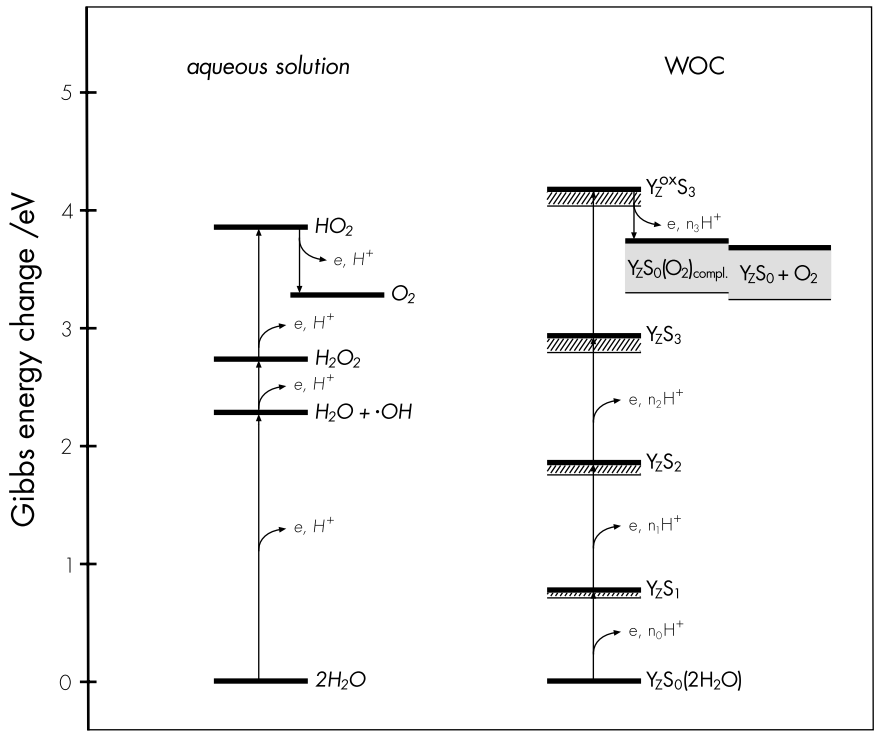

Fig. 17.7. Energetic pattern of four-step oxidative water splitting into molecular oxygen and four protons in solution (*left side*) and in the WOC (*right side*). The values for the in vitro reaction at pH 7.0 are taken from the literature (Anderson and Albu, 1999) and those of the redox steps in the WOC are calculated according to Eq. 17.3 as outlined in the text, the dashed areas indicate uncertainties in the precise energetics of the S_i -states. It must be emphasized that pH effects on the Gibbs free energy of the state of O_2 in the aqueous solution system differs from that of $Y_ZS_0 + O_2$ because pH effects in the WOC are not taken into account. Furthermore, the Gibbs free energy levels of the initial states $2\,H_2O$ in aqueous solution at pH 7 and of $Y_Z\,S_0\,(2\,H_2O)$ are not the same. Since only Gibbs free energy changes are shown both states are set to the same zero level. For the sake of direct comparability the free energy levels of water molecules in bulk solutions and in the WOC are set to zero.

The markedly smaller $\Delta G^{\circ}(S_1 / S_0)$ value implies an extra Gibbs free energy gain of about 0.25 eV during the $Y_Z^{OX}S_0 \rightarrow Y_ZS_1 + n_0H^+$ transition compared to the other redox steps. The physiological role of this peculiarity remains to be unravelled. It has been speculated that this surplus of Gibbs free energy could be transiently stored (Renger et al., 1990; Renger, 1993) but so far experimental evidence is lacking for this idea. A further consequence of the exceptional energetics of the redox steps is the virtual absence of an S_0 population in the WOC of dark-adapted samples due to the slow dark oxidation by Y_Z^{OX}. Interestingly, this feature is found in all oxygen-evolving organisms (Vermaas et al., 1984; Messinger and Renger, 1994; Isgandarova et al., 2003) including also the Chl *d*-containing cyanobacterium *Acaryochloris marina* (Shevela et al., 2006). Therefore it seems most likely to be of physiological relevance, probably in suppressing deleterious reactions that are not yet clarified. The last step of oxidative water splitting is the product/substrate exchange. In earlier model considerations this reaction was assumed to be significantly exergonic with values of 100–200 meV (Renger, 1978). Data on UV absorption changes reflecting the turnover of $Y_z^{ox}\,S_3$ (Velthuis, 1981; Renger and Weiss, 1982) led to the suggestion that the reaction $Y_Z^{OX}S_3 \Leftrightarrow [Y_ZS_2(H_XO_2)] \Leftrightarrow [Y_ZS_0(O_2)] \Leftrightarrow Y_ZS_0 + O_2 + n_3H^+$, which comprises that product substrate exchange step, is only slightly exergonic (Clausen and Junge, 2004). This conclusion, however, has been questioned on the basis of fluorescence measurements (Kolling et al., 2009) and was shown to be wrong when a straightforward mass spectrometric study (Shevela et al., 2011)

Table 17.2. Half-life times, $t_{1/2}$ (μs) and activation energies, E_A (kJ/mol) of the reactions $Y_Z^{OX}S_i \rightarrow Y_ZS_{i+1-4\delta_{i3}} + \delta_{i3}O_2 + n_iH$ ($\delta_{i3} = 1$ for i = 3, otherwise zero)

Reaction	Y_Z^{OX} reduction		S_i state oxidation					
Species	*Spinacea oleracea*[a]		*Spinacea oleracea*				*T. vulcanus*	
Preparation	Thylakoids	PS II m.f.	PS II m.f.[b]		PS II core[c]		PS II core[d]	
Temperature	8·10°C	8·10°C	20°C		20°C		25°C	
	$t_{1/2}$	$t_{1/2}$	$t_{1/2}$	E_A	$t_{1/2}$	E_A	$t_{1/2}$	E_A
i = 0	40–60	70	50	5	nd	nd	nd	nd
i = 1	85	110	100	12.0	75	14.8	70	9.6
i = 2	140	180	220	36.0	225	35.0	~130	26.8
i = 3	750	1,400	1,300	20/46*	4,100	21/67*	1,300	~16/60*

[a]Data from Razeghifard and Pace (1997)
[b]Data from Renger and Hanssum (1992)
[c]Data from Karge et al. (1997)
[d]Data from Koike et al. (1987)
*The numbers refers to E_A values above and below, respectively, the characteristic break point temperatures
m.f. = membrane fraction; nd = not determined

was performed which unambiguously reveals that oxygen evolution is not suppressed at high O_2 pressure and that ΔG^0 is indeed rather large (order of 0.2 eV) as originally proposed (Renger, 1978).

Although precise values are missing for $\Delta G^{\circ}(S_{i+1}/S_i)$ due to experimental uncertainties (symbolized by hatched areas in Fig. 17.7), the energetic considerations reveal that water splitting is thermodynamically possible down to local pH values of 5.0. Since the Gibbs free energy gap for oxidative water splitting into molecular oxygen and four protons depends on pH, the final states in Fig. 17.7 are at different energy levels for the processes in aqueous solution at pH 7.0 and in the WOC of varying local pH.

Based on the energy levels of the S_i-states as a framework, the reaction coordinate of photosynthetic water splitting can be constructed by using the values of activation barriers of the individual S_i-state transitions gathered from the temperature dependence of their kinetics. The kinetics of Y_Z^{OX} reduction and S_i-state oxidation were measured by time-resolved EPR (Babcock et al., 1976; Razeghifard and Pace, 1997) and optical UV (Dekker et al., 1984; Renger and Weiss, 1986; Rappaport et al., 1994) spectroscopy, respectively, and shown to be virtually identical. The kinetic coincidence indicates that Y_Z^{OX} is the direct oxidant of the Mn_4O_XCa cluster. Table 17.2 summarizes typical half-life times of these reactions at room temperature. A closer inspection of the data reveals that the kinetics of the reactions $Y_Z^{OX}S_i \rightarrow Y_ZS_{i+1} + n_iH^+$ are similar for i = 0, 1 and 2 (variation by factors of 2–4) while the reaction $Y_Z^{OX}S_3 \rightarrow Y_ZS_0 + O_2 + n_3H^+$ is slower by factors of 5–15 compared to the former reactions. Furthermore, the $Y_Z^{OX}S_3$ decay kinetics are particularly sensitive to different types of sample modifications; as is illustrated by a comparison between thylakoids and PS II CC from spinach. Table 17.2 shows that the rate constant $k_{0,3}^{exp}$ of PS II core complexes isolated from spinach is smaller by a factor of 3–4 compared to PS II membrane fragments while the other $k_{i+1,i}^{redox}$ values for i = 0, 1, 2 remain virtually unaffected. A striking example for this phenomenon is a *Synechocystis* sp. PCC 6803 mutant where either Ala or Asn replaces Asp61 of polypeptide D1. In these two mutants the oxidation of S_3 by Y_Z^{OX} is retarded by factors of about 8 and 10, respectively, whereas the kinetics of the other S_i-transitions are much less affected. Interestingly, mutation of Asp61 to Gln is virtually without effect (Hundelt et al., 1998). Likewise, treatment with phospholipase A2 of thylakoids from *Arabidopsis thaliana* gives rise to similar effects (Kim et al., 2007). In marked contrast to these specific modulations of the $Y_Z^{OX}S_3$ reaction the replacement of Ca^{2+} by Sr^{2+} gives rise to a retardation by factors of 3–5 of the kinetics of all S_i-state transitions in PS II membrane fragments from spinach (Westphal et al., 2000). This finding,

which was interpreted as an indication for kinetic modulations owing to structural changes (probably comprising rearrangements in a hydrogen bond network), might be indicative for a strong coupling of electron and proton transfer as originally proposed by the late Jerry Babcock in his seminal work (see Tommos and Babcock, 1998). Experiments on PS II CC isolated from *T. elongatus* cells, grown on a medium where Ca^{2+} was replaced by Sr^{2+} (Ishida et al., 2008) confirm these findings and reveal that the Sr^{2+} effect is a general phenomenon of the WOC in cyanobacteria and higher plants.

A third peculiarity of Y_Z^{OX} reduction by S_3 is the possibility of sigmoidal kinetics. This feature was observed in several studies (Dekker et al., 1984; Renger and Weiss, 1986; Rappaport et al., 1994; Clausen et al., 2004; Haumann et al., 2005b) but so far it is not clear as to what extent it is a real property of the reaction or also the result of overlapping phenomena (Karge et al., 1997). Based on a comparative study on the kinetics of Y_Z^{OX} reduction and oxygen release a possible lag phase was inferred to be no longer than 50 μs under normal in vivo conditions (Razeghifard and Pace, 1999). A recently reported lag phase of about 250 μs (Haumann et al., 2005b) is likely to be affected by artefacts because the experiments were performed on partially dehydrated samples where the transitions $Y_Z^{OX}S_2 \rightarrow Y_ZS_3 + n_2H^+$ and $Y_Z^{OX}S_3 \rightarrow Y_ZS_0 + O_2 + n_3H^+$ are severely impaired (Noguchi and Sugiura, 2002, for further discussion, see Renger, 2007, 2011).

The dependence on temperature of the kinetics of the S_i -state transitions has been analyzed in different sample types. Typical values are compiled in Table 17.2 (Koike et al., 1987; Renger and Hanssum, 1992; Karge et al., 1997). A comparison of these data reveals that the activation barrier of the oxidation steps is dependent on the redox state S_i of the WOC and virtually independent of the sample type. The highest values are found for the $S_2 \rightarrow S_3$ transition.

A striking peculiarity was observed for the reaction $Y_Z^{OX}S_3 \rightarrow Y_ZS_0 + O_2 + n_3H^+$ in PS II preparations form *Thermosynechococcus vulcanus*, i.e., a characteristic break point in the Arrhenius plot emerged at about +16°C (Koike et al., 1987). This feature was less pronounced in PS II membrane fragments from spinach (Renger and Hanssum, 1992) and not observed in another study on samples from mesophilic organisms (Clausen et al., 2004). It is possible that in the latter type of organisms the break point might be shifted to lower temperatures and/or is less pronounced so that it often escapes detection. The idea of a shift to lower temperature is in line with the finding that the S_i state transitions are blocked below characteristic threshold temperatures (see section III.C.2.d) which are higher by about 30° in thermophilic cyanobacteria (Koike et al., 1987) than in samples from higher plants (Styring and Rutherford, 1988; Gleiter et al., 1993). Existence and extent of this mechanistically important phenomenon remain to be clarified.

The close similarity of the kinetic data and their activation energies in samples from thermophilic cyanobacteria and higher plants indicate that the reaction coordinate of oxidative water splitting remained virtually invariant to evolutionary development of PS II. Figure 17.8 schematically summarizes the data. For the sake of simplicity the possible existence of a break point phenomenon will be omitted in the scheme.

Regardless of the unresolved break point phenomenon, values of 1.2–1.4 eV appear to be realistic for the reaction of $Y_Z^{OX}S_3$ (see Table 17.2).

Electron transfer steps in the WOC are coupled with protolytic reactions. The nature of the coupling of electron transfer and proton transfer of redox reactions in enzymes is a fundamental problem and the subject of numerous studies (for reviews, see Cukier, 2004; Hammes-Schiffer, 2006; Meyer et al., 2007 and references therein). Basically two different mechanisms can be distinguished: (i) separate electron transfer and proton transfer pathways and (ii) proton coupled electron transport. Straightforward analyses on the WOC are lacking so far by the current ignorance of sufficiently precise structural details at atomic resolution on the Mn_4O_XCa cluster and its protein environment but this situation is expected to become changed due to the new 1.9Å X-ray diffraction crystallography structure, (see Sections III.C.2.a and III.C.2.b).

The oxidation steps of the WOC are characterized by comparatively small kinetic isotope effects of the exchangeable protons with $k_i(H)/k_i(D)$ ratios of 1.3–1.4 for i = 1–3 in PS II membrane fragments (Renger et al., 1994; Karge et al., 1997; Lydakis-Simantiris et al., 1997) and slightly higher values of 1.5–2.5 in PS II CC from

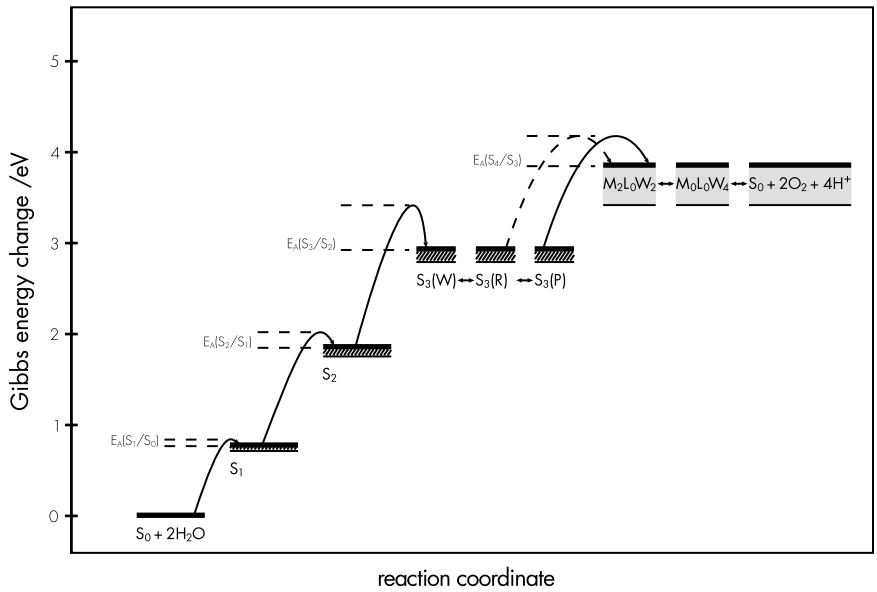

Fig. 17.8. Generalized reaction coordinates of the four-step oxidative water splitting in the photosynthetic apparatus (for details, see Fig. 17.7 and text).

spinach (Bögershausen et al., 1996; Karge et al., 1997). Interestingly, very similar numbers of about 1.4 and 2.5 have been found for two redox steps (formation of the "F-state") and a third step (transition to the "H(O) state"), respectively, in cytochrome *c* oxidase (Hallen and Nilsson, 1992; Wikström, 2004), which catalyzes the reverse reaction, i.e., O_2 reduction to water (for a review, see Babcock and Wikström, 1992). These kinetic isotope effect values exclude the breaking of O-H or N-H bonds as rate-limiting steps and rather reflect interactions between redox intermediates and protons of basic group(s) of the protein as discussed for cytochrome *c* oxidase (Hallen and Nilsson, 1992).

In order to obtain further information on the kinetic properties of the Y_Z^{OX} driven S_i state transitions in the WOC, the experimental rate constants $k_{i+1,i}^{exp}$ were compared with "theoretical" values $k_{i+1,i}^{NET}$ for nonadiabatic electron transfer (NET) calculated on the basis of the structural model of 2.9 Å resolution (Guskov et al., 2010) and an empirical rate constant-distance relationship derived on the basis of the Marcus theory (see Marcus and Sutin, 1985)

The equation

$$\log k_{i+1,i}^{NET} = 13 - (1.2 - 0.8\rho)(R_{DA} - 3.6) - 3.1(\Delta G_{i+1,i}^{0} + \lambda_{i+1,i})^2/\lambda_{i+1,i} \quad (17.8)$$

has been obtained for $k_{i+1,i}^{NET}$ when using the relation $|V_{i+1,i}(R_{DA})|^2 = |V_{i+1,i}(R_{DA}=0)|^2 \exp(-\beta \bullet R_{DA})$ for the electronic coupling of reactant and product state, where R_{DA} is the edge to edge distance between the reactants and $\beta = \rho*0.9Å^{-1} + (1 - \rho)*2.8\ Å^{-1}$ (for details, see Page et al., 1999) with ρ = packing density of protein atoms between the redox centers (ρ varies between 0 for vacuum and 1 for full package).

The values of $(\Delta G_{i+1,i}^{0} + \lambda_{i+1,i})^2 / \lambda_{i+1,i}$ in Eqn. (17.8) are gathered from the experimental activation energies $E_{A,i+1/i}$ (see Renger et al., 1998):

$$(\Delta G_{i+1,i}^{0} + \lambda_{i+1,i})^2 / \lambda_{i+1,i} = 4(E_{A,i+1,i} + 0.5RT) \quad (17.9)$$

The distances between Y_z and the individual Mn centers of the WOC vary between 7.5 Å and 7.85 Å (Guskov et al., 2010). A value of about $10^9\ s^{-1}$ is obtained for $k_{2,1}^{NET}$ (the value of $k_{1,0}^{NET}$ is of the same order of magnitude) for a typical packing density of 0.76 in proteins containing redox active cofactors (Page et al., 1999) of longest distance R_{DA} = 7.85 Å between Y_z and the redox active manganese, and an activation energy of 10 kJ/mol for $Y_Z^{OX}\ S_1 \rightarrow Y_Z S_2 + n_1 H^+$ (Koike et al., 1987; Renger and Hanssum, 1992, Karge et al., 1997). It must be emphasized that $10^9\ s^{-1}$, which is the minimum number of $k_{2,1}^{NET}$ (at shorter distances $k_{2,1}^{NET}$ is even larger), exceeds the experimental

data by a factor of about 10^5 (see Table 17.2). The same calculation performed with $E_{A,3,2} = 36$ kJ/mol for the reaction $Y_Z^{OX} S_2 \rightarrow Y_Z S_3^+ + n_2 H^+$ leads to a rate constant $k_{3,2}^{NET}$ of about 10^6 s^{-1} (Renger, 2011) which is still larger than the experimental value by a factor of more than hundred. These enormous differences between calculated $k_{i+1,i}^{NET}$ and experimental $k_{i+1,i}^{exp}$ numbers which are far beyond any reasonable experimental error drastically contrast the properties of Y_z reduction by $P680^{+\bullet}$ (see section III.C.1) where the multiple site electron proton transfer or MS-EPT mechanism perfectly satisfies the empirical rate-constant relationship as outlined in Renger et al. (1998). Therefore the rate of all Y_Z^{OX} induced oxidation steps in the WOC is inferred to be definitely not limited by a non-adiabatic electron transfer step but by another type of reaction (Renger 2007, 2011).

As a consequence, the redox steps of the WOC have to be described by a sequential reaction

$$\begin{aligned} Y_Z^{OX} S_i &\xrightarrow{k_{i+1,i}^{trigg}} (Y_Z^{OX} S_i)^* \\ &\xrightarrow{k_{i+1,i}^{NET}} Y_Z S_{i+1} + n_i H^+ \end{aligned} \qquad (17.10)$$

for i = 0, 1 and 2 (the properties of the reaction $Y_Z^{OX} S_3 \rightarrow \rightarrow Y_Z S_0 + O_2 + n_3 H^+$ will be discussed separately), where $k_{i+1,i}^{trigg}$ is the rate constant of a triggering process which precedes the electron transfer step and is rate limiting for the overall reaction, i.e., $k_{i+1,i}^{trigg} \ll k_{i+1,i}^{NET}$, and $\left(Y_Z^{OX} S_i\right)^*$ represents a "triggered" redox state S_i.

The sequence (17.10) necessarily implies that all redox transitions of the WOC are characterized by a sigmoidial time course. Therefore the deviation from a pure monoexponential kinetics would provide information on the rate of the redox step. However, this feature could not be resolved due to the limitations of the applied methods, in particular because the effect is extremely small for $k_{i+1,i}^{trigg} \ll k_{i+1,i}^{NET}$ and therefore remains buried by the error margins originating from the signal/noise ratios.

Another important consequence emerges from the relation $k_{i+1,i}^{trigg} \ll k_{i+1,i}^{NET}$: the experimentally obtained activation energies reflect the temperature dependence of the trigger reaction(s) rather than the characteristics of the redox step in the physiological range. Furthermore, the blockage of the reaction sequence (17.10) below characteristic temperatures for each S_i state (Koike et al., 1987; Styring and Rutherford, 1988; Gleiter et al., 1993) indicates that at least one breakpoint exists in the reaction coordinate. Interestingly, an analogous blockage by freezing has also been observed for the electron transfer from $Q_A^{-\bullet}$ to Q_B at the acceptor side of PS II. This effect was shown to be strictly correlated with protein dynamics (see section III.D.3). Although the nature of the triggering reactions has been neither resolved for the acceptor nor for the donor side of PS II, it appears reasonable to assume that proton transfer step(s) and/or conformational changes are involved. The idea of a role of proton shift(s) in gating the redox reactions in the WOC is in line with the markedly lower threshold temperature of the $S_1 \rightarrow S_2$ transition compared to the redox steps of the other S_i state transitions (see Table 1) because the latter reactions are, but the former reaction is not, coupled with significant proton release into the lumen (Suzuki et al., 2009). When taking into account this argument, the trigger reactions are likely to be similar for i = 0 and i = 2, but different for i = 1. Accordingly, the value of 250 µs for the $S_0 \rightarrow S_1$ transition (Rappaport et al., 1994) appears to be more realistic than the number of 30 µs (Van Leeuwen et al., 1993).

In contrast to the apparent mono-exponential kinetics (vide supra) reflecting the rate limiting triggering reactions for i = 0, 1 and 2, the time course of Y_Z^{OX} reduction by the WOC in redox state S_3 is sigmoidal (vide supra). This behaviour can be basically described by the following simplified reaction sequence:

$$\begin{aligned} &Y_Z^{OX} S_3 \xrightarrow{k_{3^*,3}^{trigg}} (Y_Z^{OX} S_3)^* \xrightarrow{k_{4,3}} \\ &Y_Z[S_2(H_xO_2)^*] \xrightarrow{k_{0,4}} \\ &Y_Z[S_0(O_2)^*] + n_3 H^+ \end{aligned} \qquad (17.11)$$

where $[S_2(H_xO_2)^*]$ and $[S_0(O_2)^*]$ reflect complexed peroxide and dioxygen, respectively, and n_3 the overall H^+ release without specifying the individual step(s) of deprotonation. Furthermore, for the sake of simplicity, the last step of water/O_2 exchange (vide infra) is omitted and back reactions are not explicitly shown.

Two different types of rate limitation of sequence (17.11) can be considered: i) the triggering process is slow compared to the redox reaction(s) and comprises a sequence of at least two steps thus giving rise to the sigmoidal time

course, or ii) a "fast" single triggering reaction similar to that of the other S_i state transitions is followed by an overall electron transfer which is slower due to the participation of a fast uphill redox equilibrium which is characterized by a rather small constant (see section III.C.2.d). At present there is no evidence for a sequence of trigger reactions and therefore the second alternative appears to be more attractive.

d. Mechanism of Oxidative Water Splitting

The unravelling of the mechanism of oxidative water splitting in photosynthesis is the topic of numerous studies. A large number of models has been proposed (for a compilation of 34 proposals during the time period from 1965 to 1996, see Ke, 2001; for reviews on recent proposals, see McEvoy and Brudvig, 2006; Meyer et al., 2007; Renger, 2007; Messinger and Renger, 2008; Sauer et al., 2008). The use of QM/MM methods have led to several proposals for the mechanism of oxidative water splitting not only in the WOC (for reviews, see Siegbahn, 2009, 2011) but also for synthetic catalysts like binuclear ruthenium complexes (Bianco et al., 2011). All current attempts on the mechanism of the WOC were based on limited structure information. It is therefore expected that the new X-ray diffraction crystallography structure of 1.9 Å resolution will open the road for a more realistic treatment and therefore new proposals are expected to be presented in the near future.

This section describes an attempt to summarize our current knowledge in a more general picture by a proposed mechanism of oxidative water splitting, which is schematically shown in Fig. 17.9.

For mechanistic considerations the following characteristics need to be taken into account: (i) water is the direct substrate for dioxygen formation (Clausen et al., 2005; Hillier et al., 2006) without any catalytic function of bicarbonate (for a proposal, see Castelfranco et al., 2007) that is not a constituent of the WOC (see Section III.C.2.b); (ii) the S_i -state transitions are coupled with proton release in a multiple site electron and proton transfer manner (for a discussion, see Meyer et al., 2007); (iii) all oxidation steps of the WOC driven by Y_Z^{OX} are not limited by the rate of NET but triggered by proton transfer and/or conformational changes (see Section III.C.2.c) and become blocked below threshold temperatures

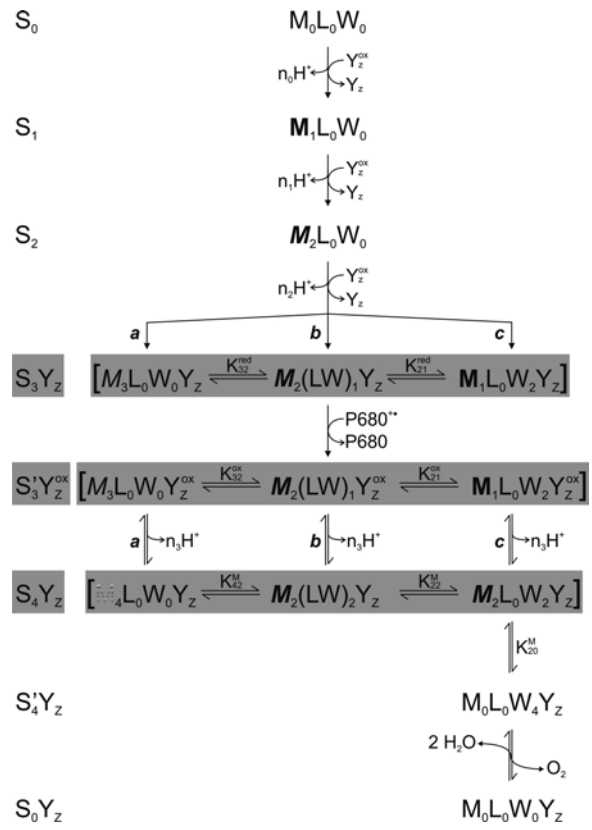

Fig. 17.9. Simplified mechanism of oxidative water splitting. The redox states S_i of the WOC are symbolized by $M_jL_kW_l$ / $M_j(LW)_m$ as outlined in the text. Different redox states of the manganese are distinguished by using different types of capital letters M and index j=0,…4. Likewise, L_k and W_l describe the redox states of ligand and substrate (water), respectively. In the case of $(LW)_m$ no distinction is made between the redox levels of L and W (for details, see text); ***a***, ***b*** and ***c*** represent different possible pathways for oxidation of S_2 by Y_Z^{OX}; K_{32}^{red}, K_{21}^{red} and K_{32}^{OX}, K_{21}^{OX} are the overall equilibrium constants between the three different states $M_3L_0W_0$, $M_2(LW)_1$ and $M_1L_0W_2$ of S_3 and of $Y_Z^{OX}S_3$, respectively, including redox isomerism and proton tautomerism, K_{42}^{M} and K_{22}^{M} (not shown) are the equilibrium constants for the possible states of S_4 and K_{20}^{M} describes the equilibration between S_4 configuration $M_2L_0W_2$ (complexed peroxide) and S_4 (complexed dioxygen), respectively. Oxidation of Y_Z by $P680^{+\bullet}$ is explicitly shown only for the transition S_3Y_Z $P680^{+\bullet} \rightarrow S_3$ Y_Z^{OX} P680, otherwise only oxidation of the WOC by Y_Z^{OX} is presented.

(for compilation of data, see Renger, 2001) and hydration levels that depend on the redox state S_i (Noguchi and Sugiura, 2002), and (iv) the redox steps $Y_Z^{OX}S_0 \rightarrow Y_ZS_1$ and $Y_Z^{OX}S_1 \rightarrow Y_ZS_2$ are metal centered reactions (see Section III.C.2.b).

With respect to the proton transfer reactions it is important to note that different protonable

groups are involved and the intrinsic proton release from the catalytic site is difficult to separate from the extrinsic pattern (Renger, 1978, 1987). A stoichiometry of $n_i = 1, 0, 1$ and 2 for $i = 0, 1, 2$ and 3, respectively, is now widely accepted to reflect the release pattern from the catalytic site (for a review, see Hillier and Messinger, 2005, see also Noguchi, 2008; Suzuki et al., 2009). Possible proton shifts and changes in hydrogen bonding of water molecules due to the S_i state transitions have been monitored by FTIR measurements. The FTIR difference spectra revealed marked band shifts and negative bands in the region of O-H modes (3,588–3,617 cm^{-1} and 3,612–3,634 cm^{-1}, respectively) and complex spectral features in the carboxylate stretching region (1,600–1,300 cm^{-1}). These findings led to suggestions on the water chemistry at the Mn_4O_XCa cluster; the proposed model of Noguchi (2008) comprises at least four water molecules. At present, however, only limited information is available on the mechanism of the protolytic reactions and the mode of substrate binding via hydrogen bonds within the protein matrix (for details on our fragmentary knowledge, see McEvoy and Brudvig, 2006; Messinger and Renger, 2008 and references therein). The X-ray diffraction crystallography structure of 1.9 Å resolution suggests that the water ligands to Mn4 and Ca^{2+} (see Fig. 17.6) are involved in hydrogen bonding. However, neither the protonation state nor changes during the S_i state transitions are known. Therefore, the coupled protolytic processes are not explicitly shown in the simplified scheme of the proposed mechanism that is summarized in Fig. 17.9.

Consensus exists on the nature of the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions of the WOC: i.e., these are metal centered reactions (see Section III.C.2.b). Based on our current knowledge (see Section III.C.2.a) these two steps most likely involve Mn3 and Mn2 (for assignment of manganese see Fig. 17.6). Furthermore the reaction $Y_Z^{OX}S_0 \rightarrow Y_ZS_1 + n_0H^+$ is assumed to be coupled with H^+-release from a protonated μ-oxo bridge whereas the oxidation of S_1 to S_2 is not compensated by H^+-release and therefore gives rise to accumulation of a positive charge at/or near the Mn_4O_XCa cluster (for a detailed discussion, see Messinger and Renger, 2008). The situation is more complex for the subsequent oxidation steps of the WOC. The reaction pathway of $Y_Z^{OX}S_2 \rightarrow Y_ZS_3 + H^+$ depends on the model used for S_3 (see Section III.C.2.b): pathway ***a*** describes the "Mn-only" model with a relation for the equilibrium constants of $K_{32}^{red} = K_{21}^{red} = 0$; pathway ***b*** reflects the "oxo-radical" model with $K_{32}^{red} \rightarrow \infty$ and $K_{21}^{red} = 0$, and pathways ***a,b,c*** symbolize the "multiple S_3 state" model. In the latter model, state $M_1L_0W_2$ of S_3 represents a complexed peroxide, which has been proposed to be the "entatic state" (for details on "entatic states" see Williams, 1995) for photosynthetic O_2 formation (Renger, 2001, 2004).

In the final redox step of the Kok cycle the state S_4 is formed through S_3 oxidation by Y_Z^{OX}. For all considerations on S_4 a clear distinction between states $Y_Z^{OX}S_3$ and Y_ZS_4 is necessary in order to avoid semantic confusion. Both states carry four oxidizing redox equivalents compared to Y_ZS_0. However $Y_Z^{OX}S_3$ should not be interpreted as a form of S_4 because structural data reveal that Y_Z is more than 5 Å away from the Mn_4O_XCa cluster (Loll et al., 2005; Kawakami et al., 2011) and cannot be part of its first coordination sphere.

The nature of S_4 is unknown but it is most likely a multistate system comprising at least two configurations: $M_2L_0W_2$ and $M_0L_0W_4$. The formation of a binuclearly complexed peroxide as intermediate of oxidative water splitting in photosynthesis was originally proposed in a mechanistic model of the Kok cycle (Renger, 1978) and assigned to redox state S_3. The vast majority of currently discussed elaborated schemes comprise the idea of a complexed peroxide but assume that this intermediate can be exclusively formed in S_4 (Mc Evoy and Brudvig, 2006; Siegbahn et al., 2009, 2011). At present it is not yet known if the state $M_1L_0W_3$ corresponding to a complexed superoxide species is also transiently populated, as was postulated by Meyer et al. (2007).

For a deeper mechanistic understanding of the entire process it is absolutely necessary to clarify as to whether or not electron abstraction by Y_Z^{OX} from the Mn_4O_XCa cluster in S_3 is the indispensable prerequisite for covalent linkage of the oxygen atoms from the two substrate molecules, i.e., if one of the configurations $M_4L_0W_0$ ("Mn-only model") or $M_3L_1W_0$ or $M_2(LW)_2$ ("oxo-radical model") is the precursor of O-O bond formation. The existence of configuration $M_4L_0W_0$ would necessarily imply the formation of Mn(V) if $M_1L_0W_0 \equiv S_1$ attains the configuration $Mn(III)_2Mn(IV)_2$ (see Section III.C.2.b). A

terminal oxo group in the form of Mn(V)=O has been discussed to be the precursor of the essential O-O bond formation (Pecoraro et al., 1998; Vrettos et al., 2001), but experimental data of Weng et al. (2004) and of Haumann et al. (2005b), along with several theoretical studies (Siegbahn, 2006, 2008, 2011; Sproviero et al., 2008), are not in favor of this suggestion. The possible population of configurations $M_3L_1W_0$ (e.g., a Mn(IV) = O$^{\bullet}$ radical, Siegbahn 2006, 2008; Sproviero et al., 2008) and $M_2(LW)_2$ (Yachandra et al., 1996; Pushkar et al., 2008) is also discussed but none of these configurations are experimentally confirmed thus far.

All of these S_4 scenarios comprise important kinetic consequences. If the population of any of the S_4 configurations $M_4L_0W_0$ or $M_3L_1W_0$ or $M_2(LW)_2$ precedes the O-O bond linkage and if the reaction is exergonic with $\Delta G^0 < -50$ meV (see III.C.2) then the formation of this precursor kinetically coincides with Y_Z^{OX} reduction. As a consequence the following reactions including the O-O bond linkage (forming of state $M_2L_0W_2$), the subsequent oxidation of peroxide to complexed dioxygen $(O_2)_{complexed}$ and the eventual O_2 / H_2O exchange altogether must be very fast because the oxidation of S_3 by Y_Z^{OX} and of O_2 release are characterized by a rather similar if not identical time course (Strzalka et al., 1990; Razeghifard and Pace, 1999; Clausen et al., 2004). On the other hand, in the case of a highly endergonic transition from Y_Z^{OX} S_3 to either $M_4L_0W_0/M_3L_1W_0$ or $M_2(LW)_2$ the overall reaction rate depends on the rather low population probability of these intermediates and the subsequent reactions can take place in a time domain of 100 µs to 1 ms without affecting the close kinetic similarity of Y_Z^{OX} reduction and O_2 release. The idea of an endergonic reaction leading to Y_Z^{OX} reduction by the WOC in redox state S_3 is supported by theoretical calculations (Siegbahn, 2009, 2011). It is important to note that the same proposal is an integral part of the "multiple S_3 state" hypothesis where the population probability $p(M_1L_0W_2)$ of the configuration $M_1L_0W_2$ is assumed to be low (Renger, 2007, 2011). The essential difference to the "S_4 dogma", however, is the postulate that the two oxygen atoms leading to the configuration of a complexed peroxide have to be linked in S_3 in order to form the "entatic state" which is the prerequisite for electron transfer to Y_Z^{OX}. This essential point of the mechanism still remains to be clarified. The rate constant $k_{0,3}^{exp}$ in the "multiple S_3 state" model depends on two parameters: the intrinsic rate constant $k_{0,3}^{redox}$ of Y_Z^{OX} reduction by S_3 in the electronic configuration $M_1L_0W_2$ and its population probability $p(M_1L_0W_2)=[M_1L_0W_2]/\{[M_3L_0W_0]+[M_2(LW)_1]+[M_1L_0W_2]\}$ where the brackets […] symbolize the concentration normalized to the PS II content.

The population probabilities of configurations $M_3L_0W_0$, $M_2(LW)_1$ and $M_1L_0W_2$ in redox state S_3 are expected to be prone to variation by different parameters and therefore $k_{0,3}^{exp}$ should be more variable than the rate constant of the other S_i-state transitions of the WOC. This phenomenon is experimentally observed in PS II preparations from both cyanobacteria and higher plants (see Section III.C.2.c).

A different feature emerges from replacement of Ca^{2+} by Sr^{2+} and/or Cl^- by Br^-, which gives rise to a kinetic retardation of all S_i state transitions by almost the same extent (Westphal et al., 2000; Ishida et al., 2008), i.e., in this case $k_{i+1,i}^{redox}$ is affected rather than $p(M_1L_0W_2)$, probably due to a slowing of the triggering processes by a modified hydrogen bond network.

If we assume that the shifts of equilibrium constants K_1^{red} and K_2^{red} to K_1^{ox} and K_2^{ox}, respectively, due to formation of Y_Z^{OX} (see Fig. 17.9) and the rate of equilibration steps between the three S_3 configurations are fast compared to $k_{0,3}^{redox}$, the observed rate constant is given by $k_{0,3}^{exp} = k_{0,3}^{redox} \bullet p(M_1L_0W_2)$ while for the other redox transitions $k_{i+1,i}^{exp} = k_{i+1,i}^{redox}$ (i=0, 1, 2) where $k_{i+1,i}^{redox} \approx k_{i+1,i}^{trigger}$ (see section III.C.2.c) is the rate constant of the triggered redox reaction. Accordingly, effects on $p(M_1L_0W_2)$ readily explain the much larger variation of $k_{0,3}^{exp}$ (vide supra). The value of $p(M_1L_0W_2)$ is estimated to be of the order of 0.1–0.2 under normal conditions provided that $k_{0,3}^{redox}$ is not significantly different from $k_{i+1,i}^{redox}$ for i=0, 1 and 2. This comparatively small probability of $M_1L_0W_2$ population, which could be even markedly smaller in Y_ZS_3 compared to $Y_Z^{OX}S_3$ within a three-configuration equilibrium, is difficult to detect. At present, the nature of the postulated configuration $M_1L_0W_2$ is not known, but it possibly involves a peroxide, which is either binuclearly linked between one Mn and the Ca of the Mn_4O_XCa cluster or mononuclearly ligated to Mn4 as proposed by Meyer et al. (2007). The idea of a direct functional role of Ca^{2+} is in line with conclusions

of (Pushkar et al., 2008; Yachandra and Yano, 2011). A role of Ca^{2+} in acting as a water substrate binding site has been considered in several models (for reviews, see McEvoy and Brudvig, 2006; Meyer et al., 2007; Messinger and Renger, 2008; Sproviero et al., 2008).

In the scheme shown in Fig. 17.9, the nature of the redox states with respect to metal-, ligand- and/or substrate oxidation is explicitly shown but the details of the changes of individual centers within the Mn_4O_XCa cluster and its ligands are not presented because reliable information is lacking (see Section III.C.2.a). Likewise the mode of coupling with proton transfer steps is not specified but instead the essential role of protons is discussed in a generalized manner by introducing two assumptions (for details see Renger, 2007): (a) the local proton gradient in the neighbourhood of the two substrate oxygen atoms in redox state S_3, $\nabla H^+(\vec{r},t,S_3)$, is the key player for linking them together to an O-O bond, and (b) the dependence on space vector $\vec{r}$ determines the population probability of the three S_3 state configurations ($M_3L_0W_0$, $M_2(LW)_1$ and $M_1L_0W_2$), and the time dependence due to the dynamics of the hydrogen bond network modulates the fast equilibration between these electronic configurations of redox state S_3. Information on $\nabla H^+(\vec{r},t,S_3)$ is lacking due to limited structure information. However, it is clear that protein dynamics and flexibility are essential for a functional competent WOC because the S_i-state transitions are thermally blocked below characteristic threshold temperatures (see section III.C.2.c). Likewise a decrease of sample hydration leads to suppression of these reactions at room temperature (vide supra).

The idea of a key mechanistic role of protons in O-O bond formation is in line with suggestions of Liu et al. (2008) and it gains further support from the wealth of information on the functional relevance of the mode of protonation for processes running in the opposite direction, i.e., cleavage of the O-O bond in oxygen enzymology (see Santoni et al., 2004; Yikilmaz et al., 2006; Blomberg and Siegbahn, 2006; Augustine et al., 2007; Wang et al., 2008; Davydov et al., 2008) and in model systems (Soper et al., 2007; Li et al., 2010).

In addition to $\nabla H^+(\vec{r},t,S_3)$, structural changes of the ligand geometry can markedly reorient the redox active orbital of the manganese thus modulating the reactivity by orders of magnitude (for a general discussion on the modulation of the properties of metal-oxo complexes by ligand fields, see Betley et al., 2008). An illustrative example of this effect is the tuning of the reactivity of the binuclear nonheme iron center in the catalytic site of some oxygenases by reorientation of a Glu ligand due to the binding of a "regulator" protein (Schwartz et al., 2008).

The postulated role of $\nabla H^+(\vec{r},t,S_3)$ and possible conformational effects on "orbital steering" implies that the local protein environment is an active part of the catalytic site and not simply the scaffold for proper fixing of the Mn_4O_XCa cluster and the substrate molecules.

The Mn_4O_XCa cluster of the WOC is shielded to the aqueous lumenal phase by several extrinsic proteins in all oxygen-evolving organisms (see Enami et al., 2008; Bricker and Frankel, 2011; Bricker et al., 2011) and references therein). Therefore questions arise on the pathways of substrate (H_2O) and products (O_2 and H^+). Based on the finding of a putative channel for the transport of the substrate O_2 to the catalytic binuclear site in cytochrome *c* oxidase (Riistama et al., 1996), an analogous pathway was speculated to exist in the WOC for release of the product O_2 (Renger, 1999). Likewise, Anderson (2001) postulated the existence of an O_2 channel in the WOC. Progress in resolution of the PS II structure has paved the way for an analysis of this idea. Theoretical studies led to proposals for both substrate and product transport channels (Murray and Barber, 2007; Ho and Styring, 2008). These calculations also suggest that in the WOC, substrate uptake and product release probably take place via different pathways. In the WOC, the transport function for O_2 and H_2O is a mirror image of the situation in cytochrome oxidases. In the case of oxidative water spitting, the substrate concentration of the environment is rather high (order of 50 M) and the product concentration rather low (order of 250 μM in air saturated water at room temperature).

Crystallographic studies on Kr and Xe binding in cytochrome ba_3 from *Thermus thermophilus* revealed the existence of a continuous Y-shaped channel that is lined by hydrophobic amino acid residues, extending from the protein surface to the catalytic binuclear heme $a_3 - Cu_B$ center. Based on these data, Luna et al. (2008) proposed

a concerted sequence for the transfer steps where the water molecule formed at the catalytic site is repelled by a hydrophobic surface of the O_2 channel. In cytochrome *c* oxidases the channelling is different and probably requires some protein motions/rearrangements to permit access of O_2 to the active site. This mode of channeling of O_2 to the catalytic site may provide adaptation to environments with different saturation levels of O_2 (Luna et al., 2008).

On the contrary, in PS II the removal of the O_2 product from the catalytic site is probably of key relevance in order to permit rapid substrate/product exchange and in addition to diminish the probability of $^1\Delta_g O_2$ formation by hampering the contact of O_2 with the ^{3}P680 state that is populated via $P680^{+\bullet}Q_A^{-\bullet}$ recombination in PS II (see Section III.C.3).

The transport of water to the catalytic site and the O_2 / H_2O exchange must be sufficiently fast in order to permit a high turnover of the WOC under saturating actinic light. The rate-limiting step of the electron transport chain is the PQH_2 reoxidation at the Cyt b_6f complex with a rate constant of the order of $(10\ ms)^{-1}$. This number exceeds the rate constant of the slowly exchanging water of $H_2{}^{16}O/H_2{}^{18}O$ replacement (see Hillier and Messinger, 2005; Hillier and Wydrzynski, 2008) by at least one order of magnitude. Therefore, the latter exchange does neither reflect the kinetics of substrate transport nor the rapid exchange reaction $M_0L_0W_4 + 2H_2O \rightleftarrows M_0L_0W_0 + O_2 + n_4H^+$. It is rather indicative of comparatively slow exchange reactions of the water molecules around the Mn_4O_XCa cluster in the intermediary redox states S_0, S_1, S_2 and S_3. Therefore, a retardation rather than acceleration of these exchange rates after removal of the extrinsic proteins PsbP and PsbQ in PS II core complexes from spinach (Hillier and Messinger 2005; Hillier and Wydrzynski, 2008) is not surprising. The substrate water transport to the catalytic site was estimated to occur within a few milliseconds (Messinger et al., 1991).

The "chemical" protons released from the catalytic site are probably funnelled via Asp61 of polypeptide D1 (McEvoy and Brudvig, 2006; Meyer et al., 2007; Guskov et al., 2010) into a pathway of the extrinsic PsbO protein that is an essential constituent of the WOC in all oxygen-evolving organisms (see Enami et al., 2008 and references therein). Based on the unusual titration behaviour of soluble PsbO, Shutova et al. (1997) concluded that Glu and Asp residues in this protein provide an exit pathway of H^+ into the lumen; a more refined analysis supported this idea (Shutova et al., 2007). Likewise theoretical calculations favor the proposal of H^+ transport pathways in the PsbO protein (Ishikita et al., 2006).

3. Alternative Reactions of $P680^{+\bullet}$: Recombination with $Q_A^{-\bullet}$ and $Pheo^{-\bullet}$ Including $^3P680$ Triplet Formation

The main electron transfer pathway from the WOC to $P680^{+\bullet}$ via Y_Z is always in competition with the recombination reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$. The lifetime of the radical pair $P680^{+\bullet}Q_A^{-\bullet}$ is limited by the tunneling rate of direct electron transfer leading to $P680Q_A$ in its ground state. Structural information and application of a rate constant-distance relationship (Page et al., 1999) based on the Marcus theory of NET (Marcus and Sutin, 1985) leads to values of about $(5\ ms)^{-1}$ (for details, see Renger and Holzwarth, 2005), which are in good correspondence with the experimental value of $(2.5\ ms)^{-1}$ (Reinman and Mathis, 1981). In systems lacking a functionally competent WOC the re-reduction of Y_Z^{OX} is comparatively slow. If Y_Z^{OX} is kept oxidized, $P680^{+\bullet}$ recombines with $Q_A^{-\bullet}$ with a dominant time constant of 150–200 μs (Haveman and Mathis, 1976; Renger and Wolff, 1976). This value is not markedly affected by the redox state of Y_Z, is virtually independent of pH between 5 and 8 and is invariant to replacement of exchangeable protons by deuterons (Christen et al., 1997). These kinetics are about one order of magnitude faster than the direct electron transfer pathway from $Q_A^{-\bullet}$ to $P680^{+\bullet}$ (vide supra) and therefore indicative of an alternative channel, which presumably involves the uphill regeneration of $P680^{+\bullet}Pheo_{D1}^{-\bullet}$ followed by either radiative or nonradiative charge recombination. The contribution to the overall $P680^{+\bullet}Q_A^{-\bullet}$ decay of this thermally activated route, which depends on the free energy gap between $P680^{+\bullet}Pheo_{D1}^{-\bullet}$ and $P680^{+\bullet}Q_A^{-\bullet}$, has been estimated to be almost 80% in wild-type cells of *Synechocystis* sp. PCC 6803 (Rappaport et al., 2002) and the green alga *Chlamydomonas reinhardtii* (Cuni et al., 2004).

The $P680^{+\bullet}Q_A^{-\bullet}$ recombination has two important physiological implications: (a) dissipation of

oxidizing equivalents that are required for the redox advancement in the WOC, thus giving rise to a miss parameter of these processes (Kok et al., 1970) and (b) recombination comprising the intermediary $P680^{+\bullet}Pheo_{D1}^{-\bullet}$ formation can lead to significant population of the triplet state $^3P680$ (vide infra). Based on an analysis of the H/D exchange effect on the damping of the period-four oscillation pattern of flash-induced oxygen evolution, the 150–200 μs kinetics are inferred to dominate the pathway of $P680^{+\bullet}Q_A^{-\bullet}$ recombination also in PS II complexes from higher plants (Christen et al., 1999).

In addition to the stabilization reaction in "open" PS II complexes leading to $P680^{+\bullet}Q_A^{-\bullet}$ formation with 300 ps kinetics (see Section III.B.1.a), essentially three decay pathways of the radical ion pair $^1[P680^{+\bullet}Pheo^{-\bullet}]$ have to be taken into consideration: (i) back reaction to the electronically excited singlet state $^1P680^*$; (ii) spin dephasing under formation of the triplet radical pair $^3[P680^{+\bullet}Pheo^{-\bullet}]$ followed by recombination into the triplet state $^3P680$, and (iii) direct recombination of $^1[P680^{+\bullet}Pheo^{-\bullet}]$ into the ground state.

Among these competing pathways the extent of $^3P680$ formation is of physiological relevance because chlorophyll triplets give rise to generation of singlet oxygen ($^1\Delta_g O_2$) via the sensitized reaction:

$$^3Chl + {}^3\Sigma_g O_2 \rightarrow {}^1Chl + {}^1\Delta_g O_2 \quad (17.12)$$

The species $^1\Delta_g O_2$ is very reactive and leads to oxidative degradation reactions (for further details, see Vass and Aro, 2008).

Chlorophyll triplets in the antenna are most efficiently quenched by rapid energy transfer to Car with a rate that is much faster than ^3Chl formation (Schödel et al., 1998). In the case of $^3P680$, however, this process is inefficient owing to the large distance between the chlorins of RC-PC and the two β-carotenes in the D1/D2 heterodimer (see Fig. 17.2) Therefore, the physiologically relevant role of these β-carotenes is probably the quenching of $^1\Delta_g O_2$ (Telfer, 2002) generated according to Eq. 17.12. In addition to $^1\Delta_g O_2$ suppression, the β-carotenes most likely participate in a protective cyclic electron transport pathway that involves Cyt b_{559} (Poulson et al., 1995; Stewart and Brudvig, 1998; Gadjeva et al., 1999; Kaminskaya et al., 2003; Faller et al., 2005; Pogson et al., 2005). This function becomes relevant when the normal function of water splitting is absent or disturbed (see Section III.C.1). Furthermore, the location of $^3P680^*$ on Chl_{D1} rather than on $P_{D1} - P_{D2}$ (see Section III.C.2.c) favours triplet quenching by $Q_A^{-\bullet}$ (Van Miegham et al., 1995; Noguchi, 2002). The implications of $^3P680$ formation for photodamage are beyond the scope of this chapter (for discussion, see Krieger-Liszkay and Rutherford, 1998; Vass and Aro, 2008).

D. PQH_2 Formation

1. Two-Step Reaction Sequence Induced by $Q_A^{-\bullet}$

The formation of PQH_2 takes place in a special pocket, the Q_B site of PS II, via a sequence of two 1-electron redox steps with $Q_A^{-\bullet}$ as reductant. This reaction pattern closely resembles that of the UQH_2 formation in PBRCs (for reviews, see Crofts and Wraight, 1983; Petrouleas and Crofts, 2005; Lancaster, 2008; Parson, 2008; Renger and Renger, 2008).

a. Kinetics and Energetics

In the first electron transfer step $Q_A^{-\bullet}$ reduces a PQ-9 molecule, bound at the Q_B pocket, to the semiquinone $Q_B^{-\bullet}$ with a time constant of 200–400 μs and this is followed by the second redox step leading to formation of PQH_2 with somewhat slower kinetics of 500–800 μs (Robinson and Crofts, 1984; Weiss and Renger, 1984; Renger et al., 1995; de Wijn and Van Gorkom, 2001). These reactions exhibit striking temperature dependences with a characteristic break point in the range of physiological temperatures, reflecting the role of the protein (and lipid?) environment. Activation energies of about 15 and 30 kJ mol^{-1} above and below, respectively, a temperature ϑ_C of about 20°C were gathered from fluorescence measurements on *Arabidopsis thaliana* leaves. This characteristic temperature decreased to 12°C in a mutant with reduced digalactosyldiacylglycerol content (Reifarth et al., 1997). A more pronounced effect was observed in cells of mesophilic and thermophilic cyanobacteria. In this case, the electron transfer from $Q_A^{-\bullet}$ to Q_B is virtually independent of temperature above a ϑ_C value that is different in both types of organisms. Measurements on different mutants of polypeptide D1 (residues at positions 209 and 212) lead to the conclusion that locally flexible domains

within the protein matrix are responsible for this effect (Shlyk-Kerner et al., 2006).

As in the case of the oxidation steps of the WOC (see Section III.C.2.c) the redox reactions at the Q_B site are also triggered reactions because an evaluation on the basis of the rate constant-distance relationship of NET (Page et al., 1999) using the structural data (Loll et al., 2005) and the values of the above activation energies leads to rate constants of the order of 10 μs, i.e., numbers that are at least one order of magnitude shorter than the experimental data (Renger and Renger, 2008).

The plastosemiquinone form is characterized by a high affinity to the Q_B site while PQH_2 is only weakly bound and exchanges with a PQ molecule from the pool (vide infra). A high efficiency of PQ reduction to PQH_2 requires a sufficiently low reduction potential of $Q_A^{-\bullet}$ and a fast PQH_2 / PQ exchange. E_m values in the range of −50 to −100 mV appear to be a reasonable estimate for PS II complexes with an intact WOC (see Section III.A.1). Under this condition the PQH_2 formation via $Q_B^{-\bullet}$ as intermediate is thermodynamically favored. An equilibrium constant of about 20 was reported for the reaction $Q_A^{-\bullet}Q_B$ <====> $Q_AQ_B^{-\bullet}$ in thylakoids from higher plants (Bowes et al., 1980; Vermaas et al., 1984). However, for systems lacking the WOC the reduction potential of $Q_A^{-\bullet}$ was found to increase by about 120–150 mV (Krieger et al., 1995; Shibamoto et al., 2009; Ito et al., 2011). As a consequence of this effect, the driving force for $Q_B(Q_B^{-\bullet})$ reduction by $Q_A^{-\bullet}$ is drastically diminished and a large fraction of PS II centers become "closed". This energetic shift is likely to be of physiological relevance for newly synthesized PS II complexes that do not contain a functionally competent WOC (Strasser and Sironval, 1972; Cheniae and Martin, 1973). In this case, the back reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$ could protect against the destructive action of the strongly oxidizing $P680^{+\bullet}$ radical (Renger and Wolff, 1976). Among the possible pathways for the back reaction one of these leads to population of the triplet state $^3P680$ which acts as sensitizer for singlet oxygen formation (see, Section III.C.3). A shift of the reduction potential of $Q_A^{-\bullet}$ towards a more positive value diminishes the probability not only of $^3P680$ population but also of superoxide formation and therefore provides additional protection against rapid photo-degradation of PS II complexes without the WOC (for further reading, see Krieger-Liszkay and Rutherford, 1998; Chow and Aro, 2005; Vass and Aro, 2008). A decrease of the energy gap between $Q_A^{-\bullet}/Q_A$ and $Q_B^{-\bullet}/Q_B$ was recently observed in a mutant where the strongly conserved D2-Q129 in the neighbourhood of $Pheo_{D2}$ was replaced by His or Leu. In this case, however, the value of $E_m(Q_B^{-\bullet}/Q_B)$ decreased while $E_m(Q_A^{-\bullet}/Q_A)$ remained virtually unaffected (Perrine and Sayre, 2011). This shift gives rise to an increased probability of $S_2\ Q_B^{-\bullet}$ recombination thus leading to higher susceptibility to photoinhibition.

b. Protolytic Reactions and Possible Role of the Nonheme Iron (NHFe) Center

The overall reaction sequence is coupled with the stepwise uptake of two protons from the cytoplasmic/stroma side (for a reaction scheme, see Petrouleas and Crofts, 2005). The H^+ transfer to $Q_B^{-\bullet}$ and Q_BH^- in PBRCs occurs via a hydrogen-bond network in the protein environment, including carboxylic groups and bound water molecules (Hienerwadel et al., 1995; Paddock et al., 1995; Tandori et al., 1999) and probably different pathways to each of the two carbonyl-oxygens of the quinone head group of Q_B (Koepke et al., 2007). A reorientation of the OH group of residue Ser-L223 (shift of hydrogen bonding with Asp-L213 to $Q_B^{-\bullet}$) is assumed to be an important step in the H^+-transfer to reduced quinone in RCs from *Rhodobacter sphaeroides* (see Nabedryk et al., 2005; Lancaster, 2008). FTIR measurements revealed that in PS II CC from *T. elongatus* the formation of $Q_B^{-\bullet}$ is not coupled with the protonation of a carboxylic amino acid residue, thus indicating that the mechanism of protonation is different in PBRCs and PS II (Suzuki et al., 2005). This phenomenon might be related to the striking difference in the coordination of the nonheme iron (NHFe) by Glu in PBRCs (see Lancaster, 2008) and HCO_3^- in PS II (see Kern and Renger, 2007; Zouni, 2008; Guskov et al., 2010). The mode of NHFe ligation probably modulates the properties of a hydrogen bond network, which is assumed to be involved in the proton transfer reactions that are necessary for the formation of

PQH_2. It also gives rise to the marked bicarbonate effect in PS II discovered by Govindjee and coworkers (Eaton-Rye and Govindjee, 1988a, b, for a review, see Govindjee and Van Rensen, 1993).

The possible functional role of the conserved NHFe center which is localized between Q_A and Q_B in both PBRCs (for a review, see Lancaster, 2008) and PS II (see Fig. 17.2 and for review, Zouni, 2008; Guskov et al., 2010) is not yet fully resolved. As a transition metal center with a partially filled 3 d electron shell, the NHFe might be a potential candidate for electronic coupling between Q_A and Q_B, thus facilitating the electron transfer from $Q_A^{-\bullet}$ to $Q_B(Q_B^{-\bullet})$. The midpoint potential E_m of the $NHFe^{2+}/Fe^{3+}$ system in PS II was found to be about +400 mV at pH 7.0 with a pH dependence of –60 mV/pH unit between pH 5.3 and 8.5 (Bowes et al., 1979; Wraight, 1985; Petrouleas and Diner, 1986; Renger et al., 1987). These E_m values seem to be unfavourable for a direct participation of the NHFe as a redox active intermediate in the electron transfer processes leading to PQH_2 formation at the Q_B site. Direct evidence for the NHFe being not required as intermediary redox carrier is the finding that replacement of the NHFe center by the redox inert Zn^{2+} in PBRCs has little or no effect on the rate of $Q_A^{-\bullet}$ reoxidation by Q_B (Debus et al., 1986; Kirmaier et al., 1986). It is therefore likely that the NHFe plays a structural or electrostatic role. QM/MM calculations reveal that the NHFe affects the spin density distribution within $Q_A^{-\bullet}$ thus modulating the E_m value of $Q_A^{-\bullet}/Q_A$. It is speculated that this effect might tune the rate of electron transfer from $Pheo_A^{-\bullet}$ to Q_A (Lin and O'Malley, 2011). Other compounds with carboxylic groups can replace the HCO_3^- ligation of PS II. Of special interest are metabolites of the Calvin-Benson cycle (see Section II), which might trigger a feedback mechanism in the control of electron transfer activity (Petrouleas et al., 1994).

2. Structure of the Q_B Site and Quinol/Quinone Exchange Reactions

Recent progress in the resolution of the X-ray diffraction crystallographic analysis of PS II CC form *T. elongatus* (Loll et al., 2005; Guskov et al., 2010; Umena et al., 2011) revealed that the Q_B pocket of PS II is exclusively formed by the D1 protein. This site is shown to be characterized by three structural elements (see Kern and Renger, 2007): (i) the C-terminal part from Gly207 to Val219 of transmembrane helix d; (ii) the cytosolic surface helices de (1) and de (2) including the connecting loop region, and (iii) the N terminus part ranging from Arg269 to Phe274 of transmembrane helix e. Amino acids in the Q_B site include Met214, His215, Leu218, Val219, Tyr246, His252, Phe255, Ser264, Phe265 and Leu271. The structural data reveal that hydrogen bonds can be formed between the substrate molecule PQ-9 and amino acid residues Ser264 and His215 as well as with the backbone amide of Phe265. The structural arrangement suggests that the environment of Q_B is flexible and lipophilic thus favoring the Q_B/Q_BH_2 exchange through the inner membrane-facing opening (for further structural details, see Zouni, 2008; Guskov et al., 2010). This arrangement could facilitate the diffusion of PQH_2 into lipid bilayer sections of the thylakoid membrane because neutron scattering experiments on the localization of UQ in artificial lipid bilayer systems support the idea that all natural polyisoprene chains lie in the central part without any significant protrusion into the fatty acid chain region (Hauß et al., 2005). Furthermore, the X-ray diffraction crystallographic data of Guskov et al. (2010) clearly illustrate that two charged lipid molecules are located next to the Q_B site: sulfoquinovosyldiacylglycerol at a distance of 10.2 Å between its sugar head group and the PQ-ring and a phosphatidylglycerol which is about 19 Å apart and positioned almost equidistant between Q_A and Q_B. It is clear that lipids are an integral part of the D1/D2 heterodimer. This feature supports conclusions gathered from studies on an essential functional role of lipids in PS II (see Gombos et al., 2002; Steffen et al., 2005b and references therein). In PBRCs the negatively charged cardiolipin was shown to cause a slight shift (30–40 mV) of the reduction potential of $Q_A^{-\bullet}$ towards more negative values (Rinyu et al., 2003).

The Q_B pocket is also the binding site for numerous herbicides that block the linear electron transport chain by suppression of $Q_A^{-\bullet}$ reoxidation by Q_B but this important topic is beyond

the scope of this chapter (for reviews, see Oettmeier 1992, 1999; Petrouleas and Crofts, 2005).

3. Electron Transfer and Protein Flexibility

The electron transfer form $Q_A^{-\bullet}$ to Q_B in PBRCs requires structural flexibility of the protein matrix (for reviews, see Lancaster, 2008; Parson, 2008). Likewise, the $Q_A^{-\bullet}$ reoxidation by Q_B (Q_B^-) in PS II also depends strongly on the conformational flexibility of the protein as is illustrated by the striking temperature dependence with characteristic thermal thresholds for the onset of $Q_A^{-\bullet}$ reoxidation. The temperature, which enables 50% activity, is higher for electron transfer to Q_B than to $Q_B^{-\bullet}$ in both spinach thylakoids and PS II membrane fragments (Renger et al., 1993; Reifarth and Renger, 1998). A similar feature was found in PS II CC from thermophilic cyanobacteria (Fufezan et al., 2005) indicating that it is a general phenomenon in PS II. Mößbauer spectroscopy studies using ^{57}Fe labelled NHFe as a probe (Garbers et al., 1998) and measurements of quasielastic neutron scattering (QENS) (Kühn et al., 2005; Pieper et al., 2007) revealed that the threshold temperature for increased protein flexibility is virtually the same as for the onset of $Q_A^{-\bullet}$ reoxidation by Q_B. In this respect it is interesting to note that a "controlled" protein dynamics rather that the overall flexibility is probably essential for function. Sacquin-Mora et al. (2007) found that in non-functional mutants of *Rhodobacter sphaeroides* the protein flexibility is even higher than in the competent wild type. In this case individual amino acid residues (the conserved Glu212 of the L subunit) seem to be essential for preserving the "functional" protein flexibility. Hydrogen bond networks most likely play a key role. This idea is supported by the finding that water molecules are essential in sustaining the required mobility. Dehydration was shown to block the electron transfer from $Q_A^{-\bullet}$ to Q_B in PBRCs (Nikolaev et al., 1980; see also Lancaster, 2008). Water molecules act as "plasticizers" and systems with low water content exhibit high conformational rigidity (Careri et al., 1980; Zaks and Klibanov, 1984). Studies on PS II membrane fragments from higher plants revealed that a decrease of the hydration levels leads to blockage of $Q_A^{-\bullet}$ reoxidation and a significant modification of the reaction pattern of Cyt b_{559} (Kaminskaya et al., 2003). The effect of dehydration on the protein dynamics in PS II is clearly illustrated by QENS data (Kühn et al., 2005; Pieper et al., 2008; Pieper and Renger 2009). Figure 17.10 shows that the sharp increase of the mobility above the threshold temperature, which correlates with the onset of $Q_A^{-\bullet}$ reoxidation activity, is completely suppressed upon dehydration.

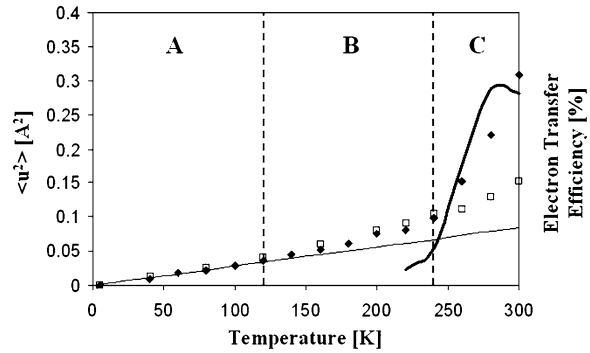

Fig. 17.10. Temperature dependence of the atomic mean square displacement $\langle u^2 \rangle$ gathered from QENS spectra of PS II membrane fragments from spinach. Samples are hydrated with D_2O at 90% (filled diamonds) or dried to values below 30% relative humidity (open squares). The straight line represents the contribution of harmonic oscillations extrapolated to high temperatures and the curved lined reflects the charge transfer efficiency from Q_A^- to Q_B (The QENS data are taken from Kühn et al. (2005), the data of $Q_A^{-\bullet}$ reoxidation from Renger et al. (1993)).

4. Turnover of PQ/PQH$_2$

The electron transfer reactions of PS II are markedly faster than the rate-limiting step of the linear electron transport chain from H_2O to $NADP^+$ (for a review, see Stiehl and Witt, 1969). Therefore, the quinol replacement by quinone at the Q_B site limits the turnover of PS II under continuous illumination. There exists a pool of PQ molecules. Values ranging from 7–40 are reported for thylakoids and cyanobacterial cells (Hauska and Hurt, 1982). As a consequence the probability of PQ occupancy at the Q_B site depends on the redox state of the pool. The overall process leading to a steady state of the PQ:PQH$_2$ ratio includes the diffusion of PQH_2 to the Cyt b_6f complex,

followed by reoxidation to PQ (for a review, see Cramer et al., 2008) and migration back to the Q_B site. Analogous reactions take place in purple bacteria where UQH_2 becomes reoxidized at the Cyt *bc* complex (for details, see Vermeglio, 2008). The lateral diffusion of quinols and quinones can be responsible for a kinetic limitation of the overall linear electron transport chain as outlined in (Haehnel, 1984). The mobility of PQ and PQH_2 depends on the array of the complexes in the membrane because the collisional encounters with proteins decrease the effective diffusion coefficient. This effect has been clearly illustrated by several studies on both purple bacteria cells and plants (Blackwell et al., 1994; Joliot et al., 1992, 1997; Lavergne et al., 1992; Verméglio et al., 1998; see also Vermeglio, 2008). Earlier experiments revealed that up to ten different PS II complexes in the thylakoid membrane are functionally coupled via a common PQ pool (Siggel et al., 1972).

In addition to the electron transfer reactions the redox state of the PQ pool also affects the regulation of the electronic excitation of PS I and PS II (for a review see Allen and Nilsson, 1997) and most likely also the ratio of cyclic to non cyclic electron transport (Iwai et al., 2010).

Recently an additional control mechanism has been proposed for the PQ/PQH_2 system. Kaminskaya et al. (2007) have postulated that a further binding site Q_C exists in the vicinity of Cyt b_{559} that controls the unique high reduction potential of its heme group. A switch of the redox properties of Cyt b_{559} is assumed to play an important role in the control of electron transfer reactions that are involved in the protection of PS II under light stress conditions (for a review on the protective role of Cyt b_{559}, see Pospisil, 2011). The Q_C site is expected to be specific for PS II because Cyt b_{559} is an integral constituent of all PS II RCs while in marked contrast none of the type II RCs of anoxygenic photosynthetic bacteria contains this special heme protein.

The existence of Q_C has been confirmed by X-ray diffraction crytallography studies and it was concluded that this PQ binding site might be involved in PQH_2/PQ exchange at the Q_B pocket (Guskov et al., 2010; Müh et al., 2011).

IV. Concluding Remarks and Future Perspectives

This chapter presents a summary of our knowledge on the kinetics, energetics and mechanisms of photosynthetic water splitting in PS II. Unresolved mechanistic details are also outlined. Unravelling of the mode of coupling between electron and proton transfer in the reactions leading to oxidative water splitting and PQ reduction to PQH_2 and in particular a resolution of the reaction coordinate of O-O bond formation are challenging tasks of future research. Of special functional relevance is a deeper understanding of the role of protein dynamics. Effects of protein relaxation on the energetics and efficiency of light-induced charge separation and the subsequent redox reactions illustrate that it is more appropriate to consider several enzyme complexes as functional and structural entities. Accordingly, PS II with its water splitting activity should be considered as a specifically tailored molecular machine (Renger, 2004). It is expected that the enormous progress achieved through the techniques of X-ray diffraction crystallography, genetic engineering and monitoring protein dynamics in combination with highly advanced theoretical approaches in the field of quantum chemistry and molecular mechanics will pave the road to a higher level of understanding of biocatalysts in general and the fascinating PS II of photosynthesis in particular.

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References

Ahlbrink R, Haumann M, Cherepanov D, Bögershausen D, Mulkidjanian O and Junge W (1998) Function of tyroszine Z in water oxidation by photosystem II: Electrostatical promoter instead of hydrogen abstractor. Biochemistry 37: 1131–1142

Åhrling KA, Peterson S and Styring S (1997) An oscillating manganese electron paramagnetic resonance signal from the S_0 state of the oxygen-evolving complex in photosystem II. Biochemistry 36: 13148–13152

Akiyama M, Miyashita H, Kise H, Watanabe T, Mimuro M, Miyachi S and Kobayashi M (2002) Quest for minor but key chlorophyll molecules in photosynthetic reaction centers – unusual pigment composition in the reaction centers of the chlorophyll *d*-dominated cyanobacterium *Acaryochloris marina*. Photosynth Res 74: 97–107

Allakhverdiev SI, Tomo T, Shimada Y, Kindo H, Nagao R, Klimov VV and Mimuro M (2010) Redox potential of pheophytin a in photosystem II of two cyanobacteria having the different special pair chlorophylls. Proc Nat Acad Sci USA 107: 3924–3929

Allen JF and Nilsson A (1997) Redox signalling and the structural basis of regulation of photosynthesis by protein phosphorylation. Physiol Plant 100: 863–868

Anderson AB and Albu TV (1999) Ab initio determination of reversible potentials and activation energies for outer-sphere oxygen reduction to water and the reverse oxidation reaction. J Am Chem Soc 121: 11855–11863

Anderson JM (2001) Does functional photosystem II complex have an oxygen channel? FEBS Lett 488: 1–4

Aoyama C, Suzuki H, Sugiura M and Noguchi T (2008) Flash-induced FTIR difference spectroscopy shows no evidence for the structural coupling of bicarbonate to the oxygen-evolving Mn cluster in photosystem II. Biochemistry 47: 2760–2765

Augustine AJ, Quintanar L, Stoj CS, Kosman, DJ, Solomon EI (2007) Spectroscopic and kinetic studies of perturbed trinuclear copper clusters: The role of protons in reductive cleavage of the O-O bond in the multicopper oxidase Fet3p. J Am Chem Soc 129: 13118–13126

Babcock GT, Blankenship RE and Sauer K (1976) Reaction kinetics for positive charge accumulation on the water side of chloroplast photosystem II. FEBS Lett 61: 286–289

Babcock GT and Wikström M (1992) Oxygen activation and the conservation of energy in cell respiration. Nature 356**:** 301–309

Bader KP, Renger G and Schmidt GH (1993) A mass spectroscopic analysis of the water-splitting reaction. Photosynth Res 38: 355–361

Bao H, Zhang C, Ren Y and Zhao J (2010) Low-temperature electron transfer suggests two types of Q_A in intact photosystem II. Biochim Biophys Acta 1797: 339–346

Barter LMC, Bianchietti M, Jeans C, Schilstra MJ, Hankamer B, Diner BA, Barber J, Durrant JR and Klug DR (2001) Relationship between excitation energy transfer, trapping, and antenna size in photosystem II. Biochemistry 40: 4026–4034

Barter LMC and Klug DR (2005) A unified picture of energy and electron transfer in primary photosynthesis. Chem Phys 319: 308–315

Bell LN and Gudkov ND (1992) Thermodynamics of light energy conversion. In: Barber J (ed) Topics in Photosynthesis, The Photosystems: Structure, Function and Molecular Biology, pp 17–43. Kluwer Academic Publishers, Dordrecht

Benson AA (2002) Following the path of carbon in photosynthesis: A personal story. Photosynth Res 73: 29–49

Bernarding J, Eckert HJ, Eichler HJ, Napiwotzki A and Renger G (1994) Kinetic studies on the stabilisation of the primary radical pair $P680^+Pheo^-$ in different photosystem II preparations from higher plants. Photochem Photobiol 59: 566–573

Berthomieu C and Hienerwadel R (2005) Vibrational spectroscopy to study the properties of redox-active tyrosines in photosystem II and other proteins. Biochim Biophys Acta 1707: 51–66

Betley TA, Wu Q, Van Voorhis T and Nocera DG (2008) Electronic design criteria for O−O bond formation via metal−oxo complexes. Inorg Chem 47: 1849–1861

Bianco R, Hay PJ and Hynes JT (2011) Theoretical study of O–O single bond formation in the oxidation of water by the ruthenium blue dimer. J Phys Chem A115: 8003–8016

Bibby TS, Nield J, Chen M, Larkum AWD and Barber J (2003) Structure of a photosystem II supercomplex isolated from *Prochloron didemni* retaining its chlorophyll *a*/*b* light-harvesting system. Proc Natl Acad Sci USA 100: 9050–9054

Biesiadka J, Loll B, Kern J, Irrgang K-D and Zouni A (2004) Crystal structure of cyanobacterial photosystem II at 3.2 Å resolution: a closer look at the Mn-cluster. Phys Chem Chem Phys 6: 4733–4736

Blackwell M, Gibas C, Gygax S, Roman D and Wagner B (1994) The plastoquinone diffusion coefficient in chloroplasts and its mechanistic implications. Biochim Biophys Acta 1183: 533–543

Blomberg MRA and Siegbahn PEM (2006) Different types of biological proton transfer reactions studied by quantum chemical methods. Biochim Biophys Acta 1757: 969–980

Bögershausen O, Haumann M and Junge W (1996) Photosynthetic oxygen evolution: H/D isotope effects and the coupling between electron and proton transfer during transitions S_2 to S_3 and S_3 to S_4 to S_0 . Ber Bun Gesell Chem Phys Phys Chem 100: 1987–1992

Boekema EJ, Hankamer B, Bald D, Kruip J, Nield J, Boonstra AF, Barber J and Rögner M (1995) Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. Proc Natl Acad Sci USA 92: 175–179

Boussac A and Etienne A-L (1984) Midpoint potential of signal II (slow) in tris-washed photosystem-II particles. Biochim Biophys Acta 766: 576–581

Boussac A and Rutherford AW (1988) Nature of the inhibition of the oxygen-evolving enzyme of photosystem II induced by NaCl washing and reversed by the addition of Ca^{2+} or Sr^{2+}. Biochemistry 27: 3476–3483

Bowes JM, Crofts AR and Itoh S (1979) High potential acceptor for photosystem II. Biochim Biophys Acta 547: 320–335

Bowes JM, Crofts AR and Arntzen CJ (1980) Redox reactions on the reducing side of photosystem II in chloroplasts with altered herbicide-binding properties. Arch Biochem Biophys 200: 303–308

Brettel K, Schlodder E and Witt HT (1984) Nanosecond reduction kinetics of photooxidized chlorophyll-a_{II} (P-680) in single flashes as a probe for the electron pathway, H^+-release and charge accumulation in the O_2-evolving complex. Biochim Biophys Acta 766: 403–415

Briantais JM, Vernotte C, Lavergne J and Arntzen CJ (1977) Identification of S_2 as the sensitive state to alkaline photoinactivation of photosystem II in chloroplasts. Biochim Biophys Acta 461: 61–74

Bricker TM and Frankel LK (2002) The structure and function of CP47 and CP43 in photosystem II. Photosynth Res 72: 131–146

Bricker TM and Frankel LK (2011) Auxiliary functions of the PsbO, PsbP and PsbQ proteins of higher plant photosystem II: A critical analysis. J Photochem Photobiol B: Biol 104: 165–178

Bricker TM, Roose JR, Fagerlund RD, Frankel LK and Eaton-Rye JJ (2011) The extrinsic proteins of photosystem II. Biochim Biophys Acta doi: 10.1016/j.bbabio.2011.07.006

Broess K, Trinkunas G, Van Hoek A, Croce R and Van Amerongen H (2008) Determination of the exciton migration time in photosystem II. Consequences for the membrane organization and charge separation parameters. Biochim Biophys Acta 1777: 404–409

Buick R (1992) The antiquity of oxygenic photosynthesis; evidence from stromatolites in sulphate-deficient Archaen lakes. Science 255: 74–77

Burda K, Bader KP and Schmid GH (2001) An estimation of the size of the water cluster present at the cleavage site of the water splitting enzyme. FEBS Lett 491: 81–84

Bylina EJ, Kirmaier C, McDowell L, Holten D and Youvan DC (1988) Influence of an amino-acid residue on the optical properties and electron transfer dynamics of a photosynthetic reaction centre complex. Nature 336: 182–184

Callahan EE, Beaker DW and Cheniae GM (1986) Studies on the photoactivation of the water-oxidizing enzyme. II. Characterization of weak light photoinhibition of PS II and its light-induced recovery. Plant Physiol 82: 79–88

Calvin M (1989) Forty years of photosynthesis and related activities. Photosynth Res 21: 3–16

Careri C, Gratton E, Yang P-H and Rupley JA (1980) Correlation of IR spectroscopic, heat capacity, diamagnetic susceptibility and enzymatic measurements on lysozyme powder. Nature 284: 572–573

Carugo O and Carugo-Djinovic K (2005) When X-rays modify the protein structure: radiation damage at work. Trends Biochem Sci 30: 213–219

Castelfranco PA, Lu Y-K and Stemler AJ (2007) Hypothesis: the peroxydicarbonic acid cycle in photosynthetic oxygen evolution. Photosynth Res 94: 235–246

Cheniae GM and Martin IF (1973) Absence of oxygen-evolving capacity in dark-grown *Chlorella*: the photoactivation of oxygen-evolving centers. Photochem Photobiol 17: 441–459

Chow WS and Aro E-M (2005) Photoinactivation and mechanism of recovery. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 627–648. Springer, Dordrecht

Christen G, Karge M, Eckert HJ and Renger G (1997) The role of protonation steps in electron transfer reactions in tris-treated PS II membrane fragments. Photosynthetica 33: 529–539

Christen G, Reifarth F and Renger G (1998) On the origin of the "35 ms kinetics" of $P680^{+\bullet}$ reduction in photosystem II with an intact water-oxidising complex. FEBS Lett 429: 49–52

Christen G, Seeliger A and Renger G (1999) $p_{680}^{+\bullet}$ reduction kinetics and redox transition probability of the water oxidising complex as a function of pH and H/D isotope exchange in spinach thylakoids. Biochemistry 38: 6082–6092

Chu HA, Hillier W and Debus RJ (2004) Evidence that the C-terminus of the D1 polypeptide of photosystem II is ligated to the manganese ion that undergoes oxidation during the S_1 to S_2 transition: An isotope-edited FTIR study. Biochemistry 43: 3152–3166

Clausen J and Junge W (2004) Detection of an intermediate of photosynthetic water oxidation. Nature 430: 480–483

Clausen J, Debus RJ and Junge W (2004) Time-resolved oxygen production by PSII: chasing chemical intermediates. Biochim Biophys Acta 1655: 184–194

Clausen J, Beckmann K, Junge W, Messinger J (2005) Evidence that bicarbonate is not the substrate in photosynthetic oxygen evolution. Plant Physiol 139: 1444–1450

Conjeaud H, Mathis P and Paillotin G (1979) The effect of pH on the reduction kinetics of P680 in tris-treated chloroplasts. Biochim Biophys Acta 48: 280–291

Cox N, Rapatskiy L, Su J-H,. Pantazis DA, Sugiura M, Kulik L, Dorlet P, Rutherford AW, Neese F, Boussac A, Lubitz W and Messinger J (2011) Effect of Ca^{2+}/Sr^{2+} substitution on the electronic structure of the oxygen-evolving complex of photosystem II: A combined multifrequency EPR, ^{55}Mn-ENDOR, and DFT study of the S_2 state. J Am Chem Soc 133: 3635–3648

Cramer WA, Zhang H, Yan J, Kurisu G, Yamashita E, Dashdorj N, Kim H, and Savikhin S (2008) Structure-function of the cytochrome b_6f complex: A design that has worked for three billion years. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 417–446. Royal Society Chemistry, Cambridge

Croce R and Van Amerongen H (2011) Light-harvesting and structural organization of photosystem II: From individual complexes to thylakoid membrane. J Photochem Photobiol B: Biol 104: 142–153

Crofts AR and Wraight CA (1983) The electrochemical domain of photosynthesis. Biochim Biophys Acta 726: 149–185

Cukier RI (2004) Theory and simulation of proton-coupled electron transfer, hydrogen-atom transfer, and proton translocation in proteins. Biochim Biophys Acta 1655: 37–44

Cuni A, Xiong L, Sayre R, Rappaport F and Lavergne J (2004) Modification of the pheophytin midpoint potential in photosystem II: Modulation of the quantum yield of charge separation and of charge recombination pathways. Chem Phys Phys Chem 6: 4825–4831

Davydov R, Osborne RL, Kim SH, Dawson JH and Hoffman BM (2008) EPR and ENDOR studies of cryoreduced compounds II of peroxidases and myoglobin. Proton-coupled electron transfer and protonation status of ferryl hemes. Biochemistry 47: 5147–5155

Debus R (2005) The catalytic manganese cluster: protein ligation. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastiquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 261–284. Springer, Dordrecht

Debus RJ, Feher G and Okamura MY (1986) Iron-depleted reaction centers from *Rhodopseudomonas sphaeroides* R-26.1: Characterization and reconstitution with Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. Biochemistry 25: 2276–2287

Debus R, Strickler MA, Walker LM and. Hillier W (2005) No evidence from FTIR difference spectroscopy that aspartate-170 of the D1 polypeptide ligates a manganese ion that undergoes oxidation during the S_0 to S_1, S_1 to S_2, or S_2 to S_3 transitions in photosystem II. Biochemistry 44: 1367–1374

Dekker JP, Plijter JJ, Ouwehand L and Van Gorkom HJ (1984) Kinetics of manganese redox transitions in the oxygen-evolving apparatus of photosynthesis. Biochim Biophys Acta 767: 176–179

de Marais DJ (2000) Evolution. When did photosynthesis emerge on Earth? Science 289: 1703–1705

de Wijn R and Van Gorkom HJ (2001) Kinetics of electron transfer from Q_A to Q_B in photosystem II. Biochemistry 40: 11912–11922

Dexheimer SL and Klein MP (1992) Detection of a paramagnetic intermediate in the S1 state of the photosynthetic oxygen-evolving complex. J Am Chem Soc 114: 2821–2826

Diner BA, Force DA, Randall DW and Britt RD (1998) Hydrogen bonding, solvent exchange, and coupled proton and electron transfer in the oxidation and reduction of redox-active tyrosine Y_Z in Mn-depleted core complexes of photosystem II. Biochemistry 37: 17931–17943

Diner BA, Schlodder E, Nixon PJ, Coleman WJ, Rappaport F, Lavergne J, Vermaas WFJ and Chisholm DA (2001) Site-directed mutations at D1-His198 and D2-His197 of photosystem II in *Synechocystis* PCC 6803: Sites of primary charge separation and cation triplet stabilization. Biochemistry 40: 9265–9281

Diner BA, Bautista JA, Nixon PJ, Berthomieu C, Hienerwadel R, Britt RD, Vermaas WFJ and Chisholm DA (2004) Coordination of proton and electron transferform the redox-active tyrosine, Y_Z, of photosystem II and examination of the electrostatic influence of oxidized tyrosine, $Y_D(H^+)$. Phys Chem Chem Phys 6: 4844–4850

Dismukes GC and Siderer Y (1981) Intermediates of a polynuclear manganese cluster involved in photosynthetic oxidation of water. Proc Natl Acad Sci USA 78: 274–278

Durrant JR, Hastings G, Joseph DM, Barber J, Porter G and Klug DR (1992) Subpicosecond equilibration of excitation energy in isolated photosystem II reaction centers. Proc Natl Acad Sci USA 89: 11632–1163

Duysens LMN and Sweers HE (1963) Mechanism of two photochemical reactions in algae as studied by means of fluorescence. In: Takamiya A and Shibata K (eds) Studies on Microalgae and Photosynthetic Bacteria, pp 353–372. Univ Tokyo Press, Tokyo

Eaton-Rye JJ and Govindjee (1988a) Electron transfer through the quinone acceptor complex of photosystem II in bicarbonate-depleted spinach thylakoid membranes as a function of actinic flash number and frequency. Biochim Biophys Acta 935: 237–247

Eaton-Rye JJ and Govindjee (1988b) Electron transfer through the quinone acceptor complex of photosystem II after one or two actinic flashes in bicarbonate-depleted spinach thylakoid membranes. Biochim Biophys Acta 935: 248–257

Eaton-Rye JJ and Putnam-Evans C (2005) The CP47 and CP43 core antenna components. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 139–175. Springer, Dordrecht

Eckert H-J and Renger G (1988) Temperature dependence of $P680^+$ reduction in O_2-evolving PS II membrane fragments at different redox states S_i of the water oxidizing system. FEBS Lett 236: 425–431

Eckert H-J, Renger G, Bernarding J, Faust P, Eichler HJ and Salk J (1987) Examination of fluorescence lifetime and radical pair decay in PS II membrane fragments from spinach. Biochim Biophys Acta 893: 208–218

Eckert H-J, Wiese N, Bernarding J, Eichler HJ and Renger G (1988) Analysis of the electron transfer from $Pheo^-$ to Q_A in PS II membrane fragments from spinach by time resolved 325 nm absorption changes in the picosecond domain. FEBS Lett 240: 153–158

Eckert H-J, Geiken B, Bernarding J, Napiwotzki A, Eichler HJ and Renger G (1991) Two sites of photoinhibition of the electron transfer in oxygen evolving and tris-treated PS II membrane fragments from spinach. Photosynth Res 27: 97–108

Enami I, Kitamura M, Tomo T, Isokawa Y Ohta H and Katoh S (1994) Is the primary course of thermal inactivation of oxygen evolution in spinach PS II membranes release of the extrinsic 33 kDa protein or of Mn? Biochim Biophys Acta 1186: 52–58

Enami I, Okumura A, Nagao R, Suzuki T, Iwai M and Shen JR (2008) Structures and functions of the extrinsic proteins of photosystem II from different species. Photosynth Res 98: 349–363

Engelmann ECM, Zucchelli Garlaschi GFM, Casazza AP and Jennings RC (2005) The effect of outer antenna complexes on the photochemical trapping rate in barley thyalkoid photosystem II. Biochim Biophys Acta 1706: 276–286

Faller P, Fufezan C and Rutherford AW (2005) Side-path electron donors: Cytochrome b_{559}, chlorophyll Z and β-carotene. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoredutase. Advances in Photosynthesis and Respiration, Vol 22, pp 347–365. Springer, Dordrecht

Ferreira K, Iverson TM, Maghlouni K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Flores M, Savitsky A, Paddock ML, Abresch EC, Dubinskii AA, Okamura MY, Lubitz W and Möbius K (2010) Electron−Nuclear and Electron−Electron Double Resonance spectroscopies show that the primary quinone acceptor Q_A in reaction centers from photosynthetic bacteria *Rhodobacter sphaeroides* remains in the same orientation upon light-induced reduction. J Phys Chem B 114: 16894–16901

Franzen LG, Styring S, Etienne AL, Hansson O and Vernotte C (1986) Spectroscopic and functional-characterization of a highly oxygen-evolving photosystem-II reaction center complex from spinach. Photobiochem Photobiophys 13: 15–28

Fromme R, Grotjohann I and Fromme P (2008) Structure and function of photosystem I. In: Renger G (ed) Primary Processes of photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 111–146. Royal Society Chemistry, Cambridge

Fufezan C, Drepper F, Juhnke HD, Lancaster CRD, Un S, Rutherford AW and Krieger-Liszkay A (2005) Herbicide-induced changes in charge recombination and redox potential of Q_A in the T4 mutant of *Blastochloris viridi*s. Biochemistry 44: 5931–5939

Fufezan C, Gross CM, Sjödin M, Rutherford AW, Krieger-Liszkay A and Kirilovsky D (2007) Influence of the redox potential of the primary quinone electron acceptor on photoinhibition in photosystem II. J Biol Chem 282: 12492–12502

Gadjeva R, Mamedov F, Renger G and Styring S (1999) Interconversion of low and high potential forms of cytochrome b_{559} in tris-washed photosystem II membranes under aerobic/anaerobic conditions. Biochemistry 38: 10578–10584

Garbers A, Kurreck J, Reifarth F, Renger G and Parak F (1998) Correlation between protein flexibility and electron transfer from $Q_A^{\bullet-}$ to Q_B in PS II membrane fragments from spinach. Biochemistry 37: 11399–11404

Gatt P, Stranger R and Pace RJ (2011) Application of computational chemistry to understanding the structure and mechanism of the Mn catalytic site in photosystem II – A review. J Photochem Photobiol B: Biol 104: 80–93

Germano M, Shkuropatov AY, Permentier H, de Wijn R, Hoff AJ, Shuvalov VA and Van Gorkom HJ (2001) Pigment organization and their interactions in reaction centers of photosystem II: Optical spectroscopy at 6 K of reaction centers with modified pheophytin composition. Biochemistry 40: 11472–11482

Gibasiewicz K, Dobek A, Breton J and Leibl W (2001) Modulation of primary radical pair kinetics and energetics in photosystem II by the redox state of the quinone electron acceptor Q_A. Biophys J 80: 1617–1630

Giera W, Ramesh VM, Webber AN, Van Stokkum I, Van Grondelle R and Gibasiewicz K (2010) Effect of the P700 pre-oxidation and point mutations near A_0 on the reversibility of the primary charge separation in photosystem I from *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1797: 106–112

Gläser M, Wolff C and Renger G (1976) Indirect evidence for a very fast recovery kinetics of chlorophyll-*a* in spinach chloroplasts. Z Naturforsch 31c: 712–721

Glatzel P, Bergmann U, Yano J, Visser H, Robblee JH, Gu WW, de Groot FMF, Christou G, Pecoraro VL, Cramer SP and Yachandra VK (2004) The electronic structure of Mn in oxides, coordination complexes, and the oxygen-evolving complex of photosystem II studied by resonant inelastic X-ray scattering. J Am Chem Soc 126: 9946–9959

Gleiter HM, Haag E, Inoue Y and Renger G (1993) New results on the functional properties of a photosystem II core complex preparation from spinach. Photosynth Res 35: 41–53

Gombos Z, Várkonyi Z, Hagio M, Iwaki M, Kovács L, Masamoto K, Itoh S, and Wada H (2002) Phosphatidylglycerol requirement for the function of electron acceptor plastoquinone Q_B in the photosystem II reaction center. Biochemistry 41: 3796–3802

Govindjee and Van Rensen JJS (1993) Photosystem II reaction centers and bicarbonate. In: Deisenhofer J and

Norris, JR (eds) Photosynthetic Reaction Centers, Vol I, pp 357–389. Academic Press, Orlando

Gray MW (1992) The endosymbiont hypothesis revisited. Int Rev Cytol 141: 233–257

Guskov A, Gabdulkhakov A, Broser M, Glöckner C, Hellmich J, Kern J, Frank J, Saenger W and Zouni A (2010) Recent progress in the crystallographic studies of photosystem II. ChemPhysChem 11: 1160–1171

Haag E, Irrgang K-D, Boekema EJ and Renger G (1990) Functional and structural-analysis of photosystem-II core complexes from spinach with high oxygen evolution capacity. Eur J Biochem 189: 47–53

Haehnel W (1984) Photosynthetic electron transport in higher plants. Annu Rev Plant Physiol 35: 659–693

Hallen S and Nilsson T (1992) Proton-transfer during the reaction between fully reduced cytochrome-*c*-oxidase and dioxygen - pH and deuterium-isotope effects. Biochemistry 31: 11853–11859

Hammes-Schiffer S (2006) Hydrogen tunneling and protein motion in enzyme reactions. Acc Chem Res 39: 93–100

Hansson Ö, Andreasson LE and Vänngard T (1986) Oxygen from water is coordinated to manganese in the S_2 state of photosystem II. FEBS Lett 195: 151–154

Hasegawa K and Noguchi T (2005) Density functional theory calculations on the dielectric-constant dependence of the oxidation potential of chlorophyll: Implication for the high potential of P680 in photosystem II. Biochemistry 44: 8865–8872

Hasegawa K, Kimura Y and Ono T (2004) Oxidation of the Mn cluster induces structural changes of NO_3^- functionally bound to the Cl^- site in the oxygen-evolving complex of photosystem II. Biophys J 86: 1042–1050

Haumann M, Müller C, Liebisch P, Iuzzolino L, Dittmer J, Grabolle M, Neisius T, Meyer-Klaucke W and Dau H (2005a) Structural and oxidation state changes of the photosystem II manganese complex in four transitions of the water oxidation cycle (S_0 -> S_1, S_1 -> S_2, S_2 -> S_3, and S_3, S_4 -> S_0) characterized by X-ray absorption spectroscopy at 20 K and room temperature. Biochemistry 44: 1894–1908

Haumann M, Liebisch P, Müller C, Barra M, Grabolle M and Dau H (2005b) Photosynthetic O_2 formation tracked by time-resolved X-ray experiments. Science 310: 1019–1021

Haumann M, Barra M, Loja P, Loscher S, Krivanek R, Grundmeier A, Andreasson L-E and Dau H (2006) Bromide does not bind to the Mn_4Ca complex in its S_1 state in Cl^--depleted and Br^--reconstituted oxygen-evolving photosystem II: Evidence from X-ray absorption spectroscopy at the Br K-edge. Biochemistry 45: 13101–13107

Hauska G and Hurt E (1982) Pool function behaviour and mobility of isoprenoid quinones. In: Trumpower B (ed) Functions of Quinones in Energy Conserving Systems, pp 87–110. Academic Press, New York

Hauß T, Dante S, Haines TH and Dencher NA (2005) Localization of coenzyme Q_{10} in the center of a deuterated lipid membrane by neutron diffraction. Biochim Biophys Acta 1710: 57–62

Havaux M, Rumeau D and Ducruet J-M (2005) Probing the FQR and NDH activities involved in cyclic electron transport around photosystem I by the 'afterglow' luminescence. Biochim Biophys Acta 1709: 203–213

Haveman J and Mathis P (1976) Flash-induced absorption changes of the primary donor of photosystem II at 820 nm in chloroplasts inhibited by low pH or tris-treatment. Biochim Biophys Acta 440: 346–355

Hayes AMA, Vassiliev IR, Golbeck JH and Debus RJ (1998) Role of D1-His 190 in proton-coupled electron transfer reactions in photosystem II: A chemical complementation study. Biochemistry 37: 11352–11365

Hienerwadel R, Grzybek S, Fogel C, Kreutz W, Okamura MY, Paddock ML, Breton J, Nabedryk E and Maentele W (1995) Protonation of Glu L212 following Q_B^- formation in the photosynthetic reaction center of *Rhodobacter sphaeroides*: Evidence from time-resolved infrared spectroscopy. Biochemistry 34: 2832–2843

Hillier W and Messinger J (2005) Mechanism of photosynthetic oxygen production. In Wydrzynski TJ and Satoh K (eds) Photosystem II. The Light-Driven Water:Plastoquinone Oxidoredutase. Advances in Photosynthesis and Respiration, Vol 22, pp 567–608. Springer, Dordrecht

Hillier W and Wydrzynski T (2008) ^{18}O Water exchange in photosystem II: Substrate binding and intermediates of the water splitting cycle. Coord Chem Rev 252: 306–317

Hillier W, McConnell I, Badger MR, Boussac A Klimov VV, Dismukes GC, Wydrzynski T (2006). Quantitative assessment of intrinsic carbonic anhydrase activity and the capacity for bicarbonate oxidation in photosystem II. Biochemistry 45: 2094–2102

Hillier W and Wydrzynski T (2000) The affinities for the two substrate water binding sites in the O_2 evolving complex of photosystem II vary independently during S-state turnover. Biochemistry 39: 4399–4405

Ho FM and Styring S (2008) Access channels and methanol binding site to the $CaMn_4$ cluster in photosystem II based on solvent accessibility simulations, with implications for substrate water access. Biochim Biophys Acta 1777: 140–153

Holzwarth AR, Müller MG, Reus M, Nowaczyk M, Sander MJ and Rögner M (2006a) Kinetics and mechanism of electron transfer in intact photosystem II and in the isolated reaction center: Pheophytin is the primary electron acceptor. Proc Natl Acad Sci USA 103: 6895–6900

Holzwarth AR, Müller MG, Niklas J and Lubitz W (2006b) Ultrafast transient absorption studies on photosystem I reaction centers from *Chlamydomonas reinhardtii*. 2: Mutations near the P700 reaction center chlorophylls provide new insight into the nature of the primary electron donor. Biophys J 90: 552–565

Hudaky P and Perczel A (2004) Conformation dependence of pK_a: ab initio and DFT investigation of histidine. J Phys Chem A108: 6195–6205

Hundelt M, Hays AMA, Debus RJ and Junge W (1998) Oxygenic photosystem II: The mutation D1 D61N in *Synechocystis* sp. PCC 6803 retards S-state transitions without affecting electron transfer from Y_Z to $P680^{+\bullet}$. Biochemistry 37: 14450–14456

Ido K, Gross CM, Guerrero F, Sedoud A, Lai T-L, Ifuku K, Rutherford AW and Krieger-Liszkay A (2011) High and low potential forms of the Q_A quinone electron acceptor in photosystem II of *Thermosynechococcus elongatus* and spinach. J Photochem Photobiol B: Biol 104: 154–157

Iizasa M, Suzuki H and Noguchi T (2010) Orientations of carboxylate groups coupled to the Mn cluster in the photosynthetic oxygen-evolving center as studied by polarized ATR-FTIR spectroscopy. Biochemistry 49: 3074–3082

Irrgang K-D, Lekauskas A, Franke P, Reifarth F, Smolian H, Karge M and Renger G (1998) Structural analysis of the water: plastoquinone oxidoreductase from spinach thylakoids. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, Vol II pp 977–980, Kluwer, Dordrecht

Isgandarova S, Renger G and Messinger J (2003) Functional differences of photosystem II from *Synechococcus elongatus* and spinach characterized by flash induced oxygen evolution patterns. Biochemistry 42: 8929–8938

Ishida N, Sugiura M, Rappaport F, Lai T-L, Rutherford AW and Boussac A (2008) Biosynthetic exchange of bromide for chloride and strontium for calcium in the photosystem II oxygen-evolving enzymes. J Biol Chem 283: 13330–13340

Ishikita H, Loll B, Biesiadka J, Saenger W and Knapp E-W (2005) Redox potentials of chlorophylls in the photosystem II reaction center. Biochemistry 44: 4118–4124

Ishikita H, Saenger W, Loll B, Biesiadka J and Knapp EW (2006) Energetics of a possible proton exit pathway for water oxidation in photosystem II. Biochemistry 45: 2063–2071

Iwai M, Takizawa K, Tokutsu R, Okamuro A, Takahashi Y and Minagawa J (2010) Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis Nature 464: 1210–1215

Jankowiak R, Hayes JM and Small GJ (2002) An excitonic pentamer model for the core Q_y states of the isolated photosystem II reaction center. J Phys Chem B 106: 8803–8814

Jeans C, Schilstra MJ, Ray N, Husain S, Minagawa J, Nugent JHA and Klug DR (2002) Replacement of tyrosine D with phenylalanine affects the normal proton transfer pathways for the reduction of $P680^+$ in oxygen-evolving photosystem II particles from *Chlamydomonas*. Biochemistry 41: 15754–15761

Johnston H, Wang J, Ruffle SV, Sayre RT and Gustafson TL (2000) Fluorescence decay kinetics of wild type and D2-H117N mutant photosystem II reaction centers isolated from *Chlamydomonas reinhardtii*. J Phys Chem B 104: 4777–4781

Joliot P and Joliot A (2005) Quantification of cyclic and linear flows in plants. Proc Natl Acad Sci USA 102: 4913–4918

Joliot P, Barbieri G and Chabaud R (1969) Un nouveau modele des centres photochimiques du systeme II. Photochem Photobiol 10: 309–329

Joliot P, Lavergne J and Béal D (1992) Plastoquinone compartmentation in chloroplasts; evidence for domains with different rates of photoreduction. Biochim Biophys Acta 1101: 1–12

Joliot P, Verméglio A and Joliot A (1997) Photo-induced cyclic electron transfer operates in frozen cells of *Rhodobacter sphaeroides*. Biochim Biophys Acta 1318: 374–384

Junge W and Jackson JB (1982) The development of electrochemical potential gradients across photosynthetic membranes. In: Govindjee (ed) Energy Conservation by Plants and Bacteria, pp 589–646. Academic Press, New York

Junge W (2008) Photophosphorylation. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 449–487. Royal Society Chemistry, Cambridge

Jursinic P and Govindjee (1977) Temperature dependence of delayed light emission in the 6-340 microsecond range after a single flash in chloroplasts. Photochem Photobiol 26: 617–628

Kaminskaya O, Kurreck J, Irrgang K-D, Renger G and Shuvalov VA (1999) Redox and spectral properties of cytochrome *b*559 in different preparations of photosystem II. Biochemistry 38: 16223–16235

Kaminskaya O, Renger G and Shuvalov VA (2003) Effect of dehydration on light induced reactions in photosystem II: Evidence for the presence of two functionally different cytochromes *b*559. Biochemistry 42: 8119–8132

Kaminskaya O, Kern J, Shuvalov VA and Renger G (2005) Difference extinction coefficients of cytochromes *b*559 and *c*550 in PSII core complex from *Thermosynechococcus elongatus.* Biochim Biophys Acta 1708: 333–341

Kaminskaya O, Shuvalov VA and Renger G (2007) Evidence for a novel quinone binding site in the photosystem II (PS II) complex which regulates the redox potential of Cyt *b*559. Biochemistry 46: 1091–1105

Kamiya N and Shen J-R (2003) Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7-Å resolution. Proc Natl Acad Sci USA 100: 98–103

Karge M, Irrgang K-D, Sellin S, Feinäugle R, Liu B, Eckert H-J, Eichler H-J and Renger G (1996) Effects of hydrogen/deuterium exchange on photosynthetic water splitting in PS II core complexes from spinach. FEBS Lett 378: 140–144

Karge M, Irrgang K-D and Renger G (1997) Analysis of the reaction coordinate of photosynthetic water oxidation

by kinetic measurements of 355 nm absorption changes at different temperatures in photosystem II preparations suspended in either H_2O or D_2O. Biochemistry 36: 8904–8913

Kasting JF and Seifert JF (2002) Life and the evolution of earth's atmosphere. Science 296: 1066–1067

Katiliene Z, Katilius E and Woodbury NW (2003) Energy trapping and detrapping in reaction center mutants from *Rhodobacter sphaeroides*. Biophys J 84: 3240–3251

Kato Y, Sugiura M, Oda A and Watanabe T (2009) Spectroelectrochemical determination of the redox potential of pheophytin *a*, the primary electron acceptor in photosystem II. Proc Nat Acad Sci USA 106: 17365–17370

Kawakami K, Umena Y, Kamiya N and J-R Shen (2011) Structure of the catalytic, inorganic core of oxygen-evolving photosystem II at 1.9 Å resolution. J Photochem Photobiol B: Biol 104: 9–18

Ke B (2001) Photosynthesis: Photobiochemistry and Photobiophysics. Advances in Photosynthesis and Respiration, Vol 12. Kluwer Academic Publishers, Dordrecht

Kern J and Renger G (2007) Photosystem II: Structure and mechanism of the water:plastoquinone oxidoreductase. Photosynth Res 94: 183–202

Kern J, Loll B, Zouni A, Saenger W Irrgang K-D and Biesiadka J (2005) Cyanobacterial photosystem II at 3.2 Å resolution – the plastoquinone binding pockets. Photosynth Res 84: 153–159

Kim EH, Razeghifard MR, Anderson JM and Chow WS (2007) Multiple sites of retardation of electron transfer in photosystem II after hydrolysis of phosphatidylglycerol. Photosynth Res 93: 149–158

Kimura Y, Mizusawa N, Yamanari T, Ishii A and Ono T (2005a) Structural changes of D1 C-terminal alpha-carboxylate during S-state cycling in photosynthetic oxygen evolution. J Biol Chem 280: 2078–2083

Kimura Y, Mizusawa N, Ishii A and Ono T (2005b) FTIR detection of structural changes in a histidine ligand during S-state cycling of photosynthetic oxygen-evolving complex. Biochemistry 44: 16072–16078

Kirmeier C, Holten D and Parson WW (1985) Temperature and detection-wavelength dependence of the picosecond electron-transfer kinetics measured in *Rhodopseudomonas sphaeroides* reaction centers. Resolution of new spectral and kinetic components in the primary charge-separation process. Biochim Biophys Acta 810: 33–48

Kirmaier C, Holten D, Debus RJ, Feher G and Okamura MY (1986) Primary photochemistry of iron-depleted and zinc-reconstituted reaction centers from *Rhodopseudomonas sphaeroides*. Proc Natl Acad Sci USA 83: 6407–6411

Klimov VV, Klevanik AV, Shuvalov VA and Krasnovsky AA (1977) Reduction of pheophytin in the primary light reaction of photosystem II. FEBS Lett 82: 183–186

Klimov VV, Allakhverdiev SI, Demeter S and Krasnovsky AA (1979) Photoreduction of pheophytin in photosystem II of chloroplasts as a function of redox potential of the medium. Dokl Akad Nauk SSSR 249: 227–230

Kobayashi M, Maeda H, Watanabe T, Nakane H and Satoh K (1990) Chlorophyll *a* and β-carotene content in the D1/D2/cytochrome b_{559} reaction center complex from spinach. FEBS Lett 260: 138–140

Koepke J, Krammer E-M, Klingen AR, Sebban P, Ullmann G.M and Fritzsch G (2007) pH modulates the quinone position in the photosynthetic reaction center from *Rhodobacter sphaeroides* in the neutral and charge separated states. J Mol Biol 371: 396–409

Koike H and Inoue Y (1987) Temperature dependence of the S-state transitions in a thermophilic cyanobacterium measured by thermoluminescence. In: Biggins J (ed) Progress in Photosynthesis Research, pp. 645–648. Nijhoff, Dordrecht

Koike H, Hanssum B, Inoue Y and Renger G (1987) Temperature dependence of the S-state transitions in a thermophilic cyanobacterium, *Synechococcus vulcanus* Copeland measured by absorption changes in the ultraviolet region. Biochim Biophys Acta 893: 524–533

Kok B, Forbush B and McGloin M (1970) Cooperation of charges in photosynthetic O_2 evolution. Photochem Photobiol 11: 457–476

Kolling DRJ, Brown TS, Ananyev G and Dismukes GC (2009) Photosynthetic oxygen evolution is not reversed at high oxygen pressures: mechanistic consequences for the water-oxidizing complex. Biochemistry 48: 1381–1389

Konermann L and Holzwarth AR (1996) Analysis of the absorption spectrum of photosystem II reaction centers: temperature dependence, pigment assignment and inhomogeneous broadening. Biochemistry 35: 829–842

Krausz E, Cox N and Peterson Årsköld S (2008) Spectral characteristics of PS II reaction centres: as isolated preparations and when integral to PS II core complexes. Photosynth Res 98: 207–217

Krieger-Liszkay A and Rutherford AW (1998) Influence of herbicide binding on the redox potential of the quinone acceptor in photosystem II: Relevance to photodamage and phytotoxicity. Biochemistry 37: 17339–17344

Krieger A, Rutherford AW and Johnson GN (1995) On the determination of the redox midpoint potential of the primary quinone acceptor, Q_A, in photosystem II. Biochim Biophys Acta 1229: 193–201

Krishtalik LI (1986) Energetics of multielectron reactions. Photosynthetic oxygen evolution. Biochim Biophys Acta 849: 162–171

Kühn P, Eckert H-J, Eichler H-J and Renger G (2004) Analysis of the $P680^{+\bullet}$ reduction pattern and its temperature dependence in oxygen-evolving PS II core complexes from thermophilic cyanobacteria and higher plants. Phys Chem Chem Phys 6: 4838–4843

Kühn P, Pieper J, Kaminskaya O, Eckert H-J, Lechner R, Shuvalov V and Renger G (2005) Reaction pattern of photosystem II: Oxidative water cleavage and protein flexibility. Photosynth Res 84: 317–323

Kulik LV, Epel B, Lubitz W and Messinger J (2005a) ^{55}Mn pulse ENDOR at 34 GHz of the S_0 and S_2 states of the

oxygen-evolving complex in photosystem II. J Am Chem Soc 127: 2392–2393

Kulik LV, Lubitz W and Messinger J (2005b) Electron spin-lattice relaxation of the S_0 state of the oxygen-evolving complex in photosystem II and of dinuclear manganese model complexes. Biochemistry 44: 9368–9374

Kulik LV, Epel B, Lubitz W and Messinger J.(2007) Electronic structure of the Mn_4O_xCa cluster in the S_0 and S_2 states of the oxygen-evolving complex of photosystem II based on pulse ^{55}Mn -ENDOR and EPR spectroscopy. J Am Chem Soc 129: 13421–13435

Kurreck J, Garbers A, Reifarth F, Andréasson LE, Parak F and Renger G (1996) Isolation and properties of PS II membrane fragments depleted of the non heme iron center. FEBS Lett 381: 53–57

Kurreck J, Liu B, Napiwotzki A, Sellin S, Eckert HJ, Eichler HJ and Renger G (1997a) Stoichiometry of pigments and radical pair formation under saturating pulse excitation in D1/D2/cyt *b*559 preparations. Biochim Biophys Acta 1318: 307–315

Kurreck J, Garbers A, Parak F and Renger G (1997b) Highly purified D1/D2/Cyt *b*559 preparations from spinach do not contain the non heme iron center. FEBS Lett 403: 283–286

Kusunoki M (2011) S_1-state Mn_4Ca complex of photosystem II exists in equilibrium between the two most-stable isomeric substates: XRD and EXAFS evidence. J Photochem Photobiol B: Biol 104: 100–110

Laible PD, Kirmaier C, Udawatte CSM, Hofman SJ, Holten D and Hanson DK (2003) Quinone reduction via secondary b-branch electron transfer in mutant bacterial reaction centers. Biochemistry 42: 1718–1730

Lancaster CRD (2008) Structures of reaction centers in anoxygenic bacteria. In: Renger G (ed) Primary Processes of Photosynthesis: Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 5–56. Royal Society Chemistry, Cambridge

Lane N (2003) Oxygen - The Molecule that Made the World, Oxford University Press

Larkum AWD (2008) The evolution of photosynthesis. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 491–521. Royal Society Chemistry, Cambridge

Lavergne L, Bouchaud JP and Joliot P (1992) Plastoquinone compartmentation inchloroplasts: 2. Theoretical aspects. Biochim Biophys Acta 1101: 13–22

Liang W, Roelofs TA, Cinco RM, Rompel A, Latimer MJ, Yu WO, Sauer K, Klein MP and Yachandra VK (2000) Structural change of the Mn cluster during the S_2 to S_3 state transition of the oxygen evolving complex of photosystem II. Does it reflect the onset of water/substrate oxidation? Determination by Mn X-ray absorption spectroscopy, J Am Chem Soc 122: 3399–3412

Li F, England J and Que Jr L (2010) Near-stoichiometric conversion of H_2O_2 to FeIV=O at a nonheme iron(II) center. Insights into the O−O bond cleavage step. J Am Chem Soc 132: 2134–2135

Lin TJ and O'Malley PJ (2011) An ONIOM study of the spin density distribution of the Q_A site plastosemiquinone in the photosystem II reaction center J Phys Chem B115: 4227–4233

Lince MT and Vermaas W (1998) Association of His117 in the D2 protein of photosystem II with a chlorophyll that affects excitation energy transfer efficiency to the reaction center. Eur J Biochem 256: 595–602

Liu F, Concepcion JJ, Jurss JW, Cardolaccia T, Templeton JL and Meyer TJ (2008) Mechanisms of water oxidation from the blue dimer to photosystem II. Inorg Chem 47: 1727–1752

Loll B, Kern J, Saenger W, Zouni A and Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. Nature 438: 1040–1044

Luna VM, Chen Y, Fee JA and Stout CD (2008) Crystallographic studies of Xe and Kr binding within the large internal cavity of cytochrome ba_3 from *Thermus thermophilus*: Structural analysis and role of oxygen transport channels in the heme−Cu oxidases Biochemistry 47: 4657–4665

Lydakis-Simantiris N, Ghanotakis DF and Babcock GT (1997) Kinetic isotope effects on the reduction of the Y_Z radical in oxygen evolving and tris washed photosystem II membranes by time resolved EPR. Biochim Biophys Acta 1322: 129–140

MacMillan F, Gleiter H, Renger G and Lubitz W (1990) EPR/ENDOR studies of plastoquinone anion radical in photosystem II (Q_A^-) and in organic solvents. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol 1, pp 849–852. Kluwer, Dordrecht

MacMillan F, Lendzian F, Renger G and Lubitz W (1995a) EPR and ENDOR investigation of the primary electron acceptor radical anion $Q_A^{-\bullet}$ in iron-depleted photosystem II membrane fragments. Biochemistry 34: 8144–8156

MacMillan F, Kurreck J, Adir N, Lendzian F, Käss H, Reifarth F, Renger G and Lubitz W (1995b) EPR, ENDOR and ESEEM investigation of the electron acceptor radical anion $Q_A^{-\bullet}$ in photosystem II (PS II) reaction centres. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, Vol I, pp 659–662. Kluwer, Dordrecht

Marcus RA and Sutin N (1985) Electron transport in chemistry and biology. Biochem Biophys Acta 811: 265–322

Matsukawa T, Mino H, Yoneda D and Kawamori A (1999) Dual-mode EPR study of new signals from the S_3 -state of oxygen-evolving complex in photosystem II. Biochemistry 38: 4072–4077

Matsuoka H, Shen J-R, Kawamori A, Nishiyama K, Ohba Y and Yamauchi S (2011) Proton-coupled electron-transfer processes in photosystem II probed by highly resolved g-anisotropy of redox-active tyrosine Y_Z. J Am Chem Soc 133: 4655–4660

McEvoy JP and Brudvig GW (2006) Water-splitting chemistry of photosystem II. Chem Rev 106: 4455–4483

Mc Fadden, GI (1999) Endosymbiosis and evolution of the plant cell. Curr Opinion Plant Biol 2: 513–519

Messinger J and Renger G (1990) The reactivity of hydrazine with photosystem II strongly depends on the redox state of the water oxidizing system. FEBS Lett 277: 141–146

Messinger J and Renger G (1994) Analysis of pH-induced modifications in the period four oscillation of flash-induced oxygen evolution reveal distinct structural changes of the photosystem II donor side at characteristic pH values. Biochemistry 33: 10896–10905

Messinger J and Renger G (2008) Photosynthetic water splitting. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 291–349. Royal Society Chemistry, Cambridge

Messinger J, Wacker U and Renger G (1991) Unusual low reactivity of the water oxidase in the redox state S_3 toward exogenous reductants. Analysis of the NH_2OH and NH_2NH_2 induced modifications of flash induced oxygen evolution in isolated spinach thylakoids. Biochemistry 30: 7852–786

Messinger J, Nugent JHA and Evans MCW (1997a) Detection of an EPR multiline signal for the S_0^* state in photosystem II. Biochemistry 36: 11055–11060

Messinger J, Robblee JH, Yu WO, Sauer K, Yachandra VK and Klein MP (1997b) The S_0 state of the oxygen-evolving complex in photosystem II is paramagnetic: Detection of an EPR multiline signal. J Am Chem Soc 119: 11349–11350

Messinger J, Seaton GR, Wydrzynski T, Wacker U and Renger G. (1997) The S_{-3} state of the water oxidase in photosystem II. Biochemistry 36: 6862–6873

Messinger J, Robblee JH, Bergmann U, Fernandez C, Glatzel P, Visser H, Cinco RM, McFarlane KL, Bellacchio E, Pizarro SA, Cramer SP, K. Sauer, Klein MP and Yachandra VK (2001) Absence of Mn centered oxidation in the S_2 to S_3 transition: Implications for the mechanism of photosynthetic water oxidation. J Am Chem Soc 123: 7804–7820

Metz JG, Nixon PJ, Rögner M, Brudvig GW and Diner BA (1989) Directed alteration of the D1 polypeptide of photosystem II: Evidence that tyrosine 161 is the redox component, Z, connecting the oxygen-evolving complex to the primary electron donor, P680. Biochemistry 28: 6960–6969

Meyer T J, Hang M, Huynh V and Thorp HH (2007) The role of proton coupled electron transfer (PCET) in water oxidation by photosystem II. Wiring for protons. Angew Chem Int Ed 46: 5284–5304

Michel-Beyerle ME, Plato M, Deisenhofer J, Michel H, Bixon M and Jortner J (1988) Unidirectionality of charge separation in reaction centers of photosynthetic bacteria. Biochim Biophys Acta 932: 52–70

Miloslavina Y, Szczepaniak M, Müller MG, Sander J, Nowaczyk M, Rögner M and Holzwarth AR (2006) Charge separation kinetics in intact photosystem II core particles is trap-limited. A picosecond fluorescence study. Biochemistry 45: 2436–2442

Mimuro M, Kobayashi M, Murakami A, Tsuchiya T and Miyashita H (2008) Oxygen-Evolving Cyanobacteria. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part I: Photophysical Principles, Pigments Light Harvesting/Adaptation/Stress, pp 261–299. Royal Society Chemistry, Cambridge

Mitchell P (1961) Coupling of photophosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191: 144–148

Miyashita H, Adachi K, Kurano N, Ikemot H, Chihara M and Miyach S (1997) Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll *d* as the major chlorophyll. Plant Cell Physiol 38: 274–281

Morovitz HJ (1978) Foundations of Bioenergetics. Academic Press, New York

Munekage Y, Hashimoto M, Miyake C, Tomizawa K-I, EndoT, Tasaka M and Shikanai T (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature 429: 579–582

Müh F, Glöckner C, Hellmich J and Zouni A (2011) Light-induced quinone reduction in photosystem II. Biochim Biophys Acta doi:10.1016/ j.bbabio.2011.05.021

Müller MG, Dorra D, Holzwarth AR, Gad'on N and Drews G (1995) Time-dependent radical pair relaxation in chromatophores of an antenna-free mutant from *Rhodobacter capsulatus*. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, Vol 1, pp 595–598. Kluwer Academic Publishers, Dordrecht

Müller MG, Hucke M, Reus M and Holzwarth AR (1996) Primary processes and structure of the photosystem II reaction center: IV. Low intensity femtosecond transient absorption spectra of D1-D2 reaction centers. J Phys Chem 100: 9527–9536

Murray JW and Barber J (2007) Structural characteristics of channels and pathways in photosystem II including the identification of an oxygen channel. J Struct Biol 159: 228–237

Murray JW, Maghlaoui K, Kargul J, Ishida N, Lai T-L, Rutherford AW, Sugoura M, Boussac A and Barber J (2008) X-ray crystallography identifies two chloride dinding sites in the oxygen evolving centre of photosystem II. Energy Environmental Sci 1: 161–166

Nabedryk E, Paddock ML, Okamura MY and Breton J (2005) An isotope-edited FTIR investigation of the role of Ser-L223 in binding quinone (Q_B) and semiquinone (Q_B^-) in the reaction center from *Rhodobacter sphaeroides*. Biochemistry 44: 14519–14527

Nanba O and Satoh K (1987) Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. Proc Natl Acad Sci USA 84: 109–112

Nikolaev GM, Knox PP, Kononenko AA, Grishanova NP and Rubin AB (1980) Photo-induced electron transport and water state in *Rhodospirillum rubrum* chromatophores. Biochim Biophys Acta 590: 194–201

Noguchi T (2002) Dual role of triplet localization on the accessory chlorophyll in the photosystem II reaction center: photoprotection and photodamage of the D1 protein. Plant Cell Physiol 43: 1112–1116

Noguchi T (2008) FTIR detection of water reactions in the oxygen-evolving centre of photosystem II. Phil Trans R Soc B 363: 1189–1195

Noguchi T and Sugiura M (2002) Flash-induced FTIR difference spectra of the water oxidizing complex in moderately hydrated photosystem II core films: Effect of hydration extent on S-state transitions. Biochemistry 41: 2322–2330

Noguchi T, Tomo T and Inoue Y (1998) Fourier transform infrared study of the cation radical of P680 in the photosystem II reaction center: Evidence for charge delocalization on the chlorophyll dimer. Biochemistry 37: 13614–13625

Noguchi T, Kurreck J, Inoue Y and Renger G (1999) Comparative FTIR analysis of the microenvironment of $Q_A^{-\bullet}$ in cyanide and high pH treated and "iron depleted" PS II membrane fragments. Biochemistry 38: 4846–4852

Novoderezhkin VI, Romero E, Dekker JP and Van Grondelle R (2011) Multiple charge-separation pathways in photosystem II: modeling of transient absorption kinetics. ChemPhysChem 12: 681–688

Nuijs AM, Van Gorkom HJ, Plijter JJ and Duysens LNM (1986) Primary-charge separation and excitation of chlorophyll *a* in photosystem II particles from spinach as studied by picosecond absorbance-difference spectroscopy. Biochim Biophys Acta 848: 167–175

Oettmeier W (1992) Herbicides of photosystem II. In: Barber J (ed) The Photosystems: Structure, Function and Molecular Biology, pp 349–408. Elsevier, Amsterdam

Oettmeier W (1999) Herbicide resistance and supersensitivity in photosystem II. Cell Mol Life Sci 55: 1255–1277

Ogrodnik A, Volk M, Letterer R, Feick R and Michel-Beyerle ME (1988) Determination of free energies in reaction centers of *Rb. sphaeroides*. Biochim Biophys Acta 936: 361–371

Olesen K and Andréasson L-E (2003) The function of the chloride ion in photosynthetic oxygen evolution. Biochemistry 42: 2025–2035

Ono T, Noguchi T, Inoue Y, Kusunoki M, Matsushita T and Oyanagi H (1992) X-ray detection of the period-four cycling of the manganese cluster in photosynthetic water oxidizing enzyme. Science 258: 1335–1337

Paddock L, Feher G and Okamura MY (1995) Pathway of proton transfer in bacterial reaction centers: Further investigations on the role of Ser-L223 studied by site-directed mutagenesis. Biochemistry 34: 15742–15750

Page CC, Moser CC, Chen X and Dutton PL (1999) Natural engineering principles of electron tunneling in biological oxidation-reduction. Nature 402: 47–52

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration, Vol 19, Springer, Dordrecht

Parson WW (2008) Functional patterns of reaction centers in anoxygenic photosynthetic bacteria. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 57–109. Royal Society Chemistry, Cambridge

Parson WW and Warshel (2004) A density-matrix model of photosynthetic electron transfer with microscopically estimated vibrational relaxation times. Chem Phys 296: 201–216

Parson WW, Chu ZT and Warshel A (1990) Electrostatic control of charge separation in bacterial photosynthesis. Biochim Biophys Acta 1017: 251–272

Paschenko VZ, Gorokhov VV, Knox PP, Krasilnikov PM, Redlin H, Renger G and Rubin AB (2003) Energetics and mechanisms of high efficiency of charge separation and electrons transfer processes in *Rhodobacter sphaeroides* reaction centers. Bioelectrochemistry 61: 73–84

Pawlowicz NP, Groot M-L and Van Stokkum IHM, Breton J, Van Grondelle R (2007) Charge separation and energy transfer in the photosystem II core complex studied by femtosecond mid-infrared spectroscopy. Biophys J 93: 2732–2742

Pecoraro VL, Baldwin MJ, Caudle MT, Hsieh W-Y and Law NA (1998) A proposal for water oxidation in photosystem II. Pure Appl Chem 70: 925–929

Peloquin JM, Campbell KA, Randall DW, Evanchik MA, Pecoraro VL, Armstrong WH and Britt RD (2000) ^{55}Mn ENDOR of the S_2 -State multiline EPR signal of photosystem II: Implications on the structure of the tetranuclear Mn cluster. J Am Chem Soc 122: 10926–10942

Perrine Z and Sayre R (2011) Modulating the redox potential of the stable electron acceptor, Q_B, in mutagenized photosystem II reaction centers. Biochemistry 50: 1454–1464

Peter GF and Thornber JP (1991) Biochemical evidence that the higher plant photosystem II core complex is organized as a dimer. Plant Cell Physiol 32: 1237–1250

Petrouleas V and Diner BA (1986) Identification of Q_{400}, a high-potential electron acceptor of photosystem II, with the iron of the quinone-iron acceptor complex. Biochim Biophys Acta 849: 264–275

Petrouleas V and Crofts AR (2005) The iron-quinone acceptor complex. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 177–206. Springer, Dordrecht

Petrouleas V, Deligiannakis Y and Diner BA (1994) Binding of carboxylate anions at the non-heme Fe(II) of PS II. II. Competition with bicarbonate and effects on the Q_A/Q_B electron transfer rate. Biochim Biophys Acta 1188: 271–277

Pieper J and Renger G (2009) Protein dynamics investigated by neutron scattering. Photosynth Res 102: 281–293

Pieper J, Hauß T, Buchsteiner A, Baczyński K, Adamiak K, Lechner RE and Renger G (2007) Temperature- and hydration-dependent protein dynamics in photosystem II of green plants studied by quasielastic neutron scattering. Biochemistry 46: 11398–11409

Pieper J, Hauß T, Buchsteiner A and Renger G (2008) The effect of hydration on protein flexibility in photosystem II of green plants studied by quasielastic neutron scattering. Eur Biophys J 37: 657–663

Pogson JB, Rissler HM and Frank HA (2005) The role of carotenoids in energy quenching. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 515–537. Springer, Dordrecht

Pospíšil P (2011) Enzymatic function of cytochrome b_{559} in photosystem II. J Photochem Photobiol B: Biol 104: 341–347

Poulson M, Samson S and Whitmarsh J (1995) Evidence that cytochrome *b*-559 protects photosystem II against photoinhibition. Biochemistry 34: 10932–10938

Pushkar Y, Yano J, Sauer K, Boussac A and Yachandra VK (2008) Structural changes in the Mn_4Ca cluster and the mechanism of photosynthetic water splitting. Proc Nat Acad Sci USA 105: 1879–1884

Rappaport F and. Diner BA (2008) Primary photochemistry and energetics leading to the oxidation of the $(Mn)_4Ca$ cluster and to the evolution of molecular oxygen in photosystem II. Coord Chem Rev 252: 259–272

Rappaport F, Blanchard-Desce M and Lavergne J (1994) Kinetics of the electron transfer and electrochromic change during the redox transitions of the photosynthetic oxygen-evolving complex. Biochim Biophys Acta 1184: 178–192

Rappaport F, Guergova-Kuras M, Nixon PJ, Diner BA and Lavergne J (2002) Kinetics and pathways of charge recombination in photosystem II. Biochemistry 41: 8518–8527

Raszewski G and Renger T (2008) Light-harvesting in photosystem II core complexes is limited by the transfer to the trap: Can the core complex turn into a photoprotective mode? J Am Chem Soc 130: 4431–4446

Raszewski G, Saenger W and Renger T (2005) Theory of optical spectra of photosystem II reaction centers: Location of the triplet state and the identity of the primary electron donor. Biophys J 88: 986–998

Raszewski G, Diner BA, Schlodder E and Renger T (2008) Spectroscopic properties of reaction center pigments in photosystem II core complexes: Revision of the multimer model. Biophys J 95: 105–119

Raymond J and Blankenship RE (2004) The evolutionary development of the protein complement of photosystem 2. Biochim Biophys Acta 1655: 133–139

Razeghifard MR and Pace RJ (1997) Electron paramagnetic resonance kinetic studies of the S states in spinach PSII membranes. Biochim Biophys Acta 1322: 141–150

Razeghifard MR and Pace RJ (1999) EPR kinetic studies of oxygen release in thylakoids in PSII membranes: A kinetic intermediate in the S_3 to S_0 transition. Biochemistry 38: 1252–1257

Razeghifard MR, Chen M, Hughes JL, Freeman J, Krausz E, and Wydrzynski T (2005) Spectroscopic studies of photosystem II in chlorophyll *d*-containing *Acaryochloris marina*. Biochemistry 44: 11178–11187

Reifarth F and Renger G (1998) Indirect evidence for structural changes coupled with $Q_B^{-\bullet}$ formation in photosystem II. FEBS Lett 428: 123–126

Reifarth F, Christen G, Seeliger AG, Dörmann P, Benning C and Renger G (1997) Modification of the water oxidising complex in leaves of the *dgd1* mutant of *Arabidopsis thaliana* deficient in the galactolipid digalactosyldiacylglycerol. Biochemistry 36: 11769–11776

Reinman S and Mathis P (1981) Influence of temperature on photosystem II electron transfer reactions. Biochim Biophys Acta 635: 249–258

Renger G (1978) Theoretical studies about the functional and structural organization of the photosynthetic oxygen evolution. In: Metzner H (ed) Photosynthetic Oxygen Evolution, pp 229–248. Academic Press, London

Renger G (1983) Biological Energy Conservation. In: Hoppe W, Lohmann W, Markl H and Ziegler H (eds) Biophysics, pp 347–371. Springer, Berlin

Renger G (1987) Mechanistic aspects of photosynthetic water cleavage. Photosynthetica 21: 203–224

Renger G (1993) Water cleavage by solar-radiation - an inspiring challenge of photosynthesis research. Photosynth Res 38: 229–247

Renger G (1997) Mechanistic and structural aspects of photosynthetic water oxidation, Physiol Plant 100: 828–841

Renger G (1999) Molecular mechanism of water oxidation. In: Singhal GS, Renger G, Govindjee, Irrgang, KD and Sopory SK (eds) Concepts in Photobiology: Photosynthesis and Photomorphogenesis, pp 292-329. Kluwer Academic Publishers, Dordrecht; and Narosa Publishing Co, New Delhi

Renger G (2001) Photosynthetic water oxidation to molecular oxygen: apparatus and mechanism. Biochim Biophys Acta 1503: 210–228

Renger G (2004) Coupling of electron and proton transfer in oxidative water splitting in photosynthesis. Biochim Biophys Acta 1655: 195–204

Renger G (2007) Oxidative photosynthetic water splitting: energetics, kinetics and mechanism. Photosynth Res 92: 407–425

Renger G (2008) Functional pattern of photosystem II in oxygen evolving organisms. In: Renger G (ed) Primary Processes of Photosynthesis: Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron

Transport Chains, Photophosphorylation and Evolution, pp 237–290. Royal Society Chemistry, Cambridge

Renger G (2011) Light induced oxidative water splitting in photosynthesis: energetics, kinetics and mechanism. J Photochem Photobiol B: Biol 104: 35–43

Renger G and Hanssum B (1992) Studies on the reaction coordinates of the water oxidase in PS II membrane fragments from spinach. FEBS Lett 299: 28–32

Renger G and Holzwarth AR (2005) Primary electron transfer. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water: Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 139–175. Springer, Dordrecht

Renger G and Renger T (2008) Photosystem II: The machinery of photosynthetic water splitting. Photosynth Res 98: 53–80

Renger G and Völker M (1982) Studies on the proton release of the donor side of system II. Correlation between oxidation and deprotonization of donor D_1 in Tris-washed inside-out thylakoids. FEBS Lett 149: 203–207

Renger G and Weiss W (1982) The detection of intrinsic 320 nm absorption changes reflecting the turnover of the water splitting enzyme system Y which leads to oxygen formation in trypsinized chloroplats. FEBS Lett 137: 217–221

Renger G and Weiss W (1986) Functional and structural aspects of photosynthetic water oxidation. Biochem Soc Trans 14: 17–20

Renger G and Wolff Ch (1976) The existence of a high photochemical turnover rate at the reaction centers of system II in tris-washed chloroplasts. Biochim Biophys Acta 423: 610–614

Renger G, Gläser M and Buchwald HE (1977) The control of the reduction kinetics in the dark of photooxidized chlorophyll- a_{II}^{+} by inner thylakoid proton concentration. Biochim Biophys Acta 461: 392–402

Renger G, Eckert H-J and Weiss W (1983) Studies on the mechanism of photosynthetic oxygen formation. In: Inoue Y, Crofts AR,, Murata N, Renger G und Satoh K (eds) The Oxygen Evolving System in Photosynthesis, pp 73–82. Academic Press, Tokyo

Renger G, Völker M and Weiss W (1984) Studies on the nature of the water-oxidizing enzyme. I. The effect of trypsin on the system II reaction pattern in inside-out thylakoids. Biochim Biophys Acta 766: 582–591

Renger G, Wacker U and Völker M (1987) Studies on the protolytic reactions coupled with water cleavage in photosystem II membrane fragments from spinach. Photosynth Res 13: 167–184

Renger G, Völker M, Eckert H-J, Fromme R, Hohm-Veit S and Gräber P (1989) On the mechanism of photosystem II deterioration by UV-B irradiation. Photochem Photobiol 49: 97–105

Renger G, Messinger J and Hanssum B (1990) Thermodynamic, kinetic and mechanistic aspects of photosynthetic water oxidation In: Baltscheffsky M (ed) Current Research in Photosynthesis, pp 845–848. Kluwer Academic Publishers, Dordrecht

Renger G, Gleiter HM, Haag E and Reifarth F (1993) Photosystem II: thermodynamics and kinetics of electron transport from $Q_A^{-\bullet}$ to Q_B ($Q_B^{-\bullet}$) and deleterious effects of copper (II). Z Naturforsch 48c: 234–240

Renger G, Bittner T and Messinger J (1994) Structure-function relationships in photosynthetic water oxidation. Biochem Soc Trans 22: 318–322

Renger G, Eckert HJ, Bergmann A, Bernarding J, Liu B, Napiwotzki A, Reifarth F and Eichler JH (1995) Fluorescence and spectroscopic studies on exciton trapping and electron transfer in photosystem II of higher plants. Austr J Plant Physiol 22: 167–181

Renger G, Christen G, Karge M, Eckert H-J and Irrgang K-D (1998) Application of the Marcus theory for analysis of the temperature dependence of the reactions leading to photosynthetic water oxidation – results and implications. J Bioinorg Chem 3: 360–366

Rhee KH (2001) Photosystem II: the solid structural era. Annu Rev Biophys Biomol Struct 30 307–328

Riistama S, Puustinen A, García-Horsman A, Iwata S, Michel H and Wikström M (1996) Channelling of dioxygen into the respiratory enzyme. Biochim Biophys Acta 12275: 1–4

Rinyu L, Marin EW, Takahashi E, Maroti P and Wraight CA (2003) Modulation of the free energy of the primary quinone acceptor (Q_A) in reaction centers from *Rhodobacter sphaeroides*: contributions from the protein and protein-lipid (cardiolipin) interactions. Biochim Biophys Acta 1655: 93–101

Robinson HH and Crofts AR (1984) Kinetics of the oxidation-reduction reactions of the photosystem II acceptor complex and the pathway for deactivation. FEBS Lett 151: 221–226

Rockley MG, Windsor MW, Cogdell RJ and Parson WW (1975) Picosecond detection of an intermediate in the photochemical reaction of bacterial photosynthesis. Proc Natl Acad Sci USA 72: 2251–2255

Roelofs TA and Holzwarth AR (1990) In search of a putative long-lived relaxed radical pair state in closed photosystem II. Kinetic modeling of picosecond fluorescence data. Biophys J 57: 1141–1153

Ruffle SV, Wang J, Johnston HG, Gustafson TL, Hutchison RS, Minagawa J, Crofts A and Sayre RT (2001) Photosystem II peripheral accessory chlorophyll mutants in *Chlamydomonas reinhardtii*. Biochemical characterization and sensitivity to photo-inhibition. Plant Physiol 127: 633–644

Santoni E, Jakopitsch C, Obinger C, and Smulevichpp G (2004) Comparison between catalase-peroxidase and cytochrome *c* peroxidase. The role of the hydrogen-bond networks for protein stability and catalysis. Biochemistry 43: 5792–5802

Sacquin-Mora S, Sebban P, Derrien V, Frick B, Lavery R and Alba-Simionesco C (2007) Probing the flexibility of the bacterial reaction center: The wild-type protein is more rigid than two site-specific mutants. Biochemistry 46: 14960–14968

Sauer K, Yano J and Yachandra VK (2008) X-ray spectroscopy of the photosynthetic oxygen-evolving complex. Coord Chem Rev 252: 318–335

Schatz GH, Brock H and Holzwarth AR (1987) Picosecond kinetics of fluorescence and absorbance changes in photosystem II particles excited by low photon density. Proc Natl Acad Sci USA 84: 8414–8418

Schatz GH, Brock H and Holzwarth AR (1988) Kinetic and energetic model for the primary processes in photosystem II. Biophys J 54: 397–405

Scheer KH (2008) Chlorophylls. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part I: Photophysical Principles, Pigments Light Harvesting/Adaptation/Stress, pp 101–149. Royal Society Chemistry, Cambridge

Schilstra MJ, Rappaport F, Nugent JHA, Barnett CJ and Klug DR (1998) Proton/hydrogen transfer affects the S-state-dependent microsecond phases of $P680^+$ reduction during water splitting. Biochemistry 37: 3974–3981

Schmidt U and Reineker P (1985) Triplet excitons in molecular pairs. Mol Phys 55: 77–95

Schödel R, Irrgang KD, Voigt J and Renger G (1998) Rate of carotenoid triplet formation in solubilized light-harvesting complex II (LHCII) from spinach. Biophys J 75: 3143–3153

Schwartz JK, Wei P, Mitchell KH, Fox BG and Solomon EI (2008) Geometric and electronic structure studies of the binuclear nonheme ferrous active site of toluene-4-monooxygenase: Parallels with methane monooxygenase and insight into the role of the effector proteins in O_2 activation. J Am Chem Soc 130: 7098–7109

Schweitzer RH, Melkozernov AN, Blankenship RE and Brudvig GW (1998) Time-resolved fluorescence measurements of photosystem II: The effect of quenching by oxidized chlorophyll Z. J Phys Chem B 102: 8320–8326

Shelaev IV, Gostev FE, Vishnev MI, Shkuropatov AYa, Ptushenko VV, Mamedov MD, Sarkisov OM, Nadtochenko VA, Semenov AYu and Shuvalov VA (2011) P_{680} ($P_{D1}P_{D2}$) and Chl_{D1} as alternative electron donors in photosystem II core complexes and isolated reaction centers. J Photochem Photobiol B: Biol 104: 44–50

Shevela D, Nöring B, Eckert H-J, Messinger J and Renger G (2006) Characterization of the water-oxidizing complex of photosystem II of the Chl *d* - containing cyanobacterium *Acaryochloris marina* via its reactivity towards endogenous electron donors and acceptors Phys Chem Chem Phys 8: 3460–3446

Shevela D, Su J-H, Klimov V and Messinger J (2008) Hydrogencarbonate is not a tightly bound constituent of the water oxidizing complex in photosystem II. Biochim. Biophys. Acta 1777: 532–539

Sheleva D, Beckmann K, Clausen J, Junge W and Messinger J (2011) Photosynthetic oxygen-evolution at elevated oxygen pressure: direct detection by membrane inlet mass spectrometry. Proc Nat Acad Sci USA 108: 3602–3607

Shi L-X and Schröder WP (2004) The low molecular mass subunits of the photosynthetic supracomplex, photosystem II. Biochim Biophys Acta 1608: 75–96

Shibamoto T, Kato Y, Sugiura M and Watanabe T (2009) Redox potential of the primary plastoquinone electron acceptor Q_A in photosystem II from *Thermosynechococcus elongatus* determined by spectroelectrochemistry. Biochemistry 48: 10682–10684

Shibamoto T, KatoY, Nagao R, Yamazaki T, Tomo T and Watanabe T (2010) Species-dependence of the redox potential of the primary quinone electron acceptor QA in photosystem II verified by spectroelectrochemistry. FEBS Lett 584: 1526–1530

Shlyk-Kerner O, Samish I, Kaftan D, Holland N, Maruthi Sai PS, Kless H, and Scherz A (2006) Protein flexibility acclimatizes photosynthetic energy conversion to the ambient temperature. Nature 442: 827–830

Shutova T, Irrgang K-D, Shubin V, Klimov VV and Renger G (1997) Analysis of pH-induced structural changes of the isolated extrinsic 33 kDa protein of photosystem II. Biochemistry 36: 6350–6358

Shutova T, Klimov VV, Andersson B and Samuelsson G (2007) A cluster of carboxylic groups in PsbO protein is involved in proton transfer from the water oxidizing complex of photosystem II. Biochim Biophys Acta 1767: 434–440

Siegbahn PEM (2006) O--O bond formation in the S_4 state of the oxygen-evolving complex in photosystem II, Chemistry - A European Journal 12: 9217–9227

Siegbahn PEM (2008) Theoretical studies of O–O bond formation in photosystem II. Inorg Chem 47: 1779–1786

Siegbahn PEM (2009) Structures and energetics for O_2 formation in photosystem II. Acc Chem Res 42: 1871–1880

Siegbahn PEM (2011) Recent theoretical studies of water oxidation in photosystem II. J Photochem Photobiol B: Biol 104: 94–99

Siggel U, Renger G, Stiehl HH and Rumberg B (1972) Evidence for electronic and ionic interaction between electron transport chains in chloroplasts. Biochim Biophys Acta 256: 328–335

Soper JD, Kryatov SV, Rybak-Akimova EV and Nocera DG (2007) Proton-directed redox control of O-O bond activation by heme hydroperoxidase models. J Am Chem Soc 129: 5069–5075

Sproviero EM, Gascon JA, McEvoy JP, Brudvig GW and Batista VS (2006) QM/MM Models of the O_2-evolving complex of photosystem II. J Chem Theory and Comput 4: 1119–1134

Sproviero EM, Gascon JA, McEvoy JP, Brudvig GW and Batista VS (2008) Quantum mechanics/molecular mechanics study of the catalytic cycle of water splitting in photosystem II. J Am Chem Soc 130: 3428–3442

Steffen R, Eckert H-J, Kelly AA, Dörmann P and Renger G (2005a) Investigations on the reaction pattern of photosystem II in leaves from *Arabidopsis thaliana* by

time-resolved fluorometric analysis. Biochemistry 44: 3123–3133

Steffen R, Kelly AA, Huyer J, Dörmann P and Renger G (2005b) Investigations on the reaction pattern of photosystem II in leaves from *Arabidopsis thaliana* wild-type plants and mutants with genetically modified lipid content. Biochemistry 44: 3134–3142

Stewart DH and Brudvig GW (1998) Cytochrome b_{559} of photosystem II. Biochim Biophys Acta 1367: 63–87

Stiehl HH and Witt HT (1969) Quantitative treatment of the function of plastoquinone in photosynthesis. Z Naturforsch 24b: 1588–1598

Strasser RJ and Sironval C (1972) Induction of photosystem II activity in flashed leaves. FEBS Lett 28: 56–60

Strickler MA, Hillier W and Debus RJ (2006) No evidence from FTIR difference spectroscopy that glutamate-189 of the D1 polypeptides ligates a Mn ion that undergoes oxidation during the S_0 to S_1, S_1 to S_2 or S_2 to S_3 transitions in photosystem II. Biochemistry 45: 8801–8811

Strzalka K, Walczak T, Sarna T and Swartz HM (1990) Measurement of time-resolved oxygen concentration changes in photosynthetic systems by nitroxide-based EPR oximetry. Arch Biochem Biophys 281: 312–318

Styring S and Rutherford AW (1988) Deactivation kinetics and temperature dependence of the S-state transitions in the oxygen evolving system of photosystem II measured by EPR spectroscopy. Biochim Biophys Acta 933 378–387

Sugiura M and Inoue Y (1999) Highly purified thermo-stable oxygen-evolving photosystem II core complex from the thermophilic cyanobacterium *Synechococcus elongatus* having His-tagged CP43. Plant Cell Physiol 40: 1219–1231

Sugiura M, Rappaport F, Brettel K, Noguchi T, Rutherford AW and Boussac A (2004) Site-directed mutagenesis of *Thermosynechococcus elongatus* photosystem II: The O_2-evolving enzyme lacking the redox-active tyrosine D. Biochemistry 43: 13549–13563

Sugiura M, Rappaport F, Hillier W, Dorlet P, Ohno Y, Hayashi H and Boussac A (2009) Evidence that D1-His332 in photosystem II from *Thermosynechococcus elongatus* interacts with the S_3-state and not with the S_2-state. Biochemistry 48: 7856–7866

Suzuki H, Nagasaka M, Sugiura M and Noguchi T (2005) Fourier transform infrared spectrum of the secondary quinone electron acceptor Q_B in photosystem II. Biochemistry 44: 11323–11328

Suzuki H, Sugiura M and Noguchi T (2009) Monitoring proton release during photosynthetic water oxidation in photosystem II by means of isotope-edited infrared spectroscopy. J Am Chem Soc 131: 7849–7857

Szczepaniak M, Sander J, Nowaczyk M, Müller MG, Rögner M and Holzwarth AR (2009) Charge separation, stabilization, and protein relaxation in photosystem II core particles with closed reaction center. Biophys J 96: 621–631

Takahashi R, Hasegawa K and Noguchi T (2008) Effect of charge distribution over a chlorophyll dimer on the redox potential of P680 in photosystem II as studied by density functional theory calculations. Biochemistry 47: 6289–6291

Tandori J, Sebban P, Michel H and Baciou L (1999) In *Rhodobacter sphaeroides* reaction centers, mutation of proline L209 to aromatic residues in the vicinity of a water channel alters the dynamic coupling between electron and proton transfer processes. Biochemistry 38: 13179–13187

Telfer A (2002) What is β-carotene doing in the photosystem II reaction centre? Phil Tans R Soc Lond B 357: 1431–1440

Theg SM, Filar LJ and Dilley RJ (1986) Photoinactivation of chloroplasts already inhibited on the oxidizing side of phototsystem II. Biochim Biophys Acta 849: 104–111

Thompson K, Blaylock R, Sturtevant JM, and Brudvig GW (1989) Molecular basis of the heat denaturation of photosystem II. Biochemistry 28: 6686–6695

Tiede DM and Dutton PL (1981) Orientation of the primary quinone of bacterial photosynthetic reaction centers contained in chromatophore and reconstituted membranes. Biochim Biophys Acta 637: 278–290

Tommos C and Babcock GT (1998) Oxygen production in nature: a light-driven metalloradical enzyme process. Acc Chem Res 31: 18–25

Tommos C and Babcock GT (2000) Proton and hydrogen currents in photosynthetic water oxidation. Biochim Biophys Acta 1458: 199–299

Tomo T, Mimuro M, Iwaki M, Kobayashi M, Itoh S and Satoh K (1997) Topology of pigments in the isolated photosystem II reaction center studied by selective extraction. Biochim Biophys Acta 1321: 21–30

Trissl HW and Lavergne J (1995) Fluorescence induction from photosystem II: analytical equations for the yields of photochemistry and fluorescence derived from analysis of a model including exciton-radical pair equilibrium and restricted energy transfer between photosynthetic units. Austral J Plant Physiol 22: 183–193

Ulas G, Olack G and Brudvig GW (2008) Evidence against bicarbonate bound in the O_2-evolving complex of photosystem II. Biochemistry 47: 3073–3075

Umena Y, Kawakami K J-R Shen and Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473 55–60

Utschig LM, Chemerisov SD, Tiede DM and Poluektov OG (2008) Electron paramagnetic resonance study of radiation damage in photosynthetic reaction center crystals. Biochemistry 47: 9251–9257

Vacha F, Durchan M and Siffel P (2002) Excitonic interactions in the reaction centre of photosystem II studied by using circular dichroism. Biochim Biophys Acta 1554: 147–152

Van Amerongen H and Dekker JP (2003) Light-harvesting in photosystem II. In: Green BR and Parson WW (eds) Light-Harvesting Antennas in Photosynthesis Advances in Photosynthesis and Respiration, Vol 13, pp 219–251. Kluwer Academic Publishers, The Netherlands

Van Amerongen H and Croce R (2008) Structure and function of photosystem II light-harvesting proteins (Lhcb) of higher plants. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part I: Photophysical Principles, Pigments Light Harvesting/ Adaptation/Stress, pp 329–367. Royal Society Chemistry, Cambridge

Van der Weij−de Wit CD, Dekker JP, Van Grondelle R and Van Stokkum IHM (2011) Charge separation is virtually irreversible in photosystem II core complexes with oxidized primary quinone acceptor. J Phys Chem A 115: 3947–3956

Van Gorkom HJ (1974) Identification of the reduced primary electron acceptor of photosystem II as a bound semiquinone anion. Biochim Biophys Acta 347: 439–442

Van Gorkom HJ and Yocum CF (2005) The calcium and chloride cofactors. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase. Advances in Photosynthesis and Respiration, Vol 22, pp 307–328. Springer, Dordrecht

Van Leeuwen PJ, Heimann CC, Gast P, Dekker JP and Gorkom HJ (1993) Flash-induced redox changes in oxygen-evolving spinach photosystem II core particles. Photosynth Res 38 169–176

Van Mieghem FJE and Rutherford AW (1993) Comparative spectroscopy of photosystem-II and purple bacterial reaction centres. Biochem Soc Trans 21: 986–991

Van Mieghem F, Nitschke W, Mathis P and Rutherford AW (1989) The influence of the quinone-iron complex on the reaction center photochemistry of photosystem II. Biochim Biophys Acta 977: 207–214

Van Miegham F, Brettel K, Hillmann B, Kamlowski A, Rutherford AW and Schlodder E (1995) Charge recombination reactions in photosystem II. 1. Yields, recombination pathways and kinetics of the primary pair. Biochemistry 34: 4798–4813

Van Niel CB (1941) The bacterial photosynthesis and their importance of the general problem of photosynthesis. Acta Enzymology 1: 263–328

Van Rensen JJS and Klimov VV (2005) Bicarbonate interactions. In: Wydrzynski TJ and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 329–346. Springer, Dordrecht

Vasil'ev S, Bergmann A, Redlin H, Eichler H-J and Renger G (1996) On the role of exchangeable hydrogen bonds for the kinetics of $P_{680}^{+\bullet}Q_A^{-\bullet}$ formation and $P_{680}^{+\bullet}Pheo^{-\bullet}$ recombination in photosystem II. Biochim Biophys Acta 1276: 35–44

Vasil'ev S, Orth P, Zouni A, Owens TG and Diner B (2001) Excited-state dynamics in photosystem II: Insights from the X-ray crystal structure. Proc Natl Acad Sci USA 98: 8602–8607

Vasil'ev S, Lee C-I, Brudvig GW and Bruce D (2002) Structure-based kinetic modeling of excited-state transfer and trapping in histidine-tagged photosystem II core complexes from *Synechocystis*. Biochemistry 41: 12236–12243

Vasil'ev S, Shen J-R, Kamiya N and Bruce D (2004) The orientations of core antenna chlorophylls in photosystem II are optimized to maximize the quantum yield of photosynthesis. FEBS Lett 561: 111–116

Vass I and Styring S (1991) pH dependent charge equilibria between tyrosine-D and the S-states in photosystem II. Estimation of relative midpoint potentials. Biochemistry 30: 830–839

Vass I and Aro E-M (2008) Photoinhibition of photosynthetic electron transport. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part 1: Photophysical Principles, Pigments Light Harvesting/ Adaptation/Stress, pp 393–425. Royal Society Chemistry, Cambridge

Vass I, Turcsányi E, Touloupakis E, Ghanotakis D and Petrouleas V (2002) The mechanism of UV-A radiation-induced inhibition of photosystem II electron transport studied by EPR and chlorophyll fluorescence. Biochemistry 41**:** 10200–10208

Vavilin D, Xu H, Lin S and Vermaas W (2002) Energy and electron transfer in photosystem II of a chlorophyll *b*-containing *Synechocyste* sp. PCC 6803 mutant. Biochemistry 42: 1731–1746

Velthuys B (1981) Spectroscopis studies of the S-state transitions of photosystem II and of the interaction of its charged donor chains with lipid-soluble anions. In: Akoyunoglou G (ed) Photosynthesis II. Electron Transport and Photophosphorylation, pp 75–85. Balaban, Philadelphia

Vermaas WFJ, Renger G and Dohnt G (1984) The reduction of the oxygen-evolving system in chloroplasts by thylakoid components. Biochim Biophys Acta 764: 194–202

Vermeglio A (2008) Anoxygenic Bacteria. In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II. Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 353–382. Royal Society Chemistry, Cambridge

Verméglio A, Joliot A and Joliot P (1998) Supramolecular organization of the photosynthetic chain in mutants of Rhodobacter capsulatus deleted in cytochrome c_2. Biochim Biophys Acta 1318: 374–38

Vos MH, Van Gorkom HJ and Van Leeuwen PJ (1991) An electroluminescence study of stabilization reactions in the oxygen-evolving complex of photosystem II. Biochim Biophys Acta 1056: 27–39

Vrettos JS, Limburg J and Brudvig GW (2001) Mechanism of photosynthetic water oxidation: combining biophysical studies of photosystem II with inorganic model chemistry. Biochim Biophys Acta 1503: 229–245

Wakeham MC, Goodwin MG, McKibbin C and Jones MR (2003) Photo-accumulation of the $P^+Q_B^-$ radical pair state in purple bacterial reaction centres that lack the Q_A ubiquinone. FEBS Lett 540: 234–240

Walz D (1997) Nonequilibrium thermodynamics applied to energy conversion in biological systems. In: Gräber P and Milazzo G (eds) Bioenergetics, pp 1–56. Birkhäuser Verlag, Basel

Wang D, Zheng J, Shaik S and Thiel W (2008) Quantum and molecular mechanical study of the first proton transfer in the catalytic cycle of cytochrome P450cam and its mutant D251N. J Phys Chem B 112: 5126–5138

Wang H, Lin S, Allen JP, Williams JAC, Blankert S, Laser C and Woodbury NW (2007) Protein dynamics control the kinetics of initial electron transfer in photosynthesis. Science 316: 747–750

Warncke K and Dutton PS (1993) Influence of Q_A site redox cofactor structure on equilibrium binding, *in situ* electrochemistry, and electron-transfer performance in the photosynthetic reaction center protein. Biochemistry 32: 4769–4779

Warshel A and Parson WW (1987) Spectroscopic properties of photosynthetic reaction centers. 1. Theory. J Am Chem Soc 109: 6143–6152

Watanabe T and Kobayashi M (1991) Electrochemistry of chlorophylls. In: Scheer H (ed) Chlorophylls, pp 287–315. CRC Press, Boca Raton

Weiss W and Renger G (1984) Analysis of the system II reaction by UV-absorption changes in tris-washed chloroplasts. In: Sybesma C (ed) Advances in Photosynthesis Research, Vol 1, pp 167–170. Marinus Nijhoff/Dr. W. Junk Publishers, The Hague

Weng TC, Hsieh WY, Uffelman ES, Gordon-Wylie SW, Collins TJ, Pecoraro VL and Penner-Hahn JE (2004) XANES evidence against a manganyl species in the S_3 state of the oxygen-evolving complex. J Am Chem Soc 126: 8070–8071

Westphal KL, Lydakis-Simantiris N, Cukier RI and Babcock GT (2000) Effects of Sr^{2+} substitution on the reduction rates of $Y_Z^{\bullet}$ in PSII membranes: Evidence for concerted hydrogen atom transfer in oxygen evolution. Biochemistry 39: 16220–16229

Wikström M (2004) Cytochrome *c* oxidase: 25 years of the elusive proton pump. Biochim Biophys Acta 1655: 241–247

Williams RJP (1995) Energised (entatic) states of groups and of secondary structures in proteins and metalloproteins. Eur J Biochem 234: 363–381

Witt HT (1971) Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. Quart Res Biophys 4: 365–477

Woodbury NW, Parson WW, Gunner MR, Prince RC and Dutton PL (1986) Radical-pair energetics and decay mechanisms in reaction centers containing anthraquinones, naphtoquinones or benzoquinones in place of ubiquinone. Biochim Biophys Acta 851: 6–22

Wraight CA (1985) Modulation of herbicide binding by the redox state of Q_{400}, an endogenous component of photosystem II. Biochim Biophys Acta 809: 320–330

Xiong J and Bauer CE (2002) Complex evolution of photosynthesis. Annu Rev Plant Biol 53: 503–521

Xiong L, Seibert M, Gusev AV, Wasielewski MR, Hemann C, Hille CR and Sayre RT (2004) Substitution of a chlorophyll into the inactive branch pheophytin-binding site impairs charge separation in photosystem II. J Phys Chem 108: 16904–16911

Yachandra VK (2005) The catalytic manganese cluster: organisation of the metal ions. In: Wydrzynski TJ and Satoh K (eds) Photosystem II. The Light-Driven Water: Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 235–260. Springer, Dordrecht

Yachandra VK and Yano J (2011) Calcium in the oxygen-evolving complex: Structural and mechanistic role determined by X-ray spectroscopy. J Photochem Photobiol B: Biol 104: 51–59

Yachandra VK, Sauer K and Klein MP (1996) Manganese cluster in photosynthesis: where plants oxidize water to dioxygen. Chem Rev 96: 2927–2950

Yamauchi T, Mino H, Matsukawa T, Kawamori A and Ono T (1997) Parallel polarization electron paramagnetic resonance studies of the S_1-state manganese cluster in the photosynthetic oxygen-evolving system. Biochemistry 36**:** 7520–7526

Yano J, Kern J, Irrgang K-D, Latimer MJ, Bergmann U, Glatzel P, Pushkar Y, Biesiadka J, Loll B, Sauer K, Messinger J, Zouni A and Yachandra VK (2005a) X-ray damage to the Mn_4Ca complex in single crystals of photosystem II: A case study for metalloprotein crystallography. Proc Natl Acad Sci USA 102: 12047–12052

Yano J, Pushkar Y, Glatzel P, Lewis A, Sauer K, Messinger J, Bergmann U and Yachandra VK (2005b) High-resolution Mn EXAFS of the oxygen-evolving complex in photosystem II: Structural implications for the Mn_4Ca cluster. J Am Chem Soc 127: 14974–14975

Yano J, Kern J, Sauer K, Latimer JM, Pushkar Y, Biesiadka J, Loll B, Saenger W, Messinger J, Zouni A and Yachandra VK (2006) Where water is oxidized to dioxygen: Structure of the photosynthetic Mn_4Ca cluster. Science 314: 821–825

Yano J, Walker LM, Strickler MA, Service RJ, Yachandra VK and RJ Debus (2011)Altered structure of the Mn4Ca cluster in the oxygen-evolving complex of photosystem II by a histidine ligand mutation. J Biol Chem 286: 9257–9267

Yikilmaz E, Rodgers DW and Miller A-F (2006) The crucial importance of chemistry in the structure-function link: Manipulating hydrogen bonding in iron-containing superoxide dismutase. Biochemistry 45: 1151–1161

Zaks A and Klibanov AM (1984) Enzymatic catalysis in organic media at 100 degrees C. Science: 1249–1251

Zamaraev KI and Parmon VN (1980) Potential methods and perspectives of solar energy conversion via photocatalytic processes. Rev Sci Eng 22: 261–324

Zein S, Kulik LV, Yano J, Kern J, Pushkar Y, Zouni A, Yachandra VK, Lubitz W, Neese F and Messinger J (2008) Focusing the view on nature's water-splitting catalyst. Phil Trans R Soc B 363: 1167–1177

Zinth W and Kaiser W (1993) Time-resolved spectroscopy of the primary electron transfer in reaction centers of *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis*. In: Deisenhofer J and Norris JR (eds) The Photosynthetic Reaction Center, Vol II, pp 71–88. Academic Press, San Diego

Zouni A (2008) In: Renger G (ed) Primary Processes of Photosynthesis: Basic Principles and Apparatus, Part II Reaction Centers/Photosystems, Electron Transport Chains, Photophosphorylation and Evolution, pp 193–236. Royal Society Chemistry, Cambridge

Zouni A, Witt HT, Kern J, Fromme P, Krauß N, Saenger W and Orth P (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. Nature 409: 739–743

Chapter 18

Fluorescence Emission from the Photosynthetic Apparatus

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Summary

Oxygenic photosynthetic organisms (plants, algae and cyanobacteria) use the energy of visible light to drive a highly complex and elaborate enzymic mechanism that decomposes water to oxygen, protons and electrons and reduces carbon dioxide to carbohydrate. Three classes of colored molecules, all complexed to protein, serve as entry points of visible light in the mechanism of photosynthesis: chlorophylls (Chls), carotenoids (Cars) and phycobilins (PBs). The most important is Chl *a*, not only because it is ubiquitous and the most abundant of all, but mainly because it is capable of donating a valence electron to an acceptor when it is electronically excited. As a matter of fact only few Chls *a* are photoactive, those in the reaction centers of photosystems (PS) I and II. The majority of them, and all the other pigments, are not photoactive. These non-photoactive pigments serve as light-harvesting antennae, absorbing photons and transferring excitation energy (EE) to reaction center Chls *a* and, also, in light protection by dissipating excessive EE as heat. While absorbed light energy is used almost quantitatively for photosynthesis, about 3% of it is re-emitted as fluorescence and even smaller fractions as delayed fluorescence and phosphorescence. The emitted fluorescence, particularly that by Chl *a*, is very rich in information about the physical states and the interactions of emitter and sensitizer molecules. Information resides not only in their stationary absorption and fluorescence signals but more so in their time variations which reflect corresponding variations in the populations of excited chromophores and their quantum efficiencies as

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fluorescence emitters. The key reporter molecule is, of course, Chl *a*. Its fluorescence emission has been exploited extensively as a diagnostic tool for the mechanism of oxygenic photosynthesis from the moment of photon capture to that of dioxygen release from water; and also as a tool for assessing the productivity of individual plants, of plant communities and of ecosystems. The present chapter addresses these themes.

I. Introduction

Visible light enters oxygenic photosynthesis as a reactant, in fixed stoichiometry to other reactants (H_2O, CO_2) and products (O_2, carbohydrates). For overall discussions on photosynthesis, see Rabinowitch and Govindjee (1969), Govindjee (1975, 1982), and Blankenship (2002). Entry points for light are at three classes of visible light absorbing molecules, or chromophores, the *chlorophylls* (Chls), the *carotenoids* (Cars) and the *phycobilins* (PBs). All are complexed to protein(s) and are referred to as *pigments*. The pigment-protein complexes, or holochromes, assemble together with non-holochromic proteins, electron transport cofactors, lipids, water and inorganic ions and form two discrete supramolecular assemblies known as Photosystem I (PS I; see Golbeck, 2006) and Photosystem II (PS II; see Wydrzynski and Satoh, 2005). Each photosystem comprises a core complex (PSI_{CC}, $PSII_{CC}$) and peripheral antenna complexes (PSI_{PAC}, $PSII_{PAC}$; see Green and Parson, 2003). The $PSII_{CC}$ consists of a reaction center complex ($PSII_{RC}$) and a core antenna complex ($PSII_{CAC}$) that are on different proteins and therefore they are resolvable biochemically. In the PSI_{CC} the core antenna part (PSI_{CAC}) and the reaction center part (PSI_{RC}) are regions on the same protein and cannot be separated.

Figure 18.1 displays the chemical structures of selected photosynthetic pigments and Table 18.1 their distribution among the three major groups of oxygenic photosynthetic organisms (oxyphototrophs), the cyanobacteria, the algae and the higher plants. A pigment traps a photon as EE in its system of conjugated double bonds and becomes a "hot" excited molecule, according to the Franck-Condon principle. EE relaxes intramolecularly within femtoseconds and hops from molecule to molecule isoenergetically, moving along optimized excitation energy transfer (EET) paths, until it hits a reaction center Chl *a*. The special proteins of PSI_{RC} and $PSII_{RC}$, highly conserved among the photosynthetic organisms, co-ordinate clusters of six chlorins *a*, which co-operate in transferring one electron to an acceptor upon receiving one exciton (or one photon). In this way, the EE is trapped in ground state cation-anion biradicals, Chl a^+–pheophytin a^- ($Pheo^-$) in $PSII_{RC}$, and as Chl a^+–Chl a^- in PSI_{RC}.

Only reaction center Chls *a* are chemically photoactive in vivo, meaning that they are capable of transferring one electron to a ground state acceptor when excited. Antenna pigments (including Chls *a*) are not photoactive, even though in solution they may be. Their function is to ensure a regulated flow of EE to the reaction centers at rates that optimize photosynthesis. Laboratory attempts to replace some or all of the reaction center Chls *a* by genetic engineering failed, and so did nature itself during the ~3 billion years of evolution of photosynthetic organisms (see Björn and Govindjee, 2009; Björn et al., 2009, and citations

Abbreviations: ANT_M – Mobile peripheral antenna elements; APC – Allophycocyanin; Car – Carotenoid; Chl – Chlorophyll; EE – Excitation energy; EET – Excitation energy transfer; FCP – Fuc-Chl protein complex; FI – Fluorescence induction; FRAP – Fluorescence recovery after photobleaching; Fuc – Fucoxanthin; HSPCP – High-salt PCP; $h\nu_{PSI}$ – Light absorbed more by PS I; $h\nu_{PSII}$ – Light absorbed more by PS II; LHC – Peripheral light-harvesting Chl – protein; LHCI – LHCs of PS I; LHCII – LHCs of PS II; MFPCP – Main form PCP; OCP – Orange Car protein; PB – Phycobilin; PBP – Phycobiliprotein; PBS – Phycobilisome; PC – Phycocyanin; Pcb – Prochlorophyte Chl *a*/*b*-binding protein; PCB – Phycocyanobilin; PCP – Per–Chl *a* protein; PE – Phycoerythrin; PEB – Phycoerythrobilin; PEC – Phycoerythrocyanin; Per – Peridinin; Pheo – Pheophytin; PQ – Plastoquinone; PSET – Photosynthetic electron transport; PS I, PS II – Photosystem I, Photosystem II; PSI_{CAC} – Core antenna complex of PS I; PSI_{CC} – Core complex of PS I; $PSII_{CAC}$ – Core antenna complex of PS II; $PSII_{CC}$ – Core complex of PS II; $PSII_{PAC}$ – Peripheral antenna complex of PS II; $PSII_{RC}$ – Reaction center complex of PS II; PSI_{PAC} – Peripheral antenna complex of PS I; PSI_{RC} – Reaction center complex of PS I; it also contains core antenna chlorophylls PUB – Phycourobilin; PVB – Phycoviolobilin; TMH – Transmembrane α–helix

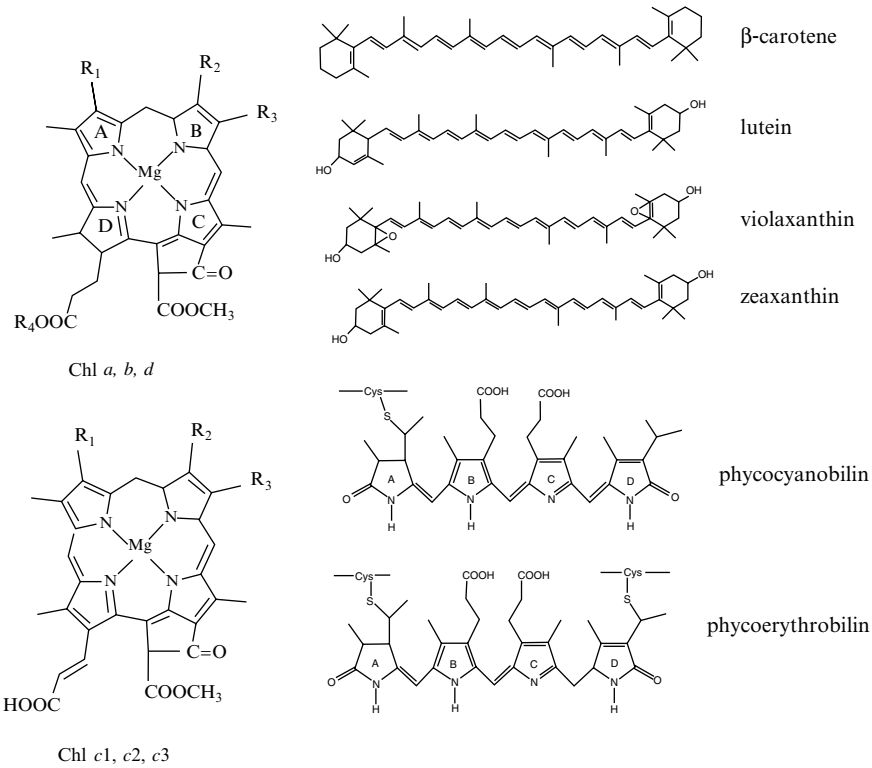

Fig. 18.1. Structural formulae of representative photosynthetic pigments: Chlorophylls (*left*), carotenoids and phycobilins (*right*). Note that Chls *c1*, *c2* and *c3* are substituted derivatives of a fully conjugated tetrapyrrole ring (porphyrin) while Chls *a*, *b*, and *d* are substituted derivatives of dihydroporphyrin. Substituents *R1*, *R2*, *R3*, and *R4* are defined as follows:

Chl	R1	R2	R3	R4
a	$-CH=CH_2$	$-CH_3$	$-CH_2CH_3$	Phytyl
b	$-CH=CH_2$	-CHO	$-CH_2CH_3$	Phytyl
c_1	$-CH=CH_2$	$-CH_3$	$-CH_2CH_3$	H
c_2	$-CH=CH_2$	$-CH_3$	$-CH=CH_2$	H
c_3	$-CH=CH_2$	$-COOCH_3$	$-CH=CH_2$	H
d	-CHO	$-CH_3$	$-CH_2CH_3$	Phytyl

therein). A rare exception is the cyanobacterium *Acaryochloris marina* whose Chls *a* in both $PSII_{RC}$ and PSI_{RC} are partially replaced by Chls *d* (see Section III).

Less than 3% of the available EE is emitted as Chl *a* fluorescence (*radiative dissipation*). The largest fraction is channeled to photosynthesis (*photochemical utilization*) and an intermediate fraction is dissipated as heat (*nonradiative-nonphotochemical dissipation*). These three dissipative routes are interdependent, because the photochemical route regulates the other two by means of ground state chemical signals that noncyclic *photosynthetic electron transport* (PSET) generates. Thus, the most conveniently measured optical signal, Chl *a* fluorescence, is extensively used to estimate the rate of photosynthesis, under the conditionally correct assumption that the complementarity relationship:

$$\text{Rate of fluorescence emission} + \text{Rate of photosynthesis} \approx \text{constant},$$

is valid. This equation implies of course a constant rate of heat dissipation.

Since Chl *a* is present in all photosynthetic organisms, and also indispensible for key photosynthetic processes (photon capture, EET and trapping), and furthermore since its EE is subject to regulation by the PSET, it appears as the ideal reporter molecule for the highly complex process of oxygenic photosynthesis. The portal to this information is the fluorescence it emits from various holochromic sites. Recent advances

Table 18.1. Occurrence of photosynthetic pigments in the major groups of oxyphototrophic organisms

Organism	Chlorophylls a	b	c	d	Carotenoids	Phycobiliproteins
Prokaryotes (Cyanobacteria)						
Chl *a*/PBP-containing	+	–	–	–	+	+
Chl *a, b*/PBP-containing (Prochlorophytes)	+	+	–	–	+	+
Chl *a, d*/PBP-containing (*Acaryochloris* genus)	+	–	(+)	+	+	(+)
Eukaryotes						
Green algae	+	+	–	(+)	+	–
Euglenoids	+	+	–	–	+	–
Red algae	+	–	–	(+)	+	+
Cryptophytes	+	–	+	–	+	+
Brown algae	+	–	+	–	+	–
Diatoms	+	–	+	–	+	–
Dinoglagellates	+	–	+	–	+	–
Higher plants	+	+	–	–	+	–

According to the hypothesis of Tomitani et al. (1999), green and red algae diversified from an ancestral photosynthetic eukaryote that was produced by the engulfment of a Chl *a*/Chl *b*/PB-containing cyanobacterium by a nonphotosynthetic eukaryote (primary endosymbiosis); euglenoids by the engulfment of a green alga by a nonphotosynthetic eukaryote, and chromophytic algae (Chl *c*-containing) correspondingly by the engulfment of a red alga (secondary endosymbioses). Higher plants are evolutionary descendants of green algae (not of the Chl *a*/Chl *b*/PCP-containing cyanobacteria, which are also known as *prochlorophytes*)

in three areas: optoelectronics, X-ray diffraction of protein crystals and genetic engineering of membrane intrinsic proteins, have enabled advanced detailed fluorometric interrogation of photosynthetic samples, from purified holochromes to whole leaves. Information resides not only in their stationary optical properties (photon absorption and emission) but also in the time variations of excited state populations that are reported by kinetic displays of the optical signals. Thus, ultrafast methods (fs to ns) enable the tracking of EE on its way to the $PSII_{RC}$ and PSI_{RC} traps; very fast methods (ns to μs) the analysis of the primary ground state electron transfers, to and from the reaction centers; fast methods (μs to s) the tracking of ground state electron transport in the direction $H_2O \rightarrow PSII_{RC} \rightarrow PSI_{RC} \rightarrow CO_2$; slow methods (s to min) the analysis of physiological adjustments of the photosynthetic apparatus; and *very slow methods* (min to h) the analysis of the genomic adjustments of photosynthetic organisms.

The relevant scientific literature is, indeed, vast. A short list of books dedicated to the theme of Chl fluorescence is: Govindjee et al. (1986), Lichtenthaler (1988), DeEll and Toivonen (2003), and Papageorgiou and Govindjee (2004) and Suggett et al. (2011).

II. Absorbers and Emitters of Visible Light

Figure 18.2 displays absorption and fluorescence spectra of Chls *a, b, c,* and *d* in vitro. In the protein environments of the PS II and PS I holochromes, absorption and emission bands shift to longer wavelengths (red shifts) and become broader (inhomogeneous broadening).

A. Photosystem II

PS II is defined as a *light-driven water-plastoquinone oxidoreductase* a term evoking its overall function that consists in the thermodynamically uphill transfer of electrons from water ($E' \approx 0.82$ V) to plastoquinone ($E' \approx 0$ V). Its core and peripheral antenna pigment-protein complexes ($PSII_{CC}$ and $PSII_{PAC}$) are biochemically resolvable. The core complex is dimeric, $(PSII_{CC})_2$, each monomer being composed of a reaction center complex ($PSII_{RC}$) and a core antenna complex ($PSII_{CAC}$): it is also biochemically resolvable, and very similar in all species of oxygenic plants. In contrast, the $PSII_{PAC}$ are quite diverse among the various taxonomic groupings reflecting evolutionary adjustment to differing environments (Wydrzynski and Satoh, 2005).

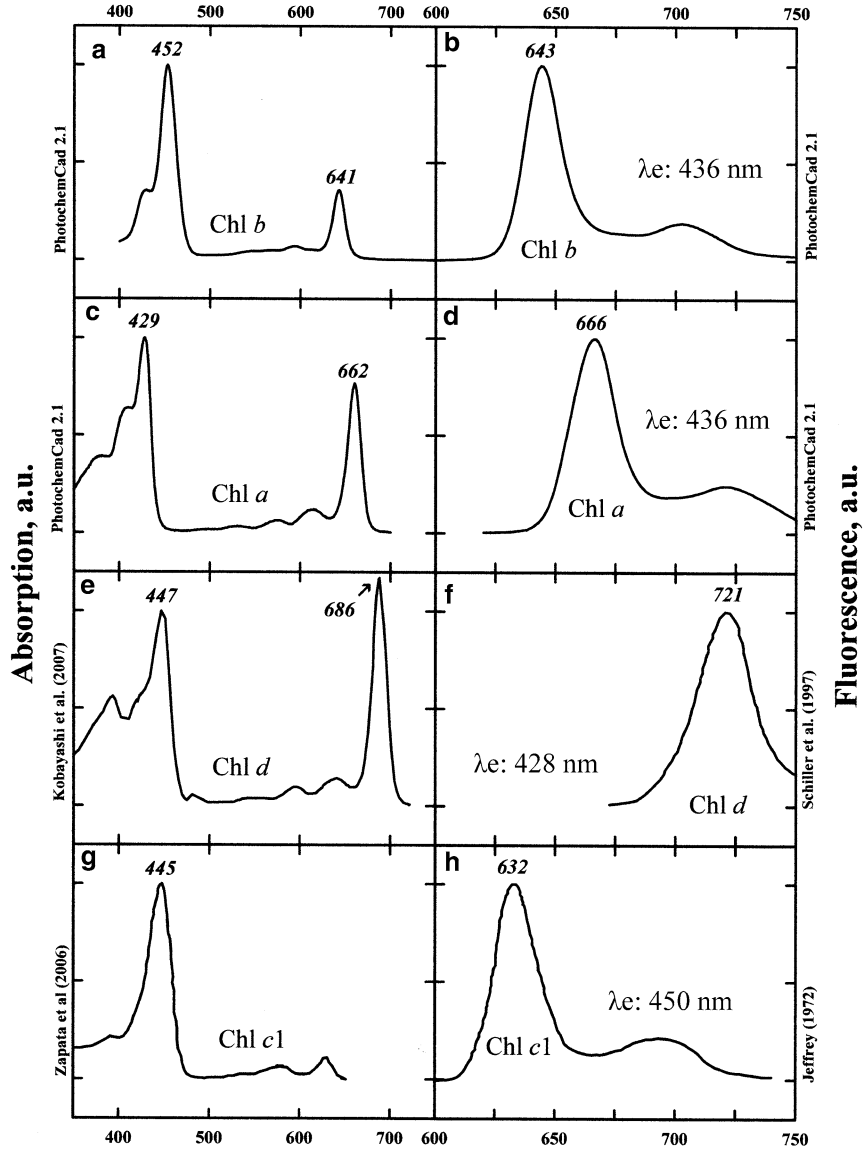

Fig. 18.2. Room temperature absorption (**a**, **b**, **c**, **g**) and fluorescence (**b**, **d**, **f**, **h**) spectra of chlorophylls in solution. Sources of the spectra: **a** to **d**, from http://www.photochemcad.com (solvent, diethyl ether); **e**, from Kobayashi et al. (2007; solvent, diethyl ether); **f**, from Schiller et al. (1997, *A. marina* cells); **g**, from Zapata et al. (2006, solvent acetone); and **h**, from Jeffrey (1972; solvent, acetone). In **b**, **d**, **f**, **h**, λ_{exc} is entered directly on the figure as follows: in **b** (λ_{exc}=436 nm); in **d** (λ_{exc}=436 nm); in **f** (λ_{exc}=428 nm); and in **h** (λ_{exc}=450 nm).

1. Core Complexes

The PS II core complex ($PSII_{CC}$) uses light energy in order to generate a high potential oxidant ($E_o' \sim 1.2$ V) with which it oxidizes water to molecular oxygen, protons and electrons, and uses the latter (at $E_o' \sim 0$ V) in order to reduce plastoquinone to plastohydroquinone. Because of that, as noted above, PS II is sometimes designated as a water-plastoquinone oxidoreductase (Wydrzynski and Satoh, 2005; Govindjee et al., 2010).

The $PSII_{CC}$ is a homodimer (700 kDa, 105 Å deep, 205 Å long and 100 Å wide). Its near-atomic structure has been resolved by X-ray diffraction only in thermophilic cyanobacteria (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004;

Loll et al., 2005; Guskov et al., 2009; Umena et al., 2011) but it is believed to be closely similar in all oxygenic photosynthetic organisms. A $PSII_{CC}$ monomer consists of a reaction center complex ($PSII_{RC}$) and a core antenna complex ($PSII_{CAC}$) and comprises 20 different proteins, including four holochromic proteins that coordinate 35 Chls *a*, 2 pheophytins *a* (Pheo) and 12 Cars modeled as all-*trans* β-Cars (Loll et al., 2005; Guskov et al., 2009). The $PSII_{RC}$ contains the holochrome heterodimer D_1/D_2, and the $PSII_{CAC}$ holochromes CP43 and CP47 that flank the D1D2 heterodimer in the order:

$$\text{CP43 [D1][D2] CP47}$$

D1 and D2 bind 3 Chls *a* and 1 Pheo *a* each. They also bind 3 Car, one on D1 and 2 on D2. CP43 binds 13 Chls *a* and 3 β–Cars and CP47 16 Chls *a* and 5 β–Cars. One β-Car is located near smaller non-holochromic $PSII_{CC}$ subunits. Four of the 6 D1D2 Chls *a*, those designated as Chl_{D1}, Chl_{D2}, P_{D1}, P_{D2}, form the primary electron donor, traditionally known as P_{680} (according to the *multimeric model* of Durrand et al., 1995) and $Pheo_{D1}$ is the primary electron acceptor of the active branch on D1. Of the remaining 2 Chls *a*, Chl_{Z1} on D_1 mediates EET from CP43 and Chl_{Z2} on D_2 EET from CP47 to the Chl *a* cluster of P680. Holochrome arrangement in the $PSII_{CC}$ homodimer is as follows:

$$\text{[CP43][D1][D2][CP47]}$$

$$\text{[CP47][D2][D1][CP43]}$$

Figure 18.3 displays typical stationary absorption and fluorescence spectra of $PSII_{CC}$ and of its subfractions $PSII_{RC}$ and CP43 and CP47. Absorption spectra were recorded at room temperature, fluorescence spectra at cryogenic temperatures (77 K, or lower). The absorption spectra encompass contributions of all chromophores in these complexes, namely Chls *a* (B_{xy} ~435 nm, Q_x ~625 nm, Q_y ~668–672 nm), Pheos *a* (B_{xy} ~416 nm, Q_x ~540 nm, Q_y ~669–681 nm) and β–Cars (~450–500 nm). Characteristic is the Soret (B_{xy}) absorption maximum of $PSII_{RC}$ at 416 nm (because of the higher Pheo *a* proportion; Tomo et al., 2008) and the higher energy Q_y band of CP43 (668 nm; Alfonso et al., 1994; Dekker et al., 1995; Groot et al., 1999) relative to those in CP47 (671 nm; Alfonso et al., 1994; Chang et al.,1994; Dekker et al., 1995; Groot et al., 1995) and in D1D2 (672 nm; Konermann and Holzwarth, 1996). At cryogenic temperatures, absorption bands become sharper and may split into two peaks. The Q_y band of isolated $PSII_{RC}$ splits to a peak at ~670 nm and another at ~680 nm (not shown here), with the former being assigned to "accessory" Chls *a* and the latter to P680 (Tetenkin et al., 1989; Braun et al., 1990). Likewise, the Qy band of CP43 splits at 4 K in two bands, at 669 nm and at 682.5 nm (Groot et al., 1999), while the Qy band of CP43 displays a peak at ~673 nm and a shoulder at ~682 nm (Groot et al., 1995).

At low temperature, accessory Chl_D has its lowest energy absorption (Q_y) at ~682–664 nm (Konermann and Holzwarth, 1996; Stewart et al., 2000), while the Q_y band of the P_{D1}, P_{D2} pair lies a little higher (~679–680 nm; Braun et al., 1990; Konermann and Holzwarth, 1996). This is consistent with the discovery that Chl_{D1} is the the primary electron donor of P680 and P_{D1} the secondary (Diner et al., 2001; Holzwarth et al., 2006). Discrepant results have been published for the reduced Pheo at low temperature. Thus $Pheo_{D1}$ has been assigned Q_y maxima at ~670–672 (Braun et al., 1990; Konermann and Holzwarth, 1996; Tomo et al., 2008), and at ~681–685 nm (Stewart et al., 2000).

The fluorescence that $PSII_{CC}$ and subcomplexes emit (Fig. 18.3) originates from Chls *a*. Low temperature emissions are centered at ~683 nm, except that of CP47 which is centered at ~690 nm. Characteristic is the narrow bandwidth of the CP43 emission at low temperature which reflects the fact that only one Chl *a* (out of 14 in this complex) emits fluorescence (Groot et al., 1999). At room temperature, the Q_y absorption maximum of this Chl *a* is at 685 nm and its fluorescence maximum is at 689 nm (Shan et al., 2001).

Various methods have been designed and used in conjunction with low temperature spectroscopic data, and X-ray crystallography models, in order to assign absorption and fluorescence transitions to individual $PSII_{CC}$ chromophores. They include computational approaches, such as band deconvolution into gaussian components and (inverted) second derivatives, biochemical, photochemical and molecular biological perturbations of samples, time-resolved spectroscopy and theoretical calculations. Reported results are not always in

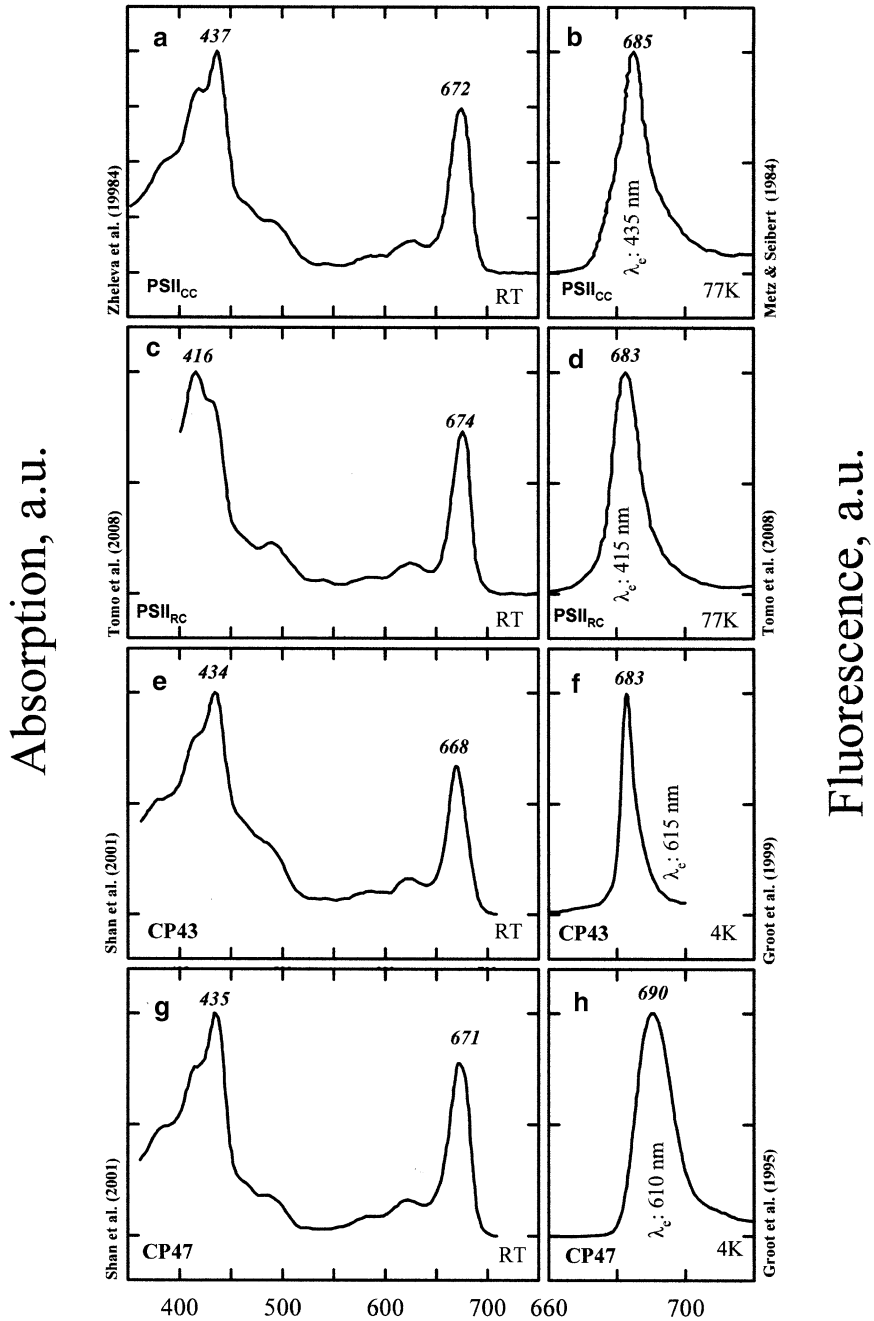

Fig. 18.3. Absorption (**a**, **c**, **e**, **g**) and fluorescence (**b**, **d**, **f**, **h**) spectra of purified PS II core complexes ($PSII_{CC}$) and of constituent holochromes (D_1, D_2, CP43, CP47) retraced from published data. Absorption was recorded at room temperature, fluorescence at low temperatures (indicated). (**a**) $PSII_{CC}$ isolated from spinach leaves (Zheleva et al., 1998); (**b**) $PSII_{CC}$ from the green alga *Scenedesmus obliquus* (Metz and Seibert, 1984); (**c**, **d**) $PSII_{RC}$ (containing holochromes D_1 and D_2) isolated from cyanobacterium *Synechocystis* sp. PCC 6803 (Tomo et al., 2008); (**e**) core antenna holochrome CP43 from spinach (Shan et al., 2001); (**f**), core antenna holochrome CP43 from spinach (Groot et al., 1999); (**g**) core antenna holochrome CP47 isolated from spinach (Shan et al., 2001); (**h**) core antenna holochrome CP47 from spinach (Groot et al., 1995).

agreement reflecting the influence, among others, of temperature, local electric field (electrochromism), sample purity and sample integrity.

2. Peripheral Antenna Complexes

Holochromes in $PSII_{PAC}$ serve basically three functions: To supply EE to core complexes, to dissipate excess EE as heat before it reaches the core complex, and to regulate the proportion of delivered EE in order to ensure equal turnover rates of $PSII_{RC}$ and PSI_{RC} (via *state transitions*; see Section III.A). In contrast to $PSII_{CC}$, whose structure and composition is conserved among oxyphototrophs, $PSII_{PAC}$ differ in structure and composition across different taxa, reflecting divergent evolutionary histories.

Among the oxygenic photosynthetic prokaryotes, the phycobilisome (PBS)–containing cyanobacteria use phycobiliproteins (PBPs) to harvest light, prochlorophytes use Chl *b*-binding proteins (Pcbs), while the Chl *d*-rich *Acaryochloris* genus uses both Pcb-like proteins (that contain mostly Chl *d* and some Chl *a*) and PBPs (Partensky and Garczarek, 2003, and citations therein). These peripheral antennae supply EE to the core complexes of both the photosystems. In contrast, peripheral antennae in green algae and higher plants, consisting of membrane-intrinsic light-harvesting Chl *a/b*-binding complexes (LHC proteins), are partly dedicated to each photosystem and partly mobile and shared by both photosystems (see Section III.A). Further, red algae use PBS on the stroma side of thylakoids to feed EE to $PSII_{CC}$, cryptophytic algae both intramembranous (Chl a/c_2/Car–containing) and intralumen proteins (PBPs), chromophytic algae intramembanous (Chl a/c_2/Fuc–containing) proteins, while dinoflagellates use additionally intralumen (Chl *a*/Per–containing) proteins (see below for details).

Figure 18.4 displays stationary absorption and fluorescence spectra of selected PS II antenna holochromes of cyanobacteria (a, b) and higher plants (c, d and e, f).

a. Cyanobacteria

Water-soluble PBPs, already known in the 19th century, are the building blocks of the PBS (Tandeau de Marsac, 2003). The principal PBPs (absorption and fluorescence bands in parentheses) are: allophycocyanin (APC, A650–655, F660), phycocyanin (PC, A615–620, F625–645), phycoerythrin (PE, A565–575, F575–581) and phycoerythrocyanin (PEC, A575–590, F625). A PBP monomer is actually a heterodimer of two hydrophobic polypeptides (α and β) that bind 1–3 PB chromophores. Their bilin chromophores are: phycocyanobilin (PCB, in PC, PEC and APC), phycoerythrobilin (PEB in PE), phycoviolobilin (PVB in PEC) and phycourobilin (PUB in some PEs; reviewed by MacColl, 1998; Adir, 2005).

PBP monomers form disk-shaped homotrimers (3-fold rotational axis plus central hole), two trimers stack face-to-face to a hexamer disk (~110 Å in diameter and ~60 Å thick) and hexamer disks, held face-to-face by linker polypeptides (mostly colorless) form cylindrical rods. The commonest PBS shape in cyanobacteria is a half-disk having a core part (2, 3, or 5 APC cylinders) and six radiating rods consisting of PC (basal disks) and PE and PEC (distal disks). PBS of other shapes, rod numbers and core cylinders have been described (Sidler, 1994; Arteni et al., 2009).

EE moves non-randomly in the direction PE → PC → APC (reviews by Glazer, 1989; Holzwarth, 1991; Brody, 2002) because of efficient intrahexamer exciton exchanges and of large interhexamer energy gaps. EET between the core APCs and the thylakoid membrane Chls *a* is mediated by the core-membrane linker protein ApcE (or L_{CM}) and the APC-B subunits ApcD and ApcF (Sidler, 1994; Dong et al., 2009). The PBS have been visualized to fit structurally to the exposed cytoplasmic surface of $PSII_{CC}$ dimers (Bald et al., 1996). However, this does not imply fixed stoichiometry since the PBS/PSII ratio exceeds unity (Manodori and Melis, 1985). Despite their large size, the PBS are possibly highly mobile on the thylakoid membrane surface (Mullineaux et al., 1997). The PBS is possibly the best described peripheral antenna complex. Historical and recent reviews are those by: Gantt, 1980, 1981; Scheer, 1981; Glazer, 1982, 1984, 1985, 1989; Zilinskas and Greenwald, 1986; Holzwarth, 1991; Sidler, 1994; Bald et al., 1996; Mimuro and Akimoto, 2003; Allen and Mullineaux, 2004; MacColl, 2004; Mimuro, 2004; Adir, 2005; Liu et al., 2005; and Scheer and Zhao, 2008.

In cyanobacteria, PBS supply EE to both photosystems, the larger fraction to PS II and a smaller one to PS I. Two possibly co-existing EET

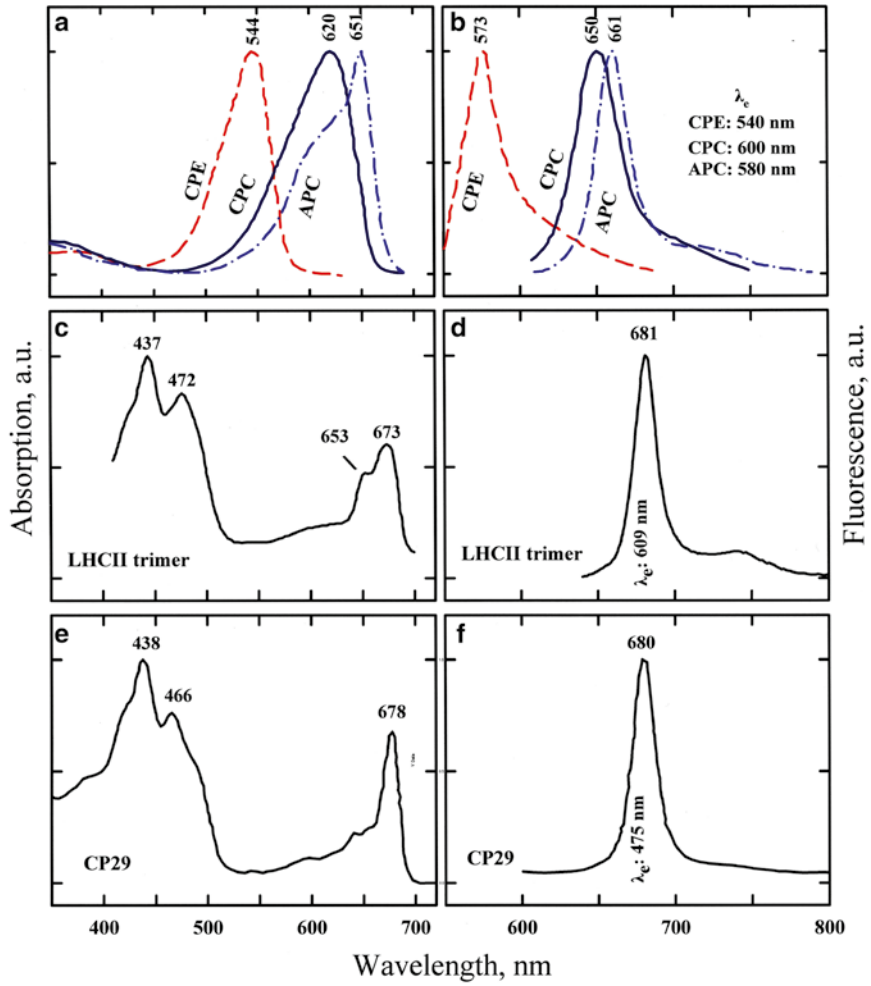

Fig. 18.4. Room temperature absorption (**a**, **c**, **e**) and fluorescence (**b**, **d**, **f**) spectra of selected PS II antenna holochromes retraced from published data. CPE and CPC, in panels **a** and **b**, were purified from *Nostoc* sp. (Wong et al., 1981); APC, in panel **a**, was purified from *Mastigocladus laminosus* (Gysi and Zuber, 1974), and in panel **b**, from *Anabaena cylidrica* (Mimuro et al., 1982); LHCII trimers, in panel **c**, were purified from pea leaves (Krikunova et al., 2002) and in panel **d**, from spinach (Palacios et al., 2002); recombinant CP29, in panels **e** and **f** (Giuffra et al., 1996).

routes have been considered: one along PE → PC → APC → ApcF → PS I (Suter and Holzwarth, 1987; Glazer, 1989; Zhao et al., 1992; Ashby and Mullineaux, 1999; Dong et al., 2009); and the second, by direct PC → PS I transfer (Glazer, 1984; Su et al., 1992). What is striking is that the PBS → PS I route appears to be subject to direct regulation, while the PBS → PS II route is only indirectly regulated, by being subordinate to the PBS → PS I route. Three regulatory mechanisms have been described: light-dark adaptation or state transitions (review by Allen and Mullineaux, 2004; Papageorgiou and Govindjee, 2011); osmotic regulation (reviewed by Papageorgiou and Stamatakis, 2004), and EE quenching at the terminal APC emitter by the so-called orange carotenoid protein (OCP; reviews by Kirilovsky, 2007 and Karapetyan, 2008). All three are complex physicochemical (not genomic) processes that are triggered by global cellular signals: state transitions by the redox poise of intersystem intermediates, osmotic regulation by the osmolality of the cytoplasm, and OCP quenching by means of a carotenoid conformational change that is induced by blue-green light.

Prochlorophytes is a separate class of cyanobacteria of which only three genera are known (*Prochloron*, *Prochlorothrix*, *Prochlorococcus*; Partensky et al., 1999; Partensky and Garczarek, 2003). The oceanic *Prochlorococcus marinus* is the most abundant genus of oxygenic photosynthetic organisms. Prochlorophytes have no PBS and no light-harvesting complex analogous to the LHC proteins of eukaryotic organisms, although they bind Chl *a* and Chl *b* (the 8-vinyl variants of them in *Prochlorococcus*). Their major peripheral light-harvesting complexes are the so-called prochlorophyte Chl *b*-binding proteins (Pcb; containg 6 transmembrane α-helices (TMHs)). They are related to the CP43 protein of the $PSII_{CC}$ and the IsiA protein of PS I in iron-stressed cyanobacteria (La Roche et al., 1996) and they supply excitation energy both to $PSII_{RC}$ and PSI_{RC}. In *Prochloron didemni*, these holochromes form two 5-subunit half rings around the $PSII_{CC}$ dimer (Bibby et al., 2003).

Unlike the PBS– and Chl *a*–containing cyanobacteria, those of the *Acaryochloris* genus (both symbiotic and free living) have Chl *d* as major pigment, and Chl *a* as minor, both being co-ordinated by Pcb-like proteins that supply excitation to both photosystems (Miyashita et al., 1996, 1997, 2003). Chl *d*/Chl *a* ratios are in the range ~30–80 (Schiller et al., 1997; Boichenko et al., 2000; Miller et al., 2005) and they depend, somehow on the culturing conditions (e.g., light intensity; Chen et al., 2002). *A. marina* contains also some PC and APC which, as 4-hexamer rods, attach to the cytoplasmic side of the thylakoid membrane (Marquardt et al., 1997; Hu et al., 1999; Boichenko et al., 2000). Unlike prochlorophytes, they have no Chl *b*. According to Chen et al. (2005a), the dimeric $PSII_{CC}$ is flanked by 4 Pcb-like subunits on each side.

$$\left[\{\text{Pcb}\}_4\{\text{CP43, CP47}\}\{\text{PSII}_{RC}\}\|\right.$$

$$\left.\|\{\text{PSII}_{RC}\}\{\text{CP47, CP43}\}\{\text{Pcb}\}_4\right]$$

This arrangement is supposed to enlarge the absorption cross-section of the $PSII_{CC}$ by 200%. Chl *d* is present also in free-living cyanobacteria (Kühl et al., 2005).

$PSII_{RC}$ preparations from cyanobacterium *A. marina* contain Chl *a*, Chl *d* and Pheo *a* but no Pheo *d*. Thus, either Chl *a* or Chl *d* can be the primary electron donor to Pheo *a*, the primary electron acceptor. determined a stoichiometric ratio of 2Chl *a*/2Pheo *a* and proposed Chl *a* to be the primary donor, either from a $(\text{Chl } a)_2$ homodimer or from a (Chl *a*/Chl *d*) heterodimer. On the other hand, on the basis of a determined stoichiometric ratio of 0.5 Chl *a*/2 Pheo *a*, Chen et al. (2005b) argued against Chl *a* being the primary electron donor, although a (Chl *a*/Chl *d*) heterodimer could be. Because of the spectral location of the delayed fluorescence band of *A. marina*, Mimuro et al. (1999, 2004) favor Chl *a* as the primary donor. A photoactive Chl *a* would imply uphill EET from antenna Chl *d* (i.e., Chl *a* ← Chl *d*). This, indeed, was observed upon exciting Chl *d* at 736 nm and observing Chl *a* fluorescence at 681 nm (Mimuro et al., 2000). However, oxidized-minus-reduced absorption spectra suggest Chl *d* as the photoactive pigment (Itoh et al., 2007). Obviously, further independent information is needed in order to reconcile this divergent phenomenology behind this crucial issue.

b. Higher Plants and Green Algae

The light-harvesting complexes of these organisms are Chl *a*–, Chl *b*– and Car–binding proteins, encoded by nuclear genes and known as LHC proteins (Green and Durnford, 1996; Jansson, 1999). Trimeric LHC complexes (or LHCII, combinations of Lcb1, 2, 3 proteins) constitute the major peripheral antenna units of these organisms, while the usually monomeric LHCs (known as CP29, CP26 and CP24) are the minor peripheral antenna units. The crystal structure of LHCII (Kühlbrandt et al., 1994; Liu et al., 2004) revealed three TMHs, 8 Chls *a*, 6 Chls *b*, 2 luteins and 1 neoxanthin per monomer, all linked to protein non-covalently. In *Chlamydomonas reinhardtii*, 9 major (LhcbM1-9), 2 minor (CP26 and CP29) and a distantly related (L1818) LHC proteins have been identified (Savard et al., 1996; Takahashi et al., 2006). Of these, CP29, CP26, LhcbM2 and LhcbM7 have been shown to shuttle between the $PSII_{CC}$ and PSI_{CC} sites during state $1 \leftrightarrows 2$ transitions (Kargul et al., 2005; Takahashi et al., 2006).

LHC proteins of roughly the same molecular size and of similar amino acid sequences serve as

peripheral light-harvesting antennae in PS II and PS I of other algae, but not in cyanobacteria. A typical PS II supercomplex (i.e., $PSII_{CC}$–$PSII_{PAC}$) is visualized to combine two core dimers (C_2, each with three minor antenna proteins), two strongly attached (S_2) and two moderately strongly attached LHCII trimers (M_2). The $C_2M_2S_2$ supercomplex has 72 Chls *a* in the core and 252 Chls (*a*+*b*) in the periphery. The number of peripheral Chls, however, may increase by the loose attachment of LHCII trimers (L), in supercomplexes such as, for example, $C_2M_2S_2L_x$ (Dekker and Boekema, 2005).

c. Other Photosynthetic Organisms

Red algae have cyanobacteria-like PS II and plant-like PS I (vide infra). In particular, their $PSII_{PAC}$ resembles that of cyanobacteria as it consists of PBS externally attached to thylakoids and lacks LHC proteins (or Chl *a*/*b*-binding proteins; Wolfe et al., 1994; Gantt et al., 2003; Bumba et al., 2004; Adachi et al., 2009; Vanselow et al., 2009).

As secondary endosymbiosis products (non-photosynthetic host cell plus red alga; Bhattacharya et al., 2003) cryptophytic algae have both membrane-intrinsic and membrane-extrinsic peripheral antenna complexes (review by Toole and Allnut, 2003). The first are Chl *a*/c_2/Car-binding LHC-type proteins, the latter PBPs located in the lumen space (Gantt et al., 1971). According to Mimuro et al. (1998) the Chl *a,* Chl c_2 and Car chromophores transfer EE directly to the Chls *a* of $PSII_{CC}$. The PBPs are either PEs or PCs (denoted as Cr-PE and Cr-PC) existing as heterodimers together with a linker polypeptide. The subunit and chromophore structure of PE545 has been studied biochemically (Martin and Hiller, 1987; Wedemayer et al., 1992; 1996) and crystallographically (Wilk et al., 1999). It is a $\alpha_1\alpha_2\beta\beta$ dimer (α_1 and $\alpha_2 \sim 10$ kDa each; $\beta \sim 20$ kDa) that binds 8 phycobilins (1 per subunit α; 3 per subunit β). Cr-PE and Cr-PC contact somehow the lumen side of the thylakoid membrane and transfer excitation to both photosystems (Van der Weij-de Wit et al., 2006, 2008). In contrast to the high mobility of PBS on the stroma-side of the cyanobacterial thylakoid membrane (see Section III.A), fluorescence recovery after photobleaching (FRAP) measurements showed very restricted mobility of Cr-PE545 in the lumen space of cryptophyte *Rhodomonas* CS24. This can be explained as the result of macromolecular crowding, of aggregation, or of membrane association, among other potential causes (Kana et al., 2009a, b; Mirkovic et al., 2009).

The light-harvesting pigments of chromophytic algae, e.g., of diatoms, brown algae and dinoflagellates are Chl *a*, Chl *c* and Car, but not Chl *b* and not PBPs (Larkum et al., 2003). Actually, Chl *c* is not a single compound but several related Mg-phytoporphyrins (see Fig. 18.1) that either lack the phytyl group of the other Chls, or have another alkyl in its place (Scheer, 2003; Zapata et al., 2006). Chromophores are linked to intramembranous FCP protein (Chl *a*/*c*/Fuc–binding protein) whose structure resembles a typical 3-TMH Chl *a*/*b*-binding LHC of higher plants. Within the thylakoid membrane, FCPs exist as multimers (Büchel, 2003). A typical chromophore ratio in an FCP monomer is 4 Chls *a* : 1 Chl *c* : 4 Fuc (Mimuro and Akimoto, 2003, and citations therein). However, FCPs of different origins differ in their pigment composition (Passaquet et al., 1991). In the FCP, EE flows in the directions Chl *c* → Chl *a* and Fuc → Chl *a*, but not in the direction Fuc → Chl *c* (Mimuro et al., 1990).

In addition to membrane-bound FCPs, dinoflagellates have a soluble antenna complex in the lumen space, known as PCP (peridinin (Per) Chl *a* binding–protein). PCPs, which exist in several isoforms, have the highest Car/Chl ratio of any light-harvesting protein. For two isoforms, the MFPCP (main form PCP) and the HSPCP (high salt PCP) of *Amphidinium carterae*, the near-atomic structures have been obtained: MFPCP is a trimeric monomer with two pigment domains of 4 Pers and 1 Chl *a* (Hofmann et al., 1996), while HSPCP is a monomer with two pigment domains of 3 Pers and 1 Chls *a* each (Schulte et al., 2009). Since absorption transitions of the type $S_0 + h\nu \rightarrow {}^*S_1$ are symmetry forbidden in polyenic carotenoids, the ${}^*S_1$ state of Per can be populated only indirectly by exciting it first to the ${}^*S_2$ state (i.e., $S_0 + h\nu \rightarrow {}^*S_2$; the blue absorption band of Cars) followed by spontaneous relaxation to the ${}^*S_1$ state. In an MFPCP-type domain, an ${}^*S_2$ exciton is delocalized over the 4 Pers. Three of them convert the ${}^*S_2$ excitation to ${}^*S_1$ and transfer it to the central Chl *a* (the Qy band). The 4th Per, transfers its EE to Chl *a* after converting first to

an $^{*}S_1$/charge transfer state (Damjanovic et al., 2000; Polivka et al., 2007).

Currently, it is under debate whether the peripheral antenna complexes of chromophytes are fully or partially shared by the two photosystems, or they are dedicated individually to each one of them (see e.g., De Martino et al., 2000; Büchel, 2003; Guglielmi et al., 2005; Veith and Büchel, 2007; and Veith et al., 2009).

B. Photosystem I

PS I is defined as a light–driven plastocyanin–ferredoxin oxidoreductase (Golbeck, 2006). In cyanobacteria, it consists of a core complex (PSI_{CC}) only, usually trimeric, but also monomeric at strong ambient light (Chitnis and Chitnis, 1993). In PBS-containing cyanobacteria, the PBS serve as external peripheral antennae to both photosystems, however, preferentially to PS I in light state 2 (or in hyperosmotic cell suspensions) and preferentially to PS II in light state 1 (or in hypoosmotic cell suspensions; see Section III.A). In higher plants (Amunts et al., 2007) and in red algae (Gardian et al., 2007; Vanselow et al., 2009) the PSI_{CC} is a monomer with four peripheral antenna proteins (Chl *a*/Car–binding LHCs) forming a belt on the PsaF side of the core complex. In Fe-deficient culture, the $(PSI_{CC})_3$ of cyanobacteria acquires a peripheral antenna ring of 18 IsiA proteins, homologues of the PS II core antenna holochrome CP43 (Burnap et al., 1993; Nield et al., 2003; review by Michel and Pistorius, 2004).

1. Core Complexes

The structure of PSI_{CC} of the cyanobacterium *Synecococcus elongatus* has been obtained by X–ray crystallography at 2.72 Å (Jordan et al., 2001; reviews by Fromme et al., 2001 and Fromme and Grotjohann, 2007). It contains 12 proteins, 96 Chls *a*, 22 Cars (carotenes and xanthophylls), 2 phylloquinones, 3 FeS centers, 4 lipids, 1 metal ion (probably Ca^{2+}) and ~200 H_2O molecules. The largest and most important proteins form the heterodimeric PsaA/PsaB which coordinates the majority of Chls *a* and Cars and the electron transport cofactors P_{700}, A_0, A_1 and F_X. Ten smaller protein subunits (PsaC–F, PsaI–M, and PsaX) complete the structure of the cyanoabactrial PSI_{CC}.

The structures of the PSI_{CC} and PSI_{PAC} of the higher plant *Pisum sativum* have been obtained by X-ray crystallography at 4.4 Å by Ben Shem et al. (2003) and at 3.4 Å by Amunts et al. (2007). Its PSI_{CC} is very similar, but not identical, to that of cyanobacteria. It contains 14 proteins, the large heterodimer PsaA/PsaB and 12 smaller subunits which are the same as those in cyanobacteria, except for 2 missing subunits (PsaM and PsaX) and for 4 new subunits (PsaG, PsaH, PsaN, PsaO). This core complex binds 102 Chls *a* (the majority at identical positions as in the cyanobacterial PSI_{CC}) and more than 20 β–Cars. The PSI_{CC} of the red alga *Galdieria sulfuraria* shares subunits PsaA, B, C, D, E, F, I, J, K, and L with plants and cyanobacteria, subunits PsaN and PsaO with plants, subunit PsaM with cyanobacteria, while the subunit PsaX of thermophilic cyanobacteria is absent (Vanselow et al., 2009).

2. Peripheral Antenna Complexes

In contrast to the PSI_{CC}, the composition of the PSI_{PAC} differs widely among various groups of photosynthetic organisms.

a. Cyanobacteria

In cyanobacteria, EE is transferred from PBS to $PSII_{CC}$ and PSI_{CC} via protein ApcF of the APC core (reviewed by Mullineaux, 2008) and to PS I alone via subunit ApcD (Dong et al., 2009). The EE share of each photosystem is regulated by the redox state of the PQ pool, but is also sensitive to the osmolality of the cell suspension. In addition, when the $PSII_{RC}$ is closed (i.e., when Q_A is reduced), Chls *a* of the PS II core antenna protein CP47 transfer EE to Chls *a* of PS I (details in Section III.A).

b. Higher Plants and Green Algae

In higher plants, PSI_{PAC} consists of 5–6 nuclear-encoded proteins (Lhca1–5; reviewed by Schmid, 2008). Proteins Lhca1–4 form heterodimers Lhca1/Lhca4 and Lhca2/Lhca3 that align in an evenly-spaced half-moon belt on the PsaF side of the PSI_{CC}. Two additional LHCI proteins have been discovered, Lhca5 and Lhca6, that had not been identified in the crystal structure. A deep

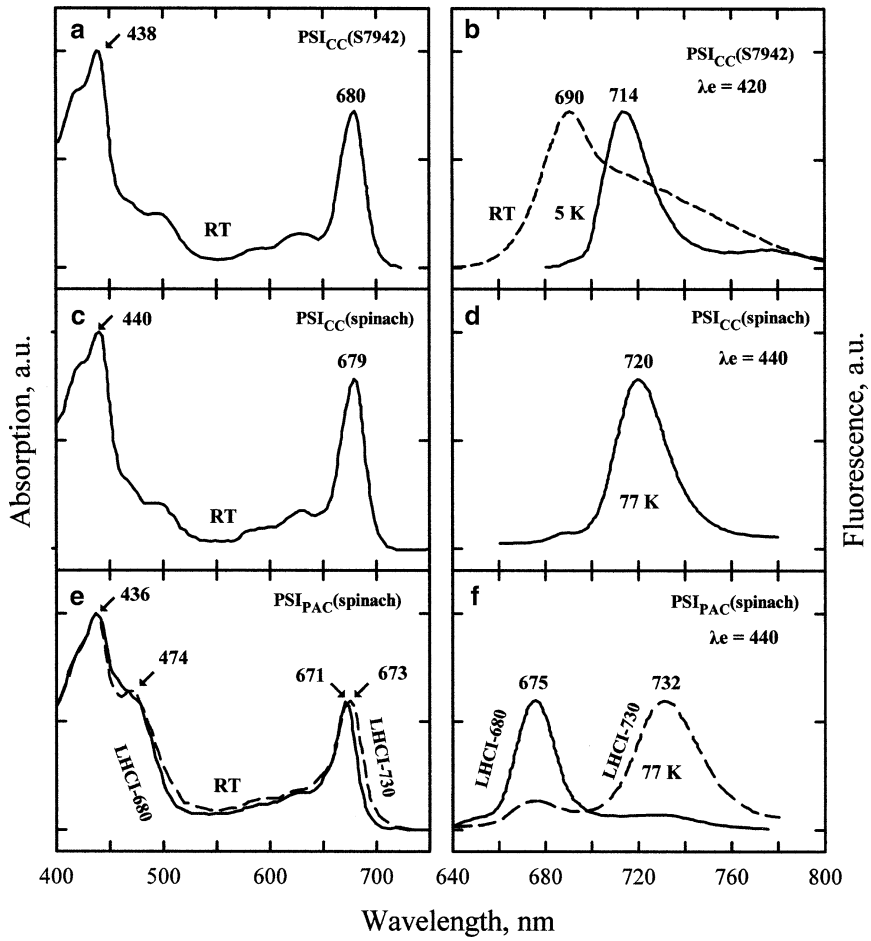

Fig. 18.5. Room temperature absorption (**a**, **c**, **e**) and low temperature fluorescence (**b**, **d**, **f**) spectra of isolated PS I subcomplexes retraced from published data. (**a**, **b**) PSI_{CC} from the unicellular freshwater cyanobacterium *Synechococcus* sp. PCC 7942; absorption trace from Andrizhiyevskaya et al. (2004), fluorescence traces from Andrizhiyevskaya et al. (2002). (**c**, **d**) PSI_{CC} from spinach leaves; absorption and fluorescence traces from Qin et al. (2006). (**e**, **f**) PSI_{PAC} from spinach leaves. LHCI-680 is the Lhca2/Lhca3 heterodimer, LHCI-730 the Lhca1/Lhca4 heterodimer; absorption and fluorescence traces from Qin et al. (2006).

cleft separates the PSI_{CC} from the Lhca1–4 antenna belt. There is a total of 66 Chls in the PSI_{PAC}, which can be subdivided into three classes: those within a Lhca protein, those exposed in the spaces in between the Lhca proteins (*linker* Chls), and those exposed in the cleft in between the antenna and the core parts (*gap* Chls). Seven or more Lhca proteins comprise the PSI_{PAC} of the green alga *Chlamydomonas reinhardtii* (Tokutsu et al., 2004). According to Stauber et al. (2009), these Lhcas are heterogeneous, since they are not present at 1:1 ratio with regard to PS I.

Room temperature absorption and low temperature fluorescence spectra of these complexes are shown in Fig. 18.5. The absorption spectra of the PSI_{CC} from a cyanobacterium (panel a) and from a higher plant (panel c) are very nearly identical, but the low temparature (77 K) fluorescence bands (panels b and d) appear to be shifted. [This is due to the different temperatures that these two spectra were recorded.] The absorption spectra of the Lhca1/Lhca4 (LHCI-730 and the Lhca2/Lhca3 (LHCI-680)) heterodimers are nearly identical but they display a hump at 474 nm due to Chl *b* which is absent from the PSI_{CC} spectra. The low temperature fluorescence spectra of these heterodimers are characteristically dissimilar, peaking at 675 nm in the case of LHCI-680 and at 732 nm in the case of LHC-730.

c. Other Photosynthetic Organisms

The PSI_{PAC} that feeds EE to the monomeric PSI_{CC} of red algae consists of 5–6 membrane-intrinsic Lhca proteins that bind Chl *a*, zeaxanthin and *β*–carotene but not Chl *b*. The Lhcas of various red algae are immunologically related, as they are also related to chlorophytic and chromophytic Lhcas, despite the fact that the red algal proteins do not bind Chl *b* or Chl *c* (Wolfe et al., 1994; Tan et al., 1997a, b). In *Cyanidium caldarium*, these Lhca proteins were shown to bind along one side of the monomeric PSI_{CC} (Gardian et al., 2007).

Two to five Chl a/c_2/Car–binding proteins have been fractionated with PS I in *Rhodomonas* CS24 (Kereïche et al., 2008) suggesting a fully, or partly, dedicated membrane-intrinsic LHCI antenna to the PS I of this cryptophyte. On the other hand, the soluble oligomeric Cr-PC and Cr-PE antennae in the cryptophyte lumen were shown to distribute their EE equally to PS I and PS II (Van der Weij-de Wit et al., 2006; 2008; see also Section II.A.2.c).

The question whether each photosystem in chromophytic algae has it own dedicated complement of FCP proteins, or whether the FCP proteins are, at least partially, shared by the two photosystems is not yet settled. The answer hinges upon the isolation of PS II–LHCII and PS I–LHCI holocomplexes and the characterization of the antenna complements in each. Results reported by Veith and coworkers (e.g., Veith and Büchel, 2007; Veith et al., 2009 and citations therein) favor the separate antennae alternative. Brakermann et al. (2006), on the other hand, obtained evidence for identical FCPs in PS II and PS I fractions.

III. Variable Chlorophyll *a* Fluorescence

The properties of Chl *a* in vivo fluorescence, such as intensity, quantum yield and spectral distribution, are subject to modulation by three global metabolic signals that the ground state PSET generates (reviewed by Govindjee, 2004): (i) the redox state of the primary quinone electron acceptor (Q_A/Q_A^-; Duysens and Sweers, 1963); (ii) the redox state of intersystem carriers and particularly of the PQ pool and the cytochrome b_6f complex, and (iii) the transthylakoid membrane proton gradient (ΔpH, inside acidic). A change in the measured fluorescence intensity may relate either to one, or both, of the following causes: (i) a change in the quantum yield of emitter molecules; and (ii) a change in the number (or the concentration) of emitter molecules in the measured sample. The first is known as *fluorescence quenching* (and *dequenching*), and its more solid criterion is a proportional change in the EE lifetime, as described by the well-known equation (see Lakowicz, 2006):

$$\tau = \tau_0 \varphi$$

Here, τ_0 is the natural lifetime of the fluorescing molecule (15.2 ns for Chl *a*; calculated from its absorption spectrum in solution) and τ the measured lifetime. The second, which is equivalent to adding or removing Chls *a* from the test tube, will be referred to as *fluorescence lowering* or *fluorescence increase*. For example, the well-known case of fluorescence decrease after a state 1 to state 2 transition is a case of fluorescence lowering and has nothing to do with quenching (although, often it is assigned erroneously a quenching coefficient, qT). On the other hand, the fluorescence changes observed by applying steady illumination to a previously darkened sample (fluorescence induction, FI) are caused both by quantum yield changes and by fluorescence lowering/increase effects.

In addition to these three metabolic signals, designated as *global* because they may affect other physicochemical and genomic processes in chloroplasts and cells, Chl *a* fluorescence is subject to regulated modulations by quenchers (e.g., the xanthophyll cycle) and holochrome conformational changes (e.g., orange carotenoid protein, OCP), but also to unregulated changes, as for example excitation quenching by ground state O_2 ($^3O_2$; Papageorgiou et al., 1972).

A. State Transitions

Light state transitions are short-term (minutes) reversible changes in the absorption cross-sections of PS I and PS II that occur when photosynthetic cells are acclimated to light that is absorbed either more by PS II ($h\nu_{PSII}$) or more by PS I ($h\nu_{PSI}$). Their chief physiological objective is to synchronize the turnovers of $PSII_{RC}$ and PSI_{RC} (presumably with a time delay to allow for electron transfer from $PSII_{RC}$ to PSI_{RC}) and, in this way, to optimize the quantum efficiency of linear PSET at weak photon fluxes. State transitions are to be contrasted to

long-term (hours) genomic adaptations of the photosynthetic apparatus to light quality (e.g., chromatic adaptation) which do affect holochrome and photosystem stoichiometries.

The changes of photosystem cross-sections occur by the exchange of mobile peripheral antenna elements (ANT_M) between the two photosystems, as shown below:

$$State\,1 \rightarrow 2$$
$$h\nu_{PSII} + PS\ II \sim ANT_M + PS\ I \rightarrow PS\ II + PS\ I \sim ANT_M$$

$$State\,2 \rightarrow 1$$
$$h\nu_{PSI} + PS\ I \sim ANT_M + PS\ II \rightarrow PS\ I + PS\ II \sim ANT_M$$

Thus, excitation with $h\nu_{PSII}$ shifts part of the PS II antenna to PS I (state 1–to–state 2 transition; from higher fluorescence to lower fluorescence) while excitation with $h\nu_{PSI}$ restores it back to PS II (state 2–to–state 1 transition; from lower fluorescence to higher fluorescence).

State transitions were discovered independently by Murata (1969) and by Bonaventura and Myers (1969) based on fluorescence spectra of red algae in the first case, and on fluorescence spectra and oxygen evolution measurements in a green alga, in the second. Light-adaptive redistribution of EE between PS II and PS I in cyanobacteria and green algae had been reported in detail earlier by Papageorgiou and Govindjee (1967, 1968a, b), but it was not recognized as evidencing a regulatory mechanism. The state transition concept proved to be a highly fertile research area, judging from the great number of research reports it has inspired and continues to inspire. Here I list only a few selected post-2001 reviews: Allen and Forsberg, 2001; Haldrup et al., 2001; Kruse, 2001; Wollman, 2001; Allen, 2002, 2004; Zer and Ohad, 2003; Allen and Mullineaux, 2004; Bruce and Vasil'ev, 2004; Finazzi and Forti, 2004; Rochaix, 2007; and Murata, 2009; Lemeille and Rochaix, 2010; Minagawa, 2011; Tikkanen et al., 2011.

State transitions are most conveniently measured in two ways: either (i) kinetically, at room temperature, from the rise or the decrease of variable Chl *a* fluorescence which is emitted mostly by PS II; or (ii) spectroscopically by resolving the emission bands of PS II (at ~685–695 nm) and of PS I (at ~715–725 nm) at low temperature (77 K, or lower). As mentioned above, state transitions are not quenching or dequenching processes (see also Holub et al., 2007; Papageorgiou et al., 2007). A laboratory-scale analog to state transitions would be the addition or removal of Chl molecules to a solution in a spectrophotometer cuvette.

In all organisms, the chemical signal that triggers the state 1–to–2 transition is the reduction of the PQ pool either photochemically (by $h\nu_{PSII}$) or nonphotochemically by metabolic reductants (Allen, 2002, and the cited literature). Beyond this first step, the molecular mechanism of state transitions in green algae and higher plants is different from that in cyanobacteria.

In the green algae and higher plants, the state 1–to–2 transition proceeds as follows:

- Reduction of the PQ pool and activation of membrane-bound kinases (Rochaix, 2007, and citations therein) by the attachment of PQH_2 to the quinol oxidation site (Q_0) site of the cytochrome b_6f complex (Vener et al., 1997, 1998; Zer and Ohad, 2003; Allen, 2004).
- Phosphorylation of surface-exposed membrane proteins, including the monomeric (CP24 and CP29) and the trimeric (LHCIIb) peripheral antenna proteins (Kargul et al., 2005; Takahashi et al., 2006; Turkina et al., 2007).
- Dissociation of the phosphorylated mobile antenna proteins from the $PSII_{CC}$, docking of CP29 (Kargul et al., 2005), or of both CP29 and CP24 (Takahashi et al., 2006) on the PsaH subunit of the PSI_{CC} (Lunde et al., 2000) and subequently docking of trimeric LHCIIb onto CP29 and CP24.

In PBS-containing cyanobacteria, the mobile antenna that the two photosystems share is the PBS. The state 1–to–2 transition begins by the reduction of the PQ pool but, unlike green algae and higher plants, no membrane proteins are phosphorylated (Pursiheimo et al., 1998). The reverse, state 2–to–1 transition requires a transmembrane ΔpH gradient since protonophores that equalize the intra-thylakoid and the extra-thylakoid pH block it (Papageorgiou and Govindjee, 1968a). Compelling evidence exists for a separate, intramembranous and PBS-independent state 1–to–2 transition mechanism, known as *spillover*, for direct EE transfer from the Chls *a* in $PSII_{CC}$ to the Chls *a* in PSI_{CC}.

Three basic models have been proposed to account for the reported state transitions phenomenology in cyanobacteria (Koblizek et al., 1998; McConnell et al., 2002; Bruce and Vasil'ev, 2004; see also Stadnichuk et al., 2009 for more models). The mobile PBS model assumes long-range random sliding movements of PBS on thylakoid surfaces (incorrectly termed as *diffusion*!) and their preferential non-covalent docking (after molecular recognition) to PS II dimers (in state 1) or to PS I trimers (in state 2; Allen and Forsberg, 2001). The evidence behind this hypothesis comes from phycobilin FRAP experiments. In these, the PBS on a membrane surface spot, are bleached and presumably disintegrated by a powerful laser beam, to be subsequently replaced by intact PBS diffusing in from non-illuminated membrane areas, as monitored by the rise of phycobilin fluorescence.

The model is attractive, as it explains more or less satisfactorily a large volume of published phenomenology (Allen and Mullineaux, 2004) but it still demands independent experimental support. Photobleaching, for example, conceivably could be simply caused by a double bond rectification in the tetrapyrrole chromophores, without PBS destruction and removal of the resulting fragments. In such a case, the subsequent fluorescence rise could reflect the restitution of the double bond, rather than a long-range random *diffusion* of the massive PBS. This local FRAP hypothesis may adequately explain the reported phenomenology with isolated PBS in vitro (embedded in an agar matrix, see Yang et al., 2007). Short-range, mostly reorientational, motions of PBS have been proposed by Bruce and Vasil'ev (2004) for state 1 to state 2 transitions by comparing the X-ray structures of $PSII_{CC}$, PSI_{CC} and APC cores in cyanobacteria. In contrast to cyanobacteria, the FRAP technique revealed very restricted mobility of PBP dimers in the lumen space of cryptophytes (Kana et al., 2009a; Mirkovic et al., 2009).

The *spillover model*, proposed for state transitions by Murata (1969), assumes fixed PBS on PS II dimers. EE residing in the Chls *a* of $PSII_{CC}$ and acquired either by direct absorption or by EET from PBS, is transferred to the Chls *a* of PS I in state 2 cells. Although a considerable volume of phenomenology supports this model (McConnell et al., 2002; Bruce and Vasil'ev, 2004, and cited literature), there has been no physicochemical elaboration of its mechanism. However, based on the recently resolved X-ray structures of PS II and PS I, and on the quasi energetic isolation of CP43, CP47 from D1 and D2, McConnell et al. (2002) proposed that, in state 2, the PS II to PS I EE spillover involves Chls *a* of CP47, of $PSII_{CC}$, as donors and Chls *a* of the PSI_{CC} as acceptors. PBS, on the other hand, transfers EE via the APC-core subunit ApcF directly to both $PSII_{CC}$ and PSI_{CC} (Mullineaux, 2008) and via the APC-core subunit ApcD directly to PS I (Dong et al., 2009).

Lastly, in the *hybrid model* (Koblizek et al., 1998; McConnell et al., 2002), PBS transfer excitation directly to PS II (in state 1 by attaching to $PSII_{CC}$) and to PS I (in state 2 by attaching to PSI_{CC}), while in state 2 excited Chls *a* in the CP47 core antenna protein of $PSII_{CC}$ may also transfer EE to Chls *a* in the PSI_{CC}.

In cyanobacterial cells, the intensity of PBS-sensitized Chl *a* fluorescence is sensitive to the osmotic pressure of the cell suspension. Fluorescence vs suspension osmolality plots and cell volume vs suspension osmolality plots are closely similar and this property has been used to estimate accurately the cytoplasmic osmolalities of several cyanobacteria (Papageorgiou and Alygizaki-Zorba, 1997; Papageorgiou et al., 1998). Cells perceive as osmotically-active molecules (osmolytes) only non-penetrating solutes. A cell suspension medium is characterized as hyper-osmotic or hypo-osmotic by reference to the cytoplasmic osmolality of the cells, which, in turn, depends on the osmolality of culturing media (Ladas and Papageorgiou, 2000a, b). In usual cyanobacterial cultures (e.g., in buffered BG-11 medium) it is approximately 200–220 mOsm kg^{-1} (reviewed by Papageorgiou and Stamatakis, 2004).

In hyperosmotic suspension, cells shrink and this favors EET from PBS to PS I, while in hypo-osmotic suspension they swell and this favors EET from PBS to PS II EE. In fact, light-induced state transitions are subordinate to osmotic regulation. Thus, dark-adapted cyanobacteria in hyperosmotic cell suspension (state 2 cells) do not transit to state 1 upon illumination with $h\nu_{PSII}$, while light-adapted cyanobacteria (state 1 cells) transit to state 2 upon given a hyper-osmotic shock (Papageorgiou and Stamatakis, 2004, and cited literature).

B. Fluorescence Induction (FI)

When dark-adapted photosynthetic samples are exposed to strong exciting light, the intensity of the Chl *a* fluorescence they emit changes with

time tracing typical and reproducible kinetic patterns. This phenomenon, known as fluorescence induction (FI), was first reported by Kautsky and Hirsch (1931; details in Govindjee, 1995, 2004), who also noted an inverse relation between fluorescence intensity and CO_2 uptake. This led to the conditionally correct inference that the quantum yield of Chl *a* fluorescence mirrors the quantum yield of photosynthesis (see Section III) and stimulated, since that time, a plethora of investigations on FI themes. Actually, however, the μs–to–min of Chl fluorescence changes relate both to quantum yield effects (quenching/de-quenching) as well as to state transition effects (lowering/increase) (reviewed by Papageorgiou et al., 2007; Papageorgiou and Govindjee, 2011).

FI has been studied quite extensively in plants and algae (but less so in cyanobacteria) as it affords a low resolution but panoramic view of the entire PSET. Total FI comprises two transients, a fast initial one lasting ~1–2 s (OPS; for Origin, Peak and Steady state) and a second slower transient lasting up to several min (SMT; for Steady state, Maximum and Terminal state). Recently, three dimensional displays of FI (fluorescence vs time of actinic illumination vs wavelength of fluorescence detection) have been published that allow for a fuller analysis of the FI phenomenology and its causes (Kana et al., 2009b).

Total FI patterns are characteristically different in plants and algae from those in PBS-containing cyanobacteria. The main reason is that, during a dark pre-adaptation of the sample, the PQ pool of plants and algae becomes oxidized (Bennoun, 1982), so they shift toward the high fluorescnce state 1, while the PQ pool of cyanobacteria becomes reduced (Hirano et al., 1980) and so they shift toward the low fluorescence state 2. As Tsimilli-Michael et al., (2009) have reported, different species of cyanobacteria may have their PQ pools poised at different potentials after dark adaptation. Thus, Fig. 18.6 shows that the FI pattern in plants and algae is dominated by the P → T decay, whose main component is the state 1 → 2 transition, while in PBS-containing cyanobacteria, it is dominated by the S → M rise, whose main component is the state 2 → 1 transition. In plants and algae maximal fluorescence output occurs at peak P, with the second peak M lying much lower or being absent. In such instances, the PSMT phases of FI are replaced by monotonous fluorescence decay from P to T. In contrast, in PBS-containing cyanobacteria, the first fluorescence maximum P is much lower than the second maximum at M. However, as Fig. 18.6 shows, the FI pattern of the PBS-less cyanobacterium *Acaryochloris marina* resembles more that of the green alga *Chlamydomonas reinhardtii* and less that of cyanobacterium *Synechococcus* sp. PCC 7942.

The time window of FI ranges from ~1 μs to tens of minutes. The first recorded signal (F_0); initial fluorescence corresponds to a physical state in which all $PSII_{RC}$ are open and ready for photochemical conversions. Operationally, F_o is usually defined as the first recorded fluorescence after a period of dark adaptation (usually, 5–20 min). The two definitions are not equivalent, and since F_o is a magnitude that enters into the calculations of quantum yields of photosynthesis and fluorescence and of nonphotochemical quenching (Baker and Oxborough, 2004; Krause and Jahns, 2004; Strasser et al., 2004), we must make sure that the value we use approximates as much as possible the true F_o value.

Further, the measured F_o is not a constant and it in no way can faithfully stand for the low limit of PS II Chl *a* fluorescence because, in addition to fluorescence emitted by active PS II complexes, F_o includes emissions from inactive (or Q_B-nonreducing) PS II complexes (Zhu et al., 2005; Vredenberg, 2008) and from PS I (Papageorgiou, 1975; Briantais et al., 1986; Pfündel, 1998; Gitelson et al., 1999; Peterson et al., 2001; Schreiber, 2004; Steffen et al., 2005). Other causes that do affect the measured value of F_o include: (i) de-quenching because of dark reduction of Q_A and the PQ pool by metabolites (Vernotte et al., 1979; Briantais et al., 1986; Haldimann and Tsimilli-Michael, 2005; Hohmann-Marriott et al., 2010) and the attendant fluorescence lowering by the state 1 → 2 transition (Malkin et al., 1980); (ii) quenching by transmembrane electrochemical gradients ($\Delta pH + \Delta\Psi$; Papageorgiou and Govindjee, 1967; Krause et al., 1982; Kramer et al., 2004; Krause and Jahns, 2004; Vredenberg, 2004; Vredenberg and Prasil, 2009); (iii) Metal ion-effected redistribution of EE between photosystems (Murata, 1969); (iv) Quenching by zeaxanthin (xanthophyll cycle; Gilmore et al., 1998; Golan et al., 2004; Holt et al., 2004), as well as by lutein (see e.g., Matsubara et al., 2011), and (v) unregulated quenching by molecular oxygen (Papageorgiou et al., 1972).

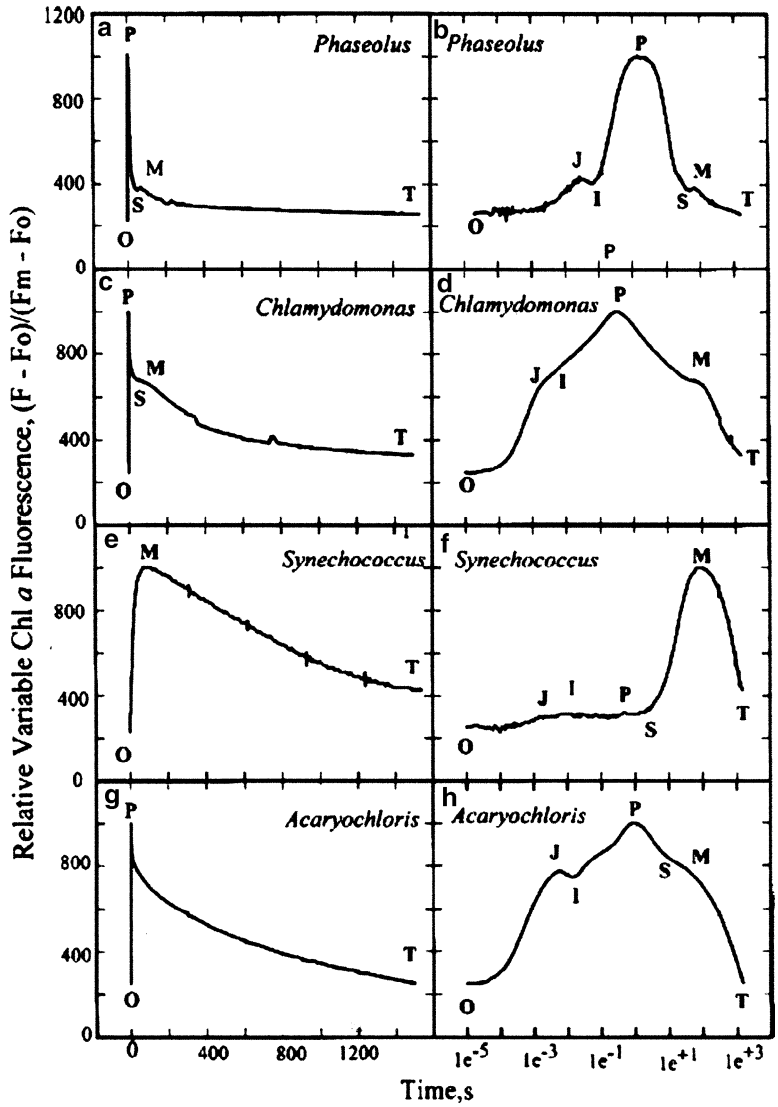

Fig. 18.6. Chl *a* fluorescence induction traces recorded with a higher plant (*Phaseolus vulgaris*) leaf, a green alga (*Chlamydomonas reinhardtii*) and a PBS/Chl *a*-containing cyanobacterium (*Synechococcus* sp. PCC 7942); also Chl *d* fluorescence induction trace recorded with a Chl d/Chl *a*–containing cyanobacterium that lacks phycobilisomes (*Acaryochloris marina*). Fluorescence data on the left are plotted against linear time scales and on the right against logarithmic time scales. All curves were recorded with the Handy PEA fluorometer (Hansatech Instruments, Ltd., UK). Measured samples were preadapted to darkness for 20 min. Fluorescence excitation, $\lambda = 650$ nm; fluorescence detection through an RG9 long pass glass filter (transmittances: threshold ~690 nm; 50% ~725 nm; maximal, ~780 nm; Schott Glass Technolgies, Inc, USA). Excitation intensities in nmol photons m^{-2} s^{-1}: *P. vulgaris*: 50; *C. reinhardtii*, *Synechococcus* sp. PCC 7942 and *A. marina*, 1,500; courtesy of Dr. K. Stamatakis.

The estimation of the fraction of the measured Fo that originates exclusively from open $PSII_{RC}$ is indeed a challenge.

The Chl *a* fluorescence rise along OJIP (Fig. 18.6) is due to the reduction of Q_A (i.e., to the removal of a quencher), and to momentary thermal equilibria between Q_A, Q_B and the PQ pool (reviews by Samson et al., 1999; Strasser et al., 2004; Zhu et al., 2005; Lazár and Schansker, 2009; Stirbet and Govindjee, 2011). OJ is a temperature-independent photochemical process which maximizes the concentration of reduced

Q_A. JIP is a temperature-dependent photochemical process during which the Q_A, Q_B and PQ pools become reduced. At peak P, Q_A^-, Q_B^{2-} and PQH_2 exist transiently in equilibrium (Zhu et al., 2005; Papageorgiou et al., 2007; Lazár and Schansker, 2009; Stirbet and Govindjee, 2011; and cited literature). The JIP phase comes the closest to a complementarity relationship between Chl *a* fluorescence and photosynthesis (O_2 evolution; see Section III), and because of that, it is used as a rapid and non-invasive diagnostic index of photosynthetic productivity (the so-called JIP-test, see Strasser et al., 2004).

The Chl *a* fluorescence decay along P to S (Fig. 18.6) is due both to the re-oxidation of reduced Q_A by PS I and to the acidification of the lumen. Protons involved in the latter process are derived from the oxidation of water at $PSII_{RC}$ and from the translocation of protons from the stroma to the lumen by phosphorylation-coupled PSET (see e.g., Krause et al., 1982). Formation of transmembrane ΔpH requires a functional and selectively permeable thylakoid membrane. The P to S decay has been observed in algae (Lavorel, 1959; Munday and Govindjee, 1969; Bannister and Rice, 1968), cyanobacteria (Bannister and Rice, 1968), leaves (Bradbury and Baker, 1981) and intact isolated chloroplasts (Krause et al., 1982), but not in thylakoid membrane fragments (Murata et al., 1966; Malkin and Kok, 1966). The P to S decay of Chl *a* fluorescence is synchronous to a rise in the rate of photosynthetic O_2 evolution (Bannister and Rice, 1968), it vanishes upon anaerobiosis (Munday and Govindjee, 1969; Schreiber et al., 1971), it is slowed down upon freezing, it is abolished by post-PS II electron transport inhibitors and by protonophoric uncouplers (Mohanty and Govindjee, 1974) and it is linearly related to the decrease of lumen pH (Briantais et al., 1979). Other contributing causes, that have been recruited to explain the P to S decay, include a state 1 to state 2 transition (Malkin et al., 1980) and an energy-dependent efflux of Mg^{2+} ions from granal to stromal compartments (Barber et al., 1974a, b; Krause, 1974; Karukstis and Sauer, 1983) that enables PS II excitation to spill over to PS I (Murata, 1969). For a recent review, see Strasser et al., 2004.

The physico-chemical causes that drive the slow FI transient (SMT) are less well known compared to those of the fast OJIPS transient, for which several mathematical simulations exist (Lazar and Shansker, 2009; Rubin and Riznicheko, 2009; Stirbet and Govindjee, 2011; and citations therein). Phenomenology relates these causes to the cell structure, to the reversible shuttling of antenna elements between PS II and PS I (state transitions), to intramembranous redox equilibria and to transmembrane pH differences. The fact that only whole cells manifest the full O(JI)PS-SMT transient emphasizes the necessity of a structural organization above that of thylakoid membranes, of closed intact thylakoids and of intact chloroplasts. Thus, broken chloroplasts are capable only of the OJIP fluorescence rise, and intact chloroplasts extend it up to OJIPS, but neither is capable of the full OJIPS-SMT transient.

Characteristic differences in the SMT fluorescence kinetic patterns exist between higher plants and chlorophytes, on the one hand, and PBS-containing cyanobacteria on the other (Fig. 18.6). In chlorophytic algae, the SM rise is blocked when linear PSET is inhibited right after $PSII_{RC}$ by DCMU; in cyanobacteria it is not. In cyanobacteria, on the other hand, the SM rise is inhibited in hyperosmotic cell suspensions (i.e., in H_2O-deficient cells), both in the absence and in the presence of DCMU. Since hyperosmotic conditions prevent the state $2 \rightarrow 1$ transition in these organisms (and maximize PBS → PS I excitation transfers; Papageorgiou and Stamatakis, 2004 and citations) it appears that the latter state transition is a major contributor to the SM fluorescence rise in cyanobacteria. This is also confirmed by the observation that a state 1-locked cyanobacterium mutant is also devoid of the SM fluorescence rise (Kaňa et al., 2011).

After maximum M, Chl *a* fluorescence decays to the stationary state T in chlorophytic algae and in higher plants, while in cyanobacteria it remains constant at moderate excitation intensities (Papageorgiou and Govindjee, 1968b) or decays incompletely at high excitation intensities (Papageorgiou et al., 2007). This disparate phenomenology evokes characteristic differences in the organization and the location of the peripheral antenna complexes in these organisms, namely the membrane-intrinsic LHC holochromes in eukaryotes vs the membrane-extrinsic PBS in cyanobacteria. Unfortunately, slow fluorescence induction data for PBS-less cyanobacteria

(*Acaryochloris* and *Prochlorophyte* species) are not yet available.

IV. Concluding Remarks

Photosynthetic organisms harvest visible sunlight photons with three pigment families, the Chls, the Cars and the PBs, all members of which form holochromes with specific proteins. One pigment, Chl *a*, occurs in every photosynthetic organism while the occurrence of all other pigments varies with the taxonomic classification of an organism and hence with its evolutionary history. Only the Chls *a* in the reaction center complexes $PSII_{RC}$ and $PSII_{CC}$ can trap electronic excitation in a ground state cation-anion pair by transferring an electron to an acceptor molecule. All other pigments are engaged either in light harvesting (PBs) or in light harvesting and excess excitation dissipation as heat (Chls, including non-photoactive Chls *a* and Cars).

Of all the in vivo photosynthetic pigments, only Chl *a* and the PBs emit fluorescence at ordinary temperatures. The fluorescence signal of Chl *a* is rich in information not only concerning the emitter molecules themselves, but also about other interacting molecules. Furthermore, the intensity of the emitted Chl *a* fluorescence is regulated by three ground state metabolic signals that PSET produces: the redox state of Q_A, the primary quinone acceptor of PS II, the redox state of the PQ pool and of the cytochrome b_6f complex, and the electrochemical potential across the thylakoid membrane ($\Delta\Psi + \Delta pH$). Because of this dependence, Chl *a* fluorescence is used to access information about the rates and the extent of slow metabolic processes, such as PSET, ion translocation across cell membranes and thylakoid membranes, light-adaptive shifts of peripheral antenna elements, and even about the physiological condition and photosynthetic productivity of individual plants and plant communities.

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References

Adachi H, Umena Y, Enami I, Henmi T, Kamiya N and Shen JR (2009) Towards structural elucidation of eukaryotic photosystem II: Purification, crystallization and preliminary X-ray diffraction analysis of photosystem II from a red alga. Biochim Biophys Acta 1787: 121–128

Adir N (2005) Elucidation of the molecular structures of components of the phycobilisome: reconstructing a giant. Photosynth Res 85: 15–32

Alfonso M, Montoya G, Cases R, Rodriguez R and Picorel R (1994) Core antenna complexes, CP43 and CP47, of higher plant photosystem II. Spectral properties, pigment stoichiometry, and amino acid composition. Biochemistry 33: 10494–10500

Allen JF (2002) Plastoquinone redox control of chloroplast thylakoid protein phosphorylation and distribution of excitation energy between photosystems: discovery, background, implications. Photosynth Res 73: 139–148

Allen JF (2004) Cytochrome b_6f: structure for signalling and vectorial metabolism. Trends Plant Sci 9: 130–137

Allen JF and Forsberg J (2001) Molecular recognition in thylakoid structure and function. Trends Plant Sci 6: 317–326

Allen JF and Mullineaux CW (2004) Probing the mechanisms of state transitions in oxygenic photosynthess by chlorophyll fluorescence spectroscopy, kinetics and imaging. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 447–461. Springer, Dordrecht

Amunts A, Drory O and Nelson N (2007) The structure of a plant photosystem I supercomplex at 3.4 Å resolution. Nature 447: 58–63

Andrizhiyevskaya EG, Schwab TME, Germano M, D'Haene S, Kruip J, Van Grondelle R and Dekker JP (2002) Spectroscopic properties of PSI–IsiA supercomplexes from the cyanobacterium *Synechococcus* PCC 7942. Biochim Biophys Acta 1556: 265–272

Andrizhiyevskaya EG, Frolov D, Van Grondelle R and Dekker JP (2004) Energy transfer and trapping in the photosystem I complex of *Synechococcus* PCC 7942 and in its supercomplex with IsiA. Biochim Biophys Acta 1656: 104–113

Arteni AA, Ajlani G and Boekema EJ (2009) Structural organisation of phycobilisomes from *Synechocystis* sp. strain PCC 6803 and their interaction with the membrane. Biochim Biophys Acta 1787: 272–279

Ashby MK and Mullineaux CW (1999) The role of ApcD and ApcE in energy transfer from phycobilisomes to PSI in a cyanobacterium. Photosynth Res 61: 169–179

Baker NR and Oxborough K (2004) Chlorophyll fluorescence as a probe of photosynthetic productivity. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 65–82. Springer, Dordrecht

Bald D, Kruip J and Rögner M (1996) Supramolecular architecture of cyanobacterial thylakoid membranes: How is the phycobilisome connected with the photosystems? Photosynth Res 49: 103–118

Bannister TT and Rice G (1968) Parallel time courses of oxygen evolution and chlorophyll fluorescence. Biochim Biophys Acta 162: 555–580

Barber J, Mills J and Nicolson J (1974a) Studies with cation specific ionophores show that within intact chloroplast Mg^{2+} acts as main exchange cation for H^+ pumping. FEBS Lett 49: 106–110

Barber J, Telfer A and Nicolson J (1974b) Evidence for divalent-cation movement within isolated whole chloroplasts from studies with ionophore A23187. Biochim Biophtys Acta 357: 161–165

Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. Proc Natl Acad Sci USA 79: 4352–4356

Ben-Shem A, Frolow F and Nelson N (2003) Crystal structure of plant photosystem I. Nature 426: 630–635

Bhattacharya D, Yoon HS and Hackett JD (2003) Photosynthetic eukaryotes unite: endosymbiosis connects the dots. BioEssays 26: 50–60

Bibby TS, Nield J, Chen M, Larkum AWD and Barber J (2003) Structure of photosystem II supercomplex isolated from *Prochloron didemni* retaining its chlorophyll *a*/*b* light-harvesting system. Proc Natl Acad Sci USA 100: 9050–9054

Björn LO and Govindjee (2009) The evolution of photosynthesis and chloroplasts. Current Sci 96: 1466–1474

Björn LO, Papageorgiou GC, Blankenship RE and Govindjee (2009) A viewpoint: Why chlorophyll *a*? Photosynth Res 99: 85–98

Blankenship RE (2002) Molecular Mechanisms of Photosynthesis. Blackwell Science, Oxford

Boichenko VA, Klimov VV, Miyashita H and Miyachi S (2000) Functional characteristics of chlorophyll *d*-predominating photosynthetic apparatus in intact cells of *Acaryochloris marina*. Photosynth Res 65: 269–277

Bonaventura C and Myers J (1969) Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. Biochim Biophys Acta 189: 366–383

Bradbury M and Baker NE (1981) Analysis of the slow phases of the in vivo chlorophyll fluorescence induction curve. Changes in the redox state of photosystem II electron acceptors and fluorescence emission from photosystems I and II. Biochim Biophys Acta 635: 542–551

Brakemann T, Schloermann W, Marquardt J, Nolte M and Rhiel E (2006) Association of fucoxanthin chlorophyll *a*/*c*-binding polypeptides with photosystems and phosphorylation in the centric diatom *Cyclotella cryptica*. Protist 157: 463–475

Braun P, Greenberg BM and Scherz A (1990) D1-D2-cytochrome *b*559 complex from the aquatic plant *Spirodela oligorrhiza*: Correlation between complex integrity, spectroscopic properties, photochemical activity, and pigment composition. Biochemistry 29**:** 10376–10387

Briantais JM, Vernotte C, Picaud M and Krause GH (1979) Quantitative study of the slow decline of chlorophyll *a* fluorescence in isolated chloroplasts 548: 128–138

Briantais JM, Vernotte C, Krause GH and Weis E (1986) Chlorophyll *a* flurescence of higher plants: chloroplasts and leaves. In: Govindjee, Amesz J and Fork DC (eds) Light Emission by Plants and Bacteria, pp 539–583. Academic Press, New York

Brody SS (2002) Fluorescence lifetime, yield, energy transfer and spectrum in photosynthesis, 1950–1960. Photosynth Res 73: 127–132

Bruce D and Vasil'ev S (2004) Excess light stress: Multiple dissipative processes of excess excitation. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 497–523. Springer, Dordrecht

Büchel C (2003) Fucoxanthin-chlorophyll proteins in diatoms: 18 and 19 kDa subunits assemble into different oligomeric states. Biochemistry 42: 13027–13034

Bumba L, Havelkova-Dousova H, Husak M and Vacha F (2004) Structural characterization of photosystem II complex from red alga *Porphyridium cruentum* retaining extrinsic subunits of the oxygen-evolving complex. Eur J Biochem 271: 2967–2975

Burnap RL, Troyan T and Sherman LA (1993) The highly abundant chlorophyll-protein complex of iron-deficient *Synechococcus* sp. PCC7942 (CP43') is encoded by the *isiA* gene. Plant Physiol 103: 893–902

Chang HC, Jankowiak R, Yocum CF, Picorel R, Alfonso M, Seibert M and Small GJ (1994) Exciton level structure and dynamics in the CP47 antenna complex of photosystem II. J Phys Chem 98: 7717–7724

Chen M, Quinnell RG and Larkum AWD (2002) The major light-harvesting pigment protein of *Acaryochloris marina*. FEBS Lett 514: 149–152

Chen M, Bibby TS, Nield J, Larkum AWD and Barber J (2005a) Structure of a large photosystem II supercomplex from *Acaryochloris marina*. FEBS Lett 579: 1306–1310

Chen M, Telfer A, Lin S, Pascal A, Larkum AWD, Barber J and Blankenship RE (2005b) The nature of the photosystem II rection centre in the chlorophyll *d*-containing prokaryote, *Acaryochloris marina*. Photochem Photobiol Sci 4: 1060–1064

Chitnis VP and Chitnis PR (1993) PsaL subunit is required for the formation of photosystem I trimers in the cyanobacterium *Synechocystis s*p. PCC 6803. FEBS Lett 336: 330–334

Damjanovic A, Ritz T and Schulten K (2000) Excitation transfer in the peridinin-chlorophyl-protein of *Amphidinium carterae*. Biophys J 79: 1695–1705

DeEll JD and Toivonen PMA (eds) (2003) Practical Applications of Chlorophyll Fluorescence in Plant Biology. Kluwer Academic Publishers, Dordrecht

Dekker JP and Boekema EJ (2005) Supramolecular organization of thylakoid membrane proteins in green plants. Biochim Biophys Acta 1706: 12–39

Dekker JP, Hassoldt A, Pettersson A, Van Roon H, Groot ML and Van Grondelle R (1995) On the nature of the F695 and F685 emissions of photosystem II. P. Mathis (ed) Photosynthesis: From Light to Biosphere 1: 53–56

De Martino A, Douady D, Quinet-Szely M, Rousseau B, Crepineau F, Apt K and Caron L (2000) The light-harvesting antenna of brown algae. Highly homologous proteins encoded by a multigene family. Eur J Biochem 267: 5540–5549

Diner BA, Schlodder E, Nixon PJ, Coleman WJ, Rappaport F, Lavergne J, Vermaas WFJ and Chisholm DA (2001) Site-directed mutations at D1-His198 and D2-His197 of photosystem II in *Synechocystis* PCC 6803: sites of primary charge separation and cation and triplet stabilization. Biochemistry 40: 9265–9281

Dong C, Tang A, Zhao, Mullineaux CW, Shen G and Bryant DA (2009) ApcD is necessary for efficient energy transfer from phycobilisomes to photosystem I and helps to prevent photoinhibition in the cyanobacterium *Synechococcus* sp. PCC 7002. Biochim Biophys Acta 1787: 1122–1128

Durrand JR, Klug DR, Kwa SLS, Van Grondelle R, Porter G and Dekker JP (1995) A multimer model for P680, the primary electron donor of photosystem II. Proc Natl Acad Sci USA 92: 4798–4802

Duysens LNM and Sweers H E (1963) Mechanism of two photochemical reactions in algae as studied by means of fluorescence. In: Japanese Society of Plant Physiologists (eds) Studies on Microalgae and Photosynthetic Bacteria, pp 353–372. University of Tokyo Press, Tokyo

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Finazzi G and Forti G (2004) Metabolic flexibility of the green alga *Chlamydomonas reinhardtii* as revealed by the link between state transitions and cyclic electron flow. Photosynth Res 82: 327–338

Fromme P, Jordan P and Krauss N (2001) Structure of photosystem I. Biochim Biophys Acta 1507: 5–31

Fromme P and Grotjohann I (2007) Structure of photosystems I and II. Results Probl Cell Differ 45: 33–72

Gantt E (1980) Structure and function of phycobilisomes: Light harvesting pigment complexes in red and blue-green algae. Int Rev Cytol 66: 45–80

Gantt E (1981) Phycobilisomes. Annu Rev Plant Physiol 32: 327–347

Gantt E, Edwards MR and Provasoli L (1971) Chloroplast structure of the *Cryptophyceae*. Evidence for phycobiliproteins within intrathylakoid spaces. J Cell. Biol 48: 280–290

Gantt E, Grabowski B and Cunningham FX Jr (2003) Antenna systems of red algae: Phycobilisomes with photosystem II and chlorophyll complexes with photosystem I. In: Green B and Parson W (eds) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 307–322. Kluwer Academic Publishers, Dordrecht

Gardian Z, Bumba L, Schrofel A, Herbstova M, Nebesarova J and Vacha F (2007) Organisation of photosystem I and photosystem II in red alga *Cyanidium caldarium*: Encounter of cyanobacterial and higher plant concepts. Biochim Biophys Acta 1767: 725–731

Gilmore A, Shinkarev VP, Hazlett TL and Govindjee (1998) Quantitative analysis of the effects of intrathylakoid pH and the xanthophyll cycle pigments on chlorophyll a fluorescence distributions and intensity in thylakoids. Biochemistry 37: 13582–13593

Gitelson AA, Buschmann C and Lichtenthaler HK (1999) The chlorophyll fluorescence ratio F735/F700 as an accurate measure of the chlorophyll content in plants. Remote Sens Environ 69: 296–302

Giuffra E, Cugini D, Croce R and Bassi R (1996) Reconstitution and pigment-binding properties of recombinant CP29. Eur J Biochem 238: 112–120

Glazer AN (1982) Phycobilisomes: Structure and dynamics. Annu Rev Microbiol 36: 173–198

Glazer AN (1984) Phycobilisome. A macromolecular complex optimized for light energy transfer. Biochim Biophys Acta 768: 29–51

Glazer AN (1985) Light harvesting by phycobilisomes. Annu Rev Bioph Biophys Chem 14: 47–77

Glazer AN (1989) Light guides: Directional energy transfer in a photosynthetic antenna. J Biol Chem 264: 1–4

Golan T, Li X-P, Müller-Moulé P and Niyogi KK (2004) Using mutants to understand light stress acclimation in plants. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 525–554. Springer, Dordrecht

Golbeck JH (ed) (2006) Photosystem I: The Light-driven Plastocyanin: Ferredoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24. Springer, Dordrecht

Govindjee (ed) (1975) Bioenergetics of Photosynthesis, pp. 319–372. Academic Press, New York

Govindjee (ed) (1982) Photosynthesis: Vol 1 Energy Conversion by Plants and Bacteria. Vol 2: Development, Carbon Metabolism, and Productivity. Academic Press, New York

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll *a* fluorescence. Aust J Plant Physiol 22: 131–160

Govindjee (2004) Chlorophyll *a* fluorescence: A bit of basics and history. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 1–42. Springer, Dordrecht

Govindjee, Amesz J and Fork DC (eds) (1986) Light Emission by Plants and Bacteria, Academic Press, Orlando

Govindjee, Kern JF, Messinger J and Whitmarssh J (2010) Photosystem II. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0000669.pub2

Green BR and Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 47: 685–714

Green BR and Parson W (eds) (2003) Light-Harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13. Kluwer Academic Publishers, Dordrecht

Groot ML, Peterman EJG, Van Stokkum IHM, Dekker JP and Van Grondelle R (1995) Triplet and fluorescing states of the CP47 antenna complex of photosystem II studied as a function of temperature. Biophys J 68: 281–290

Groot ML, Frese RN, de Weerd FL, Bromek K, Pettersson A, Peterman EJG, Van Stokkum IHM, Van Grondelle R and Dekker JP (1999) Spectroscopic properties of the CP43 core antenna protein of photosystem II. Biophys J 77: 3328–3340

Guglielmi G, Lavaud J, Rousseau B, Etienne AL, Houmard J, and Ruban AV (2005) The light-harvesting antenna of the diatom *Phaeodactylum tricornutum.* Evidence for a diadinoxanthin-binding subcomplex. FEBS J 272: 4339–4348

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A and Saenger W (2009) Cyanobacterial photosystem II and the role of quinones, lipids, channels and chloride. Nat Struct Mol Biol 16: 334–342

Gysi J and Zuber H (1974) Isolation and characterization of allophycocyanin II from the thermophilic blue-green alga *Mastigocladus laminosus* Cohn. FEBS Lett 48: 209–213

Haldimann P and Tsimilli-Michael M (2005) Non-photochemical quenching of chlorophyll *a* fluorescence by oxidised plastoquinone: new evidences based on modulation of the redox state of the endogenous plastoquinone pool in broken spinach chloroplasts. Biochim Biophys Acta 1706: 239–249

Haldrup A, Jensen PE, Lunde C and Scheller HV (2001) Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci 6: 301–306

Hirano M, Satoh K and Katoh S (1980) Plastoquinone as a common link between photosynthesis and respiration in a blue green alga. Photosynth Res 1: 149–162

Hofmann E, Wrench PM, Sharples FP, Hiller RG, Welte W and Diederichs K (1996) Structural basis of light harvesting by carotenoids: Peridinin-chlorophyll-protein from *Amphidinium carterae*. Science 272: 1788–179

Hohmann-Marriott MF, Takizawa K, Eaton-Rye JJ, Mets L and Minagawa J (2010) The redox state of the plastoquinone pool directly modulates minimum chlorophyll fluorescence yield in *Chlamydomonas reinhardtii*. FEBS Lett 584: 1021–1026

Holub O, Seufferheld MJ, Gohlke C, Govindjee, Hiss GJ and Clegg RM (2007) Fluorescence lifetime imaging microscopy of *Chlamydomonas reinhardtii*: nonphotochemical quenching mutants and the effect of photosynthetic inhibitors on the slow chlorophyll fluorescence transient. J Microscopy 30: 1–31

Holt NE, Fleming GR and Niyogi KK (2004) Toward an understanding of the mechanism of nonphotochemical quenching in green plants. Biochemistry 43: 8281–8289

Holzwarth, AR (1991) Structure-function relationships and energy transfer in phycobilisome antennae. Physiol Plant 83: 518–528

Holzwarth AR, Mueller MG, Reus M, Nowaczyk M, Sander J, and Rögner M (2006) Kinetics and mechanism of electron transfer in intact photosystem II and in the isolated reaction center: Pheophytin is the primary electron acceptor. Proc Natl Acad Sci USA 103: 6895–6900

Hu Q, Marquardt J, Iwasaki I, Miyashita H, Kurano N, Mörschel E and Miyachi S (1999) Molecular structure, localization and function of biliproteins in the chloropjyll *a*/*d* containing oxygenic photosynthetic prokaryote *Acaryochloris marina*. Biochim Biophys Acta 1412: 250–261

Itoh S, Mino H, Itoh K, Shigenaga T, Uzumaki T and Iwaki M (2007) Function of chlorophyll *d* in reaction centers of photosystems I and II of the oxygenic photosynthesis of *Acaryochloris marina*. Biochemistry 46: 12473–12481

Jansson S (1999) A guide to the Lhc genes and their relatives in *Arabidopsis*. Trends Plant Sci 4: 236–240

Jeffrey SW (1972) Preparation and some properties of crystalline chlorophyll c1 and c2 from marine algae. Biochim Biophys Acta 279: 15–33

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W and Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. Nature 411: 909–917

Kamiya N and Shen J-R (2003) Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7 Å resolution. Proc Nat Acad Sci USA 100: 98–103

Kana R, Prasil O and Mullineaux CW (2009a) Immobility of phycobilins in the thylakoid lumen of a cryptophyte suggests that protein diffusion in the lumen is very restricted. FEBS Lett 583: 670–674

Kana R, Prásil O, Komárek O, Papageorgiou GC and Govindjee (2009b) Spectral characteristic of fluorescence induction in a model cyanobacterium, *Synechococcus* sp. (PCC 7942). Biochim Biophys Acta 178: 1170–1178

Kaňa R, Komárek O, Kotabová E, Papageorgiou GC, Govindjee and Prášil O (2011) The Slow S to M fluorescence rise is missing in the RpaC mutant of *Synechocystis* sp. (PCC 6803), Proceedings of the 15th Intern. Congress of Photosynthesis, Beijing, China (2010), in press.

Karapetyan NV (2008) Protective dissipation of excess absorbed energy by photosynthetic apparatus of cyanobacteria: role of antenna terminal emitters. Photosynth Res 97: 195–204

Kargul J, Turkina MV, Nield J, Benson S, Vener AV and Barber J (2005) Light-harvesting complex II protein CP29 binds to photosystem I of *Chlamydomonas reinhardtii* under state 2 conditions. FEBS J 272: 4797–4806

Karukstis KK and Sauer K (1983) Fluorescence decay kinetics of chlorophyll in photosynthetic membranes. J Cellular Biochem 23: 131–158

Kautsky H and Hirsch A (1931) Neue Versuche zur Kohlensäureassimilation. Naturwissenschaften 19: 964

Kereïche S, Kouřil R, Oostergetel GT, Fusetti F, Boekema EJ, Doust AB, Van der Weij-de Wit CD and Dekker JP (2008) Association of chlorophyll *a*/*c*2 complexes to photosystem I and photosystem II in the cryptophyte *Rhodomonas* CS24. Biochim Biophys Acta 1777: 1122–1128

Kirilovsky D (2007) Photoprotection in cyanobacteria: the orange carotenoid protein (OCP)-related nonphotochemical quenching mechanism. Photosynth Res 93: 7–16

Kobayashi M, Ohashi S, Iwamoto K, Shirai Y, Kato Y and Watanabe T (2007) Redox potential of chlorophyll *d* *in vitro*. Biochim Biophys Acta 1767: 596–602

Koblızek M, Komenda J and Masojıdek J (1998) State transitions in the cyanobacterium *Synechococcus* PCC 7942. Mobile antenna or spillover? In G. Garab (ed) Photosynthesis: Mechanisms and Effects, Vol 1, pp 213–216. Kluwer Academic Publishers, Dordrecht

Konermann L and Holzwarth AR (1996) Analysis of the absorption spectrum of photosystem II reaction centers: Temperature dependence, pigment assignment, and inhomogeneous broadening. Biochemistry 35: 829–842

Kramer DM, Avenson TJ, Kanazawa A and Cruz JA (2004) The relationship between photosynthetic electron transfer and its regulation. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 251–278. Springer, Dordrecht

Krause GH (1974) Changes in chlorophyll fluorescence in relation to light-dependent cation transfer across thylakoid membranes. Biochim Biophys Acta 333: 301–313

Krause GH, Vernotte C and Briantais JM (1982) Photoinduced quenching of chlorophyll fluorescence in intact chloroplasts and algae: Resolution into two components. Biochim Biophys Acta 679: 116–124

Krause GH and Jahns P (2004) Nonphotochemical energy dissipation determined by chlorophyll fluorescence quenching: Characterization and function. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 463–495. Springer, Dordrecht

Krikunova M, Voigt B and Lokstein H (2002) Direct evidence for excitonically coupled chlorophylls *a* and *b* in LHC II of higher plants by nonlinear polarization spectroscopy in the frequency domain. Biochim Biophys Acta 1556: 1–5

Kruse O (2001) Light-induced short-term adaptation mechanisms under redox control in the PSII-LHCII supercomplex: LHC II state transitions and PSII repair cycle. Naturwissenschaften 88: 284–292

Kühl M, Chen M, Ralf PJ, Schreiber U and Larkum AWD (2005) A niche for cyanobacteria containing chlorophyll *d*. Nature 433: 820

Kühlbrandt W, Wang DN and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Ladas NP and Papageorgiou GC (2000a) Cell turgor: A critical factor for the proliferation of cyanobacteria at unfavorable salinity. Photosynth Res 65: 155–164

Ladas NP and Papageorgiou GC (2000b) The salinity tolerance of freshwater cyanobacterium *Synechococcus* sp. PCC 7942 is determined by its ability for osmotic adjustment and presence of osmolyte sucrose. Photosynthetica 38: 343–348

Lakowicz JR (2006) Principles of Fluorescence Spectroscopy. Springer, New York

La Roche J, Van der Staay GWM, Partensky F, Ducret A, Aebersold R, Li R, Golden SS, Hiller RG, Wrench PM, Larkum AWD and Green BR (1996) Independent evolution of the prochlorophyte and green plant chlorophyll *a*/*b* light-harvetsing proteins. Proc Natl Acad Sci USA 93: 15244–15248

Larkum WD, Douglas SE and Raven JA (eds) (2003) Photosynthesis in Algae, Advances in Photosynthesis and Respiration, Vol 14. Kluwer Academic Publishers, Dordrecht

Lavorel J (1959) Induction of fluorescence in quinone-poisoned *Chlorella* cells. Plant Physiol 34: 204–209

Lazár D and Schansker G (2009) Models of Chl *a* fluorescence transients. In: Laisk A, Nedbal L and Govindjee (eds) Photosynthesis in Silico. Understanding Complexity from Molecules to Ecosystems, Advances in Photosynthesis and Respiration, Vol 29, pp 85–123. Springer, Dordrecht

Lemeille S and Rochaix J-D (2010) State transitions at the crossroad of thylakoid signalling pathways. Photosynth Res 106: 33–46

Lichtenthaler HK (ed) (1988) Applications of Chlorophyll Fluorescence in Photosynthesis Research. Stress Physiology, Hydrobiology and Remote Sensing. Kluwer Academic Publishers, Dordrecht

Liu LN, Chen XL, Zhang YZ and Zhou BC (2005) Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: An overview. Biochim Biophys Acta 1708: 133–142

Liu ZF, Yan HC, Wang KB, Kuang TY, Zhang JP, Gul LL, An XM and Chang WR (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. Nature 428: 287–292

Loll B, Kern JF, Saenger W, Zouni A and Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. Nature 438: 1040–1044

Lunde C, Jensen PE, Haldrup A, Knoetzel J and Scheller HV (2000) The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis. Nature 408: 613–615

MacColl R (1998) Cyanobacterial phycobilisomes. J Struct Biol 124: 311–334

MacColl R (2004) Allophycocyanin and energy transfer. Biochim Biophys Acta 1657: 73–81

Malkin S and Kok B (1966) Fluorescence induction studies in isolated chloroplasts. I. Number of components involved in the reaction and quantum yields. Biochim Biophys Acta 126: 413–432

Malkin S, Wong D, Govindjee and Merkelo H (1980) Parallel measurements on fluorescence lifetime and intensity from leaves during fluorescence induction. Photobiochem Photobiophys 1: 83–89

Manodori A and Melis A (1985) Phycobilisome – photosystem II association in *Synechococcus* 6301 (Cyanophyceae) FEBS Lett 181: 79–82

Marquardt J, Senger H, Miyashita H, Miyachi S and Mörschel E (1997) Isolation and characterization of biliprotein aggregates from *Acaryochloris marina*, a Prochloron-like prokaryote containing mainly chlorophyll *d*. FEBS Lett 410: 428–432

Martin CD and Hiller RG (1987) Subunits and chromophores of a type I phycoerythrin from a *Chroomonas* sp. (Cryptophyceae). Biochim Biophys Acta 923: 88–97

Matsubara S, Chen Y-C, Caliandro R, Govindjee and Clegg RM (2011) Photosystem II fluorescence lifetime imaging in avocado leaves: contributions of the lutein-epoxide and violaxanthin cycles to fluorescence quenching. J Photochem Photobiol. B: Biol 104: 271–284

McConnell MD, Koop R, Vasil'ev S and Bruce D (2002) Regulation of the distribution of chlorophyll and phycobilin-absorbed excitation energy in cyanobacteria. A structure-based model for the light state transition. Plant Physiol 130: 1201–1212

Metz JG and Seibert M (1984) Presence in photosystem II core complexes of a 34-kilodalton polypeptide required for water photolysis. Plant Physiol 76: 829–832

Michel KP and Pistorius EK (2004) Adaptation of the photosynthetic electron transport chain in cyanobacteria to iron deficiency: The function of IdiA and IsiA. Physiol Plant 120: 36–50

Miller SR, Augustine S, Olson TL, Blankenship RE, Selker J and Wood AM (2005) Discovery of a free-living chlorophyll *d*-producing cyanobacterium with a hybrid proteobacterial/cyanobacterial small-subunit rRNA gene. Proc Natl Acad Sci USA 102: 850–855

Mimuro M (2004) Photon capture, exciton migration and trapping and fluorescence ermission in cyanobacteria and red algae. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 173–195. Springer, Dordrecht

Mimuro M and Akimoto S (2003) Carotenoids of light harvesting systems: Energy transfer processes from fucoxanthin and peridinin to chlorophyll. In: Larkum AWD, Douglas SE and Raven JA (eds) Photosynthesis in Algae, Advances in Photosynthesis and Respiration, Vol 14, pp 335–349. Kluwer Academic Publishers, Dordrecht

Mimuro M, Murakami A and Fujita Y (1982) Studies on spectral characteristics of allophycocyanin isolated from *Anabaena cylidrica*: Curve fitting analysis. Arch Biochem Biophys 215: 266–273

Mimuro M, Tamai N, Ishimaru T and Yamazaki I (1990) Excitation energy flow in the marine dinoflagellate *Protogonyalux tamarensis*. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol 2, pp 309–312. Kluwer Academic Publishers, Dordrecht

Mimuro M, Tamai N, Murakami A, Watanabe M, Erata M, Watanabe MM, Tokutomi M and Yamazaki I (1998) Multiple pathways of excitation energy flow in the photosynthetic pigment system of a cryptophyte, *Cryptomonas sp.* (CR-1). Phycological Res 46: 155–164

Mimuro M, Akimoto S, Yamazaki I, Miyashita H and Miyachi S (1999) Fluorescence prioperties of chlorophyll *d*-containing prokaryotic alga *Acaryochloris marina*: studies using time-resolved fluorescence spectroscopy on intact cells. Biochim Biophys Acta 1412: 37–46

Mimuro M, Hirayama K, Yamazaki I, Uezono K, Miyashita H and Miyachi S (2000) Uphill energy transfer in a chlorophyll *d*-containing oxygenic photosynthetic prokaryote, *Acaryochloris marina*. Biochim Biophys Acta 1456: 27–34

Mimuro M, Akimoto S, Gotoh T, Yokono M, Akiyama M, Tsuchiya T, Miyashita H, Kobayashi M and Yamazaki I (2004) Identification of the primary electron donor in PS II of the Chl *d*-dominated cyanobacterium *Acaryochloris marina*. FEBS Lett 556: 95–98

Minagawa J (2011) State transitions – the molecular remodelling of photosynthetic supercomplexes that controls energy flow in the chloroplast. Biochim Biophys Acta 1807: 897–905

Miyashita H, Ikemoto H, Kurano N, Adachi K, Chihara M and Miyachi S (1996) Chlorophyll *d* as a major pigment. Nature 383: 402

Miyashita H, Adachi K, Kurano N, Ikemoto H, Chihara M and Miyachi S (1997) Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll *d* as the major chlorophyll. Plant Cell Physiol 38: 274–281

Miyashita H, Ikemoto H, Kurano N, Miyachi S and Chihara M (2003) *Acaryochloris marina* gen. et sp. nov. (cyanobacteria), an oxygenic photosynthetic prokaryote containing Chl *d* as a major pigment. J Phycol 39: 1247–1253

Mirkovic T, Wilk KE, Curmi PMG and Scholes G (2009) Phycobiliprotein diffusion in chloroplasts of cryptophyte *Rhodomonas* CS24. Photosynth Res 100: 7–17

Mohanty P and Govindjee (1974) The slow decline and the subsequent rise of chlorophyll fluorescence transients in intact algal cells. Plant Biochem J 1: 78–106

Mullineaux CW (2008) Phycobilisome-reaction centre interaction in cyanobacteria. Photosynth Res 95:175–182

Mullineaux CW, Tobin MJ and Jones GR (1997) Mobility of photosynthetic complexes in thylakoid membranes. Nature 390: 421–424

Munday JCM Jr and Govindjee (1969) Light-induced changes in the fluorescence yield of chlorophyll *a* in vivo. III. The dip and the peak in the fluorescence transient of *Chlorella pyrenoidosa*. Biophys J 9:1–21

Murata N (1969) Control of excitation transfer in photosynthesis. Light-induced change of chlorophyll *a* fluorescence in *Porhyridium cruentum*. Biochim Biophys Acta 172: 242–251

Murata N (2009) The discovery of state transitions in photosynthesis 40 years ago. Photosynth Res 99: 155–160

Murata N, Nishimura M and Takamiya A (1966) Fluorescence of chlorophyll in photosynthetic systems. II. Induction of fluorescence in isolated spinach chloroplasts. Biochim Biophys Acta 120: 23–33

Nield J, Morris EP, Bibby TS and Barber J (2003) Structural analysis of the photosystem I supercomplex of cyanobacteria induced by iron deficiency. Biochemistry 42: 3180–3188

Palacios MA, de Weerd FL, Ihalainen JA, Van Grondelle R and Van Amerongen H (2002) Superradiance and exciton (de)localization in light-harvesting complex II from green plants. J Phys Chem 106: 5782–5787

Papageorgiou GC (1975) Chlorophyll fluorescence: an intrinsic probe of photosynthesis. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 319–372. Academic Press, New York

Papageorgiou GC and Alygizaki-Zorba A (1997) A sensitive method for the estimation of the cytoplasmic osmolality of cyanobacterial cells using chlorophyll *a* fluorescence. Biochim Biophys Acta 1335: 1–4

Papageorgiou GC and Govindjee (1967) Changes in intensity and spectral distribution of fluorescence effect of light treatment on normal and DCMU-poisoned *Anacystis nidulans*. Biophys J 7: 375–389

Papageorgiou GC and Govindjee (1968a) Light-induced changes in the fluorescence yield of chlorophyll *a* in vivo I. *Anacystis nidulans*. Biophys J 8: 1299–1315

Papageorgiou GC and Govindjee (1968b) Light-induced changes in the fluorescence yield of chlorophyll *a* in vivo II. *Chlorella pyrenoidosa*. Biophys J 8: 1299–1315

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19. Springer, Dordrecht

Papageorgiou GC and Govindjee (2011) Photosystem II fluorescence: Slow changes – scaling from the past. J Photochem Photobiol B: Biol 104: 258–270

Papageorgiou GC and Stamatakis K (2004) Water and solute transport in cyanobacteria as probed by chlorophyll fluorescence. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 663–678. Springer, Dordrecht

Papageorgiou GC, Isaakidou J and Argoudelis C (1972) Structure dependent control of chlorophyll *a* excitation density. The role of oxygen. FEBS Lett 25: 130–142

Papageorgiou GC, Alygizaki-Zorba A, Ladas N and Murata N (1998) A method to probe the cytoplasmic osmolality and water and solute fluxes across the cell membrane of cyanobacteria with chlorophyll *a* fluorescence: Experiments with *Synechococcus* sp. PCC 7942. Physiol Plant 103: 215–224

Papageorgiou GC, Tsimilli-Michael M and Stamatakis K (2007) The fast and slow kinetics of chlorophyll *a* fluorescence induction in plants, algae and cyanobacteria: a viewpoint. Photosynth Res 94: 275–290

Partensky F and Garczarek L (2003) The photosynthetic apparaus of chlorophyll *b*– and *d*–containing oxyphotobacteria. In: Larkum, AWD, Douglas SE and Raven JA (eds) Photosynthesis in Algae, Advances in Photosynthesis and Respiration, Vol 14, pp 29–62. Kluwer Academic Publishers, Dordrecht

Partensky F, Hess WR and Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol Mol Biol Rev 63: 106–127

Passaquet C, Thomas JC, Carlon L, Hauswirth N, Puel F and Berkaloff C (1991) Light-harvesting complexes of brown algae: Biochemical characterization and immunological relationships. FEBS Lett 280: 21–26

Peterson RB, Oja V and Laisk A (2001) Chlorophyll fluorescence at 680 and 730 nm and leaf photosynthesis. Photosynth Res 56:185–195

Polivka T, Hiller RG and Frank HA (2007) Spectroscopy of the peridinin-chlorophyll *a* protein: Insight into light-harvesting strategy of marine algae. Arch Biochem Biophys 458: 111–120

Pfündel E (1998) Estimating the contribution of photosystem I to total leaf chlorophyll fluorescence. Photosynth Res 56: 185–195

Pursiheimo S, Rintamaki E, Baena-Gonzalez E and Aro E-M 1998) Thylakoid protein phosphorylation in evolutionally divergent species with oxygenic photosynthesis. FEBS Lett 423: 178–182

Qin X, Kebin Z, Chen X, Qu Y, Li L and Kuang T (2006) Rapid purification of photosystem I chlorophyll-binding proteins by differential centrifugation and vertical rotor. Photosynth Res 90: 195–204

Rabinowitch E and Govindjee (1969) Photosynthesis. John Wiley & Sons, New York [Available free at: http://www.life.illinois.edu/govindjee/photosynBook.html]

Rochaix J-D (2007) Role of thylakoid protein kinases in photosynthetic acclimation. FEBS Lett 581: 2758–2775

Rubin A and Riznichenko G (2009) Modeling of the primatry processes in photosynthesis. In: Laisk A, Nedbal L and Govindjee (eds) Photosynthesis in Silico, Advances in Photosynthesis and Respiration, Vol 29, pp 151–176. Springer, Dordrecht –

Samson G, Prasil O and Yaakoubd B (1999) Photochemical and thermal phases of chlorophyll *a* fluorescence. Photosynthetica 37: 163–182

Savard F, Richard C, and Guertin M (1996) The *Chlamydomonas reinhardtii* L1818 gene represents a distant relative of the Cab I/II gene that is regulated during the cell cycle and in response to illumination. Plant Mol Biol 32: 461–473

Scheer H (1981) Biliproteins. Angew Chem Int Ed Engl 20: 241–261

Scheer H (2003) The pigments. In: Green BR and Parson WW (eds) Light-harvesting Antennas in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 13, pp 29–81. Kluwer Aademic Publishers, Dordrecht

Scheer H and Zhao KH (2008) Biliprotein maturation: the chromophore attachment. Mol Microbiol 68: 263–276

Schiller H, Senger H, Miyashita H, Miyachi S and Dau H (1997) Light-harvesting in *Acaryochloris marina* – spectroscopic characterization of a chlorophjyll *d*-dominated photosynthetic antenna system. FEBS Lett 410: 433–436

Schmid VHR (2008) Light-harvesting complexes of vascular plants. Cell Mol Life Sci 65: 3619–3639

Schreiber U (2004) Pulse-amplitude-modulation (PAM) and saturation pulse method: an overview. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 279–319. Springer, Dordrecht

Schreiber U, Bauer R and Franck UF (1971) Chlorophyll fluorescence induction in *Scenedesmus* at oxygen deficiency. Z Naturforsch 26b: 1195–1196

Schulte T, Sharples FP, Frank P, Hiller RG and Hofman E (2009) X-ray structure of the high-salt form of the peridinin-chlorophyll *a*-protein from the dinoflagellate *Amphidinium carterae*: Modulation of the spectral properties of pigments by the protein environment. Biochemistry 48: 4466–4475

Shan J, Wang J, Ruan X, Li L, Gong Y, Zhao N and Kuang T (2001) Changes of absorption spectra during heat-induced denaturation of photosystem II core antenna complexes CP43 and CP47: revealing the binding states of chlorophyll molecules in these two complexes. Biochim Biophys Acta 1504: 396–408

Sidler WA (1994) Phycobilisome and phycobiliprotein structures. In: Bryant DA (ed) The Molecular Biology of Cyanobacteria, Advances in Photosynthesis, Vol 1, pp 139–216. Kluwer Academic Publishers, Dordrecht

Stadnichuk IN, Lukashev EP and Elanskaya IV (2009) Fluorescence changes accompanying short-term light adaptations in photosystem I and photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803 and phycobiliprotein-impaired mutants: State 1/State 2 transitions and carotenoid-induced quenching of phycobilisomes. Photosynth Res 99: 227–241

Stauber EJ, Busch A, Naumann B, Svatos A and Hippler M (2009) Proteotypic profiling of LHCI from *Chlamydomonas reinhardtii* provides new insights into structure and function of the complex. Proteomics 9: 398–408

Steffen R, Eckert HJ, Kelly AA, Doermann P and Renger G (2005) Investigations on the reaction pattern of photosystem II in leaves from *Arabidopsis thaliana* by time-resolved fluorometric analysis. Biochemistry 44: 3123–3133

Stewart DH, Nixon PJ, Diner BA and Brudvig GW (2000) Assignment of the Qy absorbance bands of photosystem II chromophores by low-temperature optical spectroscopy of wild-type and mutant reaction centers. Biochemistry 39*:* 14583–14594

Stirbet A and Govindjee (2011) On the relation between the Kautsky Effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. J Photochem Photobiol B: Biol 104: 236–257

Strasser RJ, Tsimilli-Michael M and Srivastava A (2004) Analysis of the chlorophyll *a* fluorescence transient. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 321–362. Springer, Dordrecht

Su X, Fraenkel PG and Bogorad L (1992) Excitation energy transfer from phycocyanin to chlorophyll in an *apcA*-defective mutant of *Synechocystis* sp. PCC 6803. J Biol Chem 267: 22944–22950

Suggett DJ, Prášil O and Borowitzka A (2011) Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer, Dortrecht

Suter GW and Holzwarth AR (1987) A kinetic method for the energy transfer in phycobilisomes. Biophys J 52: 673–683

Takahashi H, Iwai M, Takahashi Y and Minagawa J (2006) Identification of the mobile light-harvesting complex II polypeptides for state transitions in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 103: 477–482

Tan S, Ducret A, Aebersold R and Gantt E (1997a) Red algal LHC I genes have similarities with both Chl *a*/*b*- and *a*/*c*-binding proteins: A 21 kDa polypeptide encoded by LhcaR2 is one of the six LHC I polypeptides. Photosynth Res 53: 129–140

Tan S, Cunningham FX, Jr. and Elisabeth Gantt E (1997b) LhcaR1 of the red alga *Porphyridium cruentum* encodes a polypeptide of the LHCI complex with seven potential chlorophyll *a*-binding residues that are conserved in most LHCs. Plant Mol Biol 33: 157–167

Tandeau de Marsac N (2003) Phycobiliproteins and phycobilisomes: The early observations. Photosynth Res 76: 193–205

Tetenkin VI, Gulyaev BA, Seibert M and Rubin AB (1989) Spectral properties of stabilized D 1/D2/cytochrome *b*-559 photosystem II reaction center complex. Effects of

Triton X-100, the redox state of pheophytin, and β–carotene. FEBS Lett 250: 459–463

Tikkanen M, Grieco M and Aro EM (2011) Novel insights into plant light-harvesting complex II phosphorylation and "state transitions". Trends Plant Sci 16: 126–131

Tokutsu R, Teramoto H, Takahashi Y, Ono TA and Minagawa J (2004) The light-harvesting complex of photosystem I in *Chlamydomonas reinhardtii*: Protein composition, gene structure and phylogenic implications. Plant Cell Physiol 45: 138–145

Tomitani A, Okada K, Miyashita H, Matthijs HCP, Ohno T and Tanaka A (1999) Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400: 159–162

Tomo T, Seiji Akimoto S, Tsuchiya T, Fukuya M, Tanaka K and Mimuro M (2008) Isolation and spectral characterization of photosystem II reaction center from *Synechocystis* sp. PCC 6803. Photosynth Res 98: 293–302

Toole CA and Allnut FCT (2003) Red, cryptomonad and glaucocystophyte algal phycobiliproteins. In: Larkum WD, Douglas SE and Raven JA (eds) Photosynthesis in Algae, Advances in Photosynthesis and Respiration, Vol 14, pp 305–334. Kluwer Academic Publishers, Dordrecht

Tsimilli-Michael M, Stamatakis K and Papageorgiou GC (2009) Dark-to-light transition in *Synechococcus* sp. PCC 7942 cells studied by fluorescence kinetics assesses plastoquinone redox poise in the dark and photosystem II fluorescence component and dynamics during state 2 to state 1 transition. Photosynth Res 99: 243–255

Turkina MV, Kargul J, Blanco-Rivero A, Villarejo A, Barber J and Vener AV (2007) Environmentally modulated phosphoproteome of photosynthetic membranes in the green alga *Chlamydomonas reinhardtii*. Mol Cell Proteomics 5: 1412–1425

Umena Y, Kawakami K, Shen J-R and Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473: 55–60

Van der Weij-De Wit CD, Doust AB, Van Stokkum IHM, Dekker JP, Wilk KE, Curmi PMG, Scholes GD and Van Grondelle R (2006) How energy funnels from the phycoerythrin antenna complex to photosystem I and photosystem II in cryptophyte *Rhodomonas* CS24 Cells. J Phys Chem B 110: 25066–25073

Van der Weij-De Wit CD, Doust AB, Van Stokkum IHM, Dekker JP, Wilk KE, Curmi PMG and Van Grondelle R (2008) Phycocyanin sensitizes both photosystem I and photosystem II in cryptophyte *Chroomonas* CCMP270 cells. Biophys J 94: 2423–2433

Vanselow C, Weber APM, Krause K and Fromme P (2009) Genetic analysis of the photosystem I subunits from the red alga, *Galdieria sulphuraria*. Biochim Biophys Acta 1787: 46–59

Veith T and Büchel C (2007) The monomeric photosystem I-complex of the diatom *Phaeodactylum tricornutum* binds specific fucoxanthin chlorophyll proteins (FCPs) as light-harvesting complexes. Biochim Biophys Acta 1767: 1428–1435

Veith T, Brauns J, Weisheit W, Mittag M and Büchel C (2009) Identification of a specific fucoxanthin-chlorophyll protein in the light-harvesting complex of photosystem I in the diatom *Cyclotella meneghiniana.* Biochim Biophys Acta 1787: 905–912

Vener AV, Van Kan PJM, Rich PR, Ohad I and Andersson B (1997) Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: Thylakoid protein kinase deactivation by a single-turnover flash. Proc Natl Acad Sci USA 94: 1585–1590

Vener AV, Ohad I and Andersson B (1998) Protein phosphorylation and redox sensing in chloroplast thylakoids. Cur Opin Plant Biol 1: 217–223

Vernotte C, Etienne AL and Briantais J-M (1979) Quenching of the photosystem II chlorophyll fluorescence by the plastoquinone pool. Biochim Biophys Acta 545: 519–527

Vredenberg WJ (2004) System analysis and photoelectrochemical control of chlorophyll fluorescence in terms of trapping models of Photosystem II: A challenging view. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 133–172. Springer, Dordrecht

Vredenberg WJ (2008) Kinetic models of photosystem II should incorporate a role for Q_B-nonreducing reaction centers. Biophys J 95: 3113–3114

Vredenberg WJ and Prasil O (2009) Modelling of chlorophyll *a* fluorescence in plant cells: Derivation of a descriptive algorithm. In: Laisk A, Nedbal N, Govindjee (eds) Photosynthesis in Silico, Advances in Photosynthesis and Respiration, Vol 29, pp 125–149. Springer, Dordrecht

Wedemayer GJ, Kidd DG, Wemmer DE and Glazer AN (1992) Phycobilins of cryptophycean algae – occurrence of dihydrobiliverdin and mesobiliverdin in cryptomonad biliproteins. J Biol Chem 267: 7315–7331

Wedemayer GJ, Kidd DG and Glazer AN (1996) Cryptomonad biliproteins: Bilin types and locations. Photosynth Res 48: 163–70

Wilk KE, Harrop SJ, Jankova L, Edler D, Keenan G, Sharples F, Hiller RG and Curmi PMG (1999) Evolution of a light-harvesting protein by addition of new subunits and rearrangement of conserved elements: Crystal structure of a cryptophyte phycoerythrin at 1.63-Å resolution. Proc Natl Acad Sci USA 96: 8901–8906

Wolfe GR, Cunningham FX, Grabowski B, Gantt E (1994) Isolation and characterization of photosystems I and II from the red alga *Porphyridium cruentum*. Biochim Biophys Acta 1188: 357–366

Wollman F-A (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J 20: 3633–3630

Wong D, Pellegrino F, Alfano RR and Zilinskas BA (1981) Fluorescence relaxation kinetics and quantum yield from

the isolated phycobiliproteins of the blue-green alga *Nostoc* sp. measured as a function of single picosecond pulse intensity. Photochem Photobiol 33: 651–662

Wydrzynski TJ and Satoh K (eds) (2005) Photosystem II: The Light-Driven Water: Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22. Springer, Dordrecht

Yang S, Su Z, Li H, Feng J, Xie J, Xia A, Gong Y and Zhao J (2007) Demonstration of phycobilisome mobility by the time- and space-correlated fluorescence imaging of a cyanobacterial cell. Biochim Biophys Acta 1767: 15–21

Zapata M, Garrido JL and Jeffrey SW (2006) Chlorophyll *c* pigments. Current status. In: Grimme B, Porra RJ, Ruediger W and Scheer H (eds) Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications, Advances in Photosynthesis and Respiration, Vol 25, pp. 39–53. Springer, Dordrecht

Zer H and Ohad I (2003) Light, redox state, thylakoid-protein phosphorylation and signaling gene expression. Trends Biol Sci 28: 467–470

Zhao J, Zhou J and Bryant DA (1992) Energy transfer processes in phycobilisomes as deduced from analyses of mutants of *Synechococcus* sp. PCC 7002. In: Murata N (ed) Research in Photosynthesis, Vol 1, pp 25–32. Kluwer Academic Publishers, Dordrecht

Zheleva D, Sharma J, Panico M, Morris HR and Barber J (1998) Isolation and characterization of monomeric and dimeric CP47-reaction center photosystem II complexes. J Biol Chem 273: 16122–16127

Zhu X-G, Govindjee, Baker NR, de Sturler E, Ort DR and Long SP (2005) Chlorophyll *a* fluorescence induction kinetics in leaves predicted from a model describing each discrete step of excitation energy and electron transfer associated with photosystem II. Planta 223: 114–133

Zilinskas BA and Greenwald LS (1986) Phycobilisome structure and function. Photosynth Res 10: 7–35

Zouni, A., Witt HT, Kern J, Fromme P, Krauss N, Saenger W and Orth P (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 angstrom resolution. Nature 409: 739–743

Chapter 19

Thermoluminescence

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Summary

Thermoluminescence (TL) of photosynthetic membranes was discovered by William Arnold and Helen Sherwood in 1957. In the last half century, several studies have elucidated the mechanism of TL emission, which showed that the recombination of different charge pairs generated and trapped during pre-illumination are responsible for the observed light emission. Since most of the TL bands originate within Photosystem II (PS II), the technique of TL has become a useful complementary tool to chlorophyll *a* fluorescence to probe subtle changes in PS II photochemistry. The technique is simple and non-invasive; it has been successfully used to study leaf, cells, thylakoids and even reaction center preparations. The TL technique provides quick information about the redox potential changes of the bound primary quinone (Q_A) and the secondary quinone (Q_B) acceptors of PS II; TL has been extensively used to study the effects of photoinhibition, mutations, stresses and myriad responses of the photosynthetic apparatus during acclimation and adaptation. This chapter reviews crucial evidence for the identification of charge pairs responsible for the generation of different TL bands; the relationship of these bands to the components of delayed light emission; responses to excitation pressure arising out of environmental factors; methodology, and instrumentation. A model based on the detailed analysis of the redox shifts of the PS II electron acceptors Q_A and Q_B, explaining the possibility of non-radiative dissipation of excess light energy within the reaction center of PS II (reaction center quenching) and its physiological significance in photoprotection of the photosynthetic membranes has been suggested. Developments in the analysis of biophysical parameters and the non-adherence of photosynthetic TL to the analysis by the 1945 theory of J.T. Randall and M.H.F. Wilkins have been briefly reviewed.

Abbreviations: ATP – Adenosine triphosphate; CAM – Crassulacean acid metabolism; Cyt b_{559} – Cytochrome b_{559}; D1 – 32 kDa Photosystem II reaction center polypeptide; D2 – 34 kDa Photosystem II reaction center polypeptide; DCMU – 3-(3,4-dichlorophenyl)-l,l-dimethylurea; DHAP – Dihydroxyacetone phosphate; EGTA – Ethyleneglycol bis (beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid; DLE – Delayed light emission; EPR – Electron paramagnetic resonance; LHCII – Light-harvesting complex of Photosystem II; MDA – Malondialdehyde; NAD(P)H – Nicotinamide adenine dinucleotide phosphate, the reduced form; P680 – Primary electron donor chlorophyll of Photosystem II; PGA – 3-phosphoglycerate; Pheo – Pheophytin; PS I – Photosystem I; PS II – Photosystem II; PQ – Plastoquinone; Q_A – Primary electron-accepting quinone in Photosystem II reaction centers; Q_B – Secondary electron-accepting quinone in Photosystem II reaction centers; S states – Oxidation states of the manganese cluster of PS II; TL – Thermoluminescence; T_M – Temperature of maximum thermoluminescence emission; Tris – Tris(hydroxymethyl) aminomethane; Y_D^+ – Redox active tyrosine-160 of the D2 protein; Y_Z^+ – Redox active tyrosine-161 of the D1 protein

I. Introduction

A. Definition

Thermoluminescence (TL) in semiconductors has been well studied and has been shown to originate in a recombination of the hole and the free electron that is generated during the exposure of the sample to electromagnetic radiation. Exposure of a dielectric specimen to ionizing or non-ionizing radiation results in the production of holes and free electrons that get trapped in the lattice of the material because of defects. When they acquire enough energy, the probability of escape of the trapped charge carriers is increased and their recombination produces electromagnetic radiation. Because the energy for the release of the electrons is provided by heating of the specimen the resultant luminescence is called *thermoluminescence*. When pre-illuminated oxygenic

photosynthetic membranes are cooled and then heated in the dark from low temperature, they emit light at distinct temperatures yielding what is known as the glow curve(s) or TL. The TL observed in photosynthetic membranes was initially explained on the basis of a mechanism in semiconductors. However, it became apparent that TL in photosynthetic membranes occurs as a result of a recombination between the oxidized donors and reduced acceptors produced in the preceding light exposure. Furthermore, there are several recombination events that are involved in generating the complex glow curves since several peaks are produced that are susceptible to electron transport inhibitors, modifiers of the water-oxidizing complex or compounds that interfere with the light-induced electron transport.

B. Historical

The discovery of delayed light emission (DLE) by photosynthetic organisms that later led to the study of TL was, in a way, a chance event. When Strehler and Arnold (1951) were attempting to demonstrate the photosynthetic formation of energy rich phosphate (ATP) (adenosine tri phosphate) using a firefly luminescent assay, they observed that light was given off by the chloroplasts even in the absence of the firefly extract. They studied different characteristics of this light emission and concluded that this DLE was a reflection of the reversibility of certain early reactions of photosynthesis. Several interesting observations were made and they were subsequently confirmed and extended by other studies. These included, e.g., the effect of inhibitors of photosynthesis, the action spectra and its dark decay.

Arnold and Sherwood (1957) discovered TL in dried chloroplasts and had used the term glow curve. Tollin and Calvin (1957) also showed that there are several components of DLE decaying with different half lives. Furthermore, they also showed TL from dried chloroplasts yielding two peaks, one between 50°C and 60°C and another between 140°C and 150°C. A mechanism involving the recombination of holes and electrons trapped during illumination was proposed by Arnold (1965). In subsequent studies, Arnold (1966) and Arnold and Azzi (1968) reported TL glow curves in green algal species belonging to the genera *Chlorella* and *Scenedesmus*, including *Scenedesmus* mutants. However, the first well resolved glow curves were published by Rubin and Venediktov (1969) and Shuvalov and Litvin (1969).

It was clear that not only are there several bands that appear at different temperatures but also that most of these originated in PS II. Furthermore, there were indications that TL and DLE are related to each other. These important observations were confirmed and further elaborated in the 1970s by P.V. Sane, V.G. Tatake and T.S. Desai using continuous illumination of the sample during freezing and by Yorinao Inoue and Kazuo Shibata using both flash and continuous illumination (see Inoue and Shibata, 1982; Sane and Rutherford, 1986). The flash excitation studies provided a greater insight into the mechanism of origin of PS II-related TL bands. These and subsequent studies during the 1980s by several research groups demonstrated the potential of the TL technique in probing PS II. In more recent years the bands that appear at somewhat higher temperatures have also been characterized. We now know that TL can be used not only to probe PS II but in addition it can provide information on several other physiological aspects. Several excellent reviews have appeared on these aspects (Inoue and Shibata, 1982; Sane and Rutherford, 1986; Demeter and Govindjee, 1989; Inoue, 1996; Vass and Govindjee, 1996). Vass (2003) has also provided an account of the history of photosynthetic TL that also includes photographs of the many researchers in the field of TL in photosynthetic systems. In this chapter we will emphasize those studies that have appeared since 1995 and were not covered by the previous reviews (cf., however, Ducruet and Vass, 2009; and Rappaport and Lavergne, 2009).

Earlier and subsequent studies (see previous reviews cited above for detailed discussion) from different laboratories have provided strong evidence to suggest that several recombination events emit light generating characteristic TL bands occurring at various temperatures during warming in darkness of the illuminated sample. Apart from the very low temperature peaks (the Z peaks) and those that appear at higher temperatures (above 50°C) all other bands are either sensitive to inhibitors such as 3-(3,4-dichlorophenyl)-l,l-dimethylurea (DCMU)

or to procedures that inhibit the water-oxidizing complex. Therefore, it is evident that these bands are all associated with recombination events occurring within PS II involving either of the two quinone (electron) acceptors (Q_A and Q_B) and one or the other S states of the water-oxidizing complex. The emission spectra of the TL bands are similar to the fluorescence spectra from chloroplasts (see e.g., Sonoike et al., 1991) and therefore the light emission must occur from the PS II antenna systems.

Light emission by plants and algae has provided a very valuable tool in our hands to probe the function of the light-associated reactions. Chlorophyll *a* fluorescence has been used for several decades to monitor the overall function of the photosynthetic apparatus, including that of PS II, quenching mechanisms and intersystem electron transport, as well as the effects of stress conditions (for reviews on chlorophyll *a* fluorescence see Papageorgiou and Govindjee, 2004). Thermoluminescence which provides information on the back reactions and recombinations of oxidized and reduced electron donors and acceptors of PS II has proven to be an excellent complement to the fluorescence studies. Although TL was discovered almost five decades ago its extensive use in probing PS II function has increased dramatically in the past three decades or so. With a better understanding of the mechanism and origin of different TL bands, its utility has been extended from merely probing the reactions occurring within PS II to understanding energy charge and stress related responses including lipid oxidation under in vivo conditions. It is emerging as a technique with some unique applications.

C. Instrumentation

Thermoluminescence measurements have usually been made by easily assembled, home-made instruments using a cold finger or sample holder that can be quickly cooled and heated at a predetermined rate of heating, a temperature sensor located in the sample holder for an accurate measurement of the sample temperature, and an end window type red sensitive photomultiplier equipped with high voltage power supply and signal amplifier. The apparatus is then coupled to a computer data acquisition and signal processing system that can display the signal from the photomultiplier and temperature sensor on Y and X, axes, respectively. To obtain the best signal, the photomultiplier is located as close as possible to the sample; the cooling of the photomultiplier tube reduces the noise further. In many instruments a strong lens is used to focus the light emitted from the sample onto the photomultiplier tube to improve the signal to noise ratio. The earlier versions of the set ups were somewhat cumbersome although quite sensitive with respect to the temperature of the sample (Tatake et al., 1971). With enormous improvements in electronics, compact PCs and very powerful software for signal processing, the present day TL instruments are very versatile and yet quite simple. Furthermore, if one is interested only in measuring PS II-related TL peaks, cooling of the sample to liquid nitrogen temperature is not needed and the cold finger can be even simpler. Our research group has been extensively using a simple set up described and built by Zeinalov and Maslenkova (1996) for routine measurements of glow curves of leaf and algal samples. It has a small heat capacity sample holder that can be very quickly cooled to −80°C and heated at a very fast rate of 36°C min^{-1}. Several samples per hour can be studied with such a set up. A versatile and efficient system for measurements of glow curves using flash excitation and luminescence decay has been described by Ducruet and Miranda (1992). Bhatnagar et al. (2002), Ducruet (2003) and Gilbert et al. (2004b) have described improved versions for specific uses. A flash lamp that can excite the sample with a single turnover saturating pulse is essential for flash induced TL studies. Such flash lamps are readily available commercially. The first commercially available computerized thermoluminescence system (Z700) has been introduced recently by Photon Systems Instruments (Brno, Czech Republic) and is distributed worldwide by Qubit Systems Inc. (Kingston, Ontario, Canada).

The use of programs such as THERMO Lite (Verion 1.5/2005) developed by Jean-Marc Ducruet (Ducruet and Miranda, 1992) permit deconvolution and resolution of the complex glow curves obtained. This is particularly important when glow curves are obtained from a sample that was illuminated continuously during freezing. The flash induced glow curves are usually simple and contain a single major peak. Numerical analysis of glow curves has been described by

Ducruet and Miranda (1992). Care should be taken during deconvolution of glow curves (Sane, 2004) so that one does not generate false peaks through deconvolution.

Samples such as leaf discs are directly placed on the sample holder while a suspension of algal cells, chloroplast or thylakoid membranes is usually spotted on a filter disc. Some laboratories use a plastic holder to place the leaf or filter disc to prevent damage from the plant/algal material to the cold finger. In all the cases one needs to know the difference in the actual temperature of the sample and the one indicated by the set up. Necessary corrections need to be applied to ensure that the corrected peak temperatures are reported. This is essential particularly when biophysical constants are to be calculated from the obtained data. The excitation of the sample either by continuous light during freezing of the sample or by flash excitation with a single flash, series of flashes or a combination of continuous light followed by flashes at specific temperature is done on the stage of the sample holder at a predetermined temperature prior to quickly cooling the sample. (For a review, see Ducruet and Vass, 2009)

II. Glow Curves and their Characteristics

A. Nomenclature

In their initial studies, different research groups had used different nomenclatures for the TL bands observed. This led to some confusion, but at present there is unanimity regarding the nomenclature of different TL bands. We will use the existing nomenclature in describing the TL bands (Inoue, 1996).

B. Description of Different Thermoluminescence Bands

There are over 13 TL bands that have been detected and described under different conditions. Of these, eight that appear between −50°C and +50°C are most important as these originate within PS II. A set of three bands that appear at higher temperatures are associated with lipid oxidation and provide an indication of the oxidative damage to the thylakoid membrane in response to various stress conditions. A list of these bands is given in Table 19.1 along with their respective DLE components.

1. Low Temperature Bands

The low temperature bands that appear below 77 K and the one appearing at 110–120 K have been designated as Z bands. Those that appear at 20, 50 and 70 K have been studied and described by Noguchi et al. (1993) by exciting photosynthetic pigment protein complexes and purified pigments at liquid helium temperatures and are called Z-α, Z-β and Z-γ, respectively. They do not originate in charge separation or electron transport reactions but are associated with the charge storage in chlorophyll aggregates and their interactions with their ligands. These bands therefore

Table 19.1. Thermoluminescence peak temperatures (T_M), charge pairs responsible for their emission, and related DLE components

Peak	Approximate peak position T_M (°C)	Charge pairs	Related DLE component
Very low temperature peaks	⁻250, ⁻220, ⁻200	Aggregated chlorophylls	
Z peak	⁻160	Chl^+Chl^-	
High temperature bands	+50 to +160	Oxidative chemiluminescence	
Photosystem II related bands			
Zv (variable)	−80 to −30	$P680^+Q_A^-$	Approx. 150 μs
A band	−10	$S_3Q_A^-$	4 ms
A_T band	−10	$His^+Q_A^-$	4 ms?
Q band	+5	$S_2Q_A^-$	2 s
B1 band	+20	$S_3Q_B^-$	30 s
B2 band	+30	$S_2Q_B^-$	60 s
C band	+50	$TyrD^+Q_A^-$	10 min
A_G band	+ 40 to 50	$S_2/S_3Q_B^-$ (see text)	

are of little importance for understanding the photochemistry of PS II.

Of the low temperature TL bands, the Z band that appears at −160°C has been well studied (Arnold and Azzi, 1968; Shuvalov and Litvin, 1969; Sane et al., 1974; Sonoike et al., 1991). Under continuous illumination at liquid nitrogen temperature this band is more efficiently excited by blue than by red light. It emits with a peak around 730–740 nm and was suggested to arise in chlorophyll triplets (Sane et al., 1974), or possibly in charge pairs between two Chl molecules (Sonoike et al., 1991). This band can also be excited by gamma rays. The band arises in isolated chlorophylls and is not important in understanding photosynthetic electron transport.

2. Bands Related to Photosystem II

There are at least eight bands that seem to arise within PS II. The reducing equivalents for all these bands are contributed by the two quinone acceptors of PS II, namely Q_A and Q_B. The oxidizing species involved contain the two charged S states, namely the S_2 and S_3 states, and the three electron donors closer to the reaction center II. These eight bands have proved very useful in understanding the function of the PS II complex.

a. The Variable Z_v Band

This band is called Z_v as it occurs at a variable temperature depending upon the temperature of excitation. Ichikawa et al. (1975) showed that this band appears at a temperature that is about 10–20°C higher than the temperature of its excitation. It appears in a sample preparation that has no active water-oxidizing complex (Vass et al., 1989) indicating that S states are not involved in its origin. The oxidizing equivalent for its origin is suggested to be $P680^+$. Studies by Chapman et al. (1991) using reconstitution experiments showed that the reducing entity involved is the reduced primary quinone electron acceptor Q_A^-. It appears that the charge pair responsible for its origin is $P680^+Q_A^-$. However, it is not clear why its temperature of emission is variable. Desai et al. (1977) proposed that the band may arise from charging of both the Z band and the A band and this could explain its dependence on the temperature of excitation. Klevanik (1995), using isolated PS II reaction center preparations, suggested that the charge pair responsible is oxidized tyrosine Tyr^+ and a reduced plastoquinone.

b. The A Band

A band is known to appear between −10°C and −40°C when continuous illumination is used during freezing of the sample (see Sane and Rutherford, 1986). The recombining charge species appears to reside in PS II as the band is excited by red but not far-red light (absorbed in PS I only). It appears in a PS II fraction but not in a PS I fraction and is absent in mutants lacking PS II (Arnold and Azzi, 1968; Shuvalov and Litvin, 1969; Lurie and Bertsch, 1974; Desai et al., 1975; Ichikawa et al., 1975; Sane et al., 1977). Based on its sensitivity to DCMU, Sane et al. (1977) suggested that the A band either may arise in a recombination of charges involving an acceptor beyond Q_B with the S_3 state or it may arise in the Q_A^- and S_3 state.

Demeter et al. (1985a) assigned this A band to the charge recombination of Q_A^- with the oxidized donor Z^+ based on its insensitivity to Tris-washing and NH_2OH treatment but it was present in DCMU-treated thylakoids that were exposed to two pre-flashes before the addition of DCMU and subsequent illumination at low temperature. The involvement of the S_3 state was explained by Koike et al. (1986). They used flash excitation at 15°C to create different S states followed by the addition of DCMU. The sample was then cooled to liquid nitrogen and exposed to continuous illumination. The excitation at liquid nitrogen in the presence of DCMU reduced Q_A by oxidizing Cyt b_{559} without changing the previously created S states and without reducing Q_B. The results and oscillations obtained for this band using the above approach suggested that this band arises in $S_3Q_A^-$.

c. The A_T Band

Another band that is insensitive to inhibitors of the water-oxidizing complex also appears at around −10°C (Inoue et al., 1977; Demeter et al., 1979; Sane et al., 1983a). It is different from

the A band since the S states do not participate in its production. However, it does arise in PS II fractions. Using chemical modification of histidine in Mn–depleted PS II preparations, Ono and Inoue (1991) suggested that the band arises in samples with oxidized histidine and Q_A^-. Using PS II particles from site-directed mutants of *Chlamydomonas reinhardtii* which involved His195 and His190 of the PS II D1 polypeptide, Kramer et al. (1994) demonstrated that modification of His190 to phenylalanine completely abolished the A_T band. Modification of His195 did change the intensity of the peak but did not shift the peak temperature. These data established that the positive charge was donated by His190.

d. The Q Band

The Q band appearing around 5°C has been very well studied and is related to the recombination of $S_2Q_{A.}^-$. The intensification of this band by DCMU addition (Rubin and Venediktov, 1969; Lurie and Bertsch, 1974; Desai et al., 1975; Ichikawa et al., 1975) suggested that the reducing equivalents may originate in Q_A^-. Desai et al. (1975) demonstrated that the decrease in fluorescence yield of the sample during warming was associated with the appearance of this band suggesting that the oxidation of Q_A^- was responsible for its production. This was probably the first direct evidence that related the oxidation of Q_A^- with the appearance of a TL band. The requirement of a functional water-oxidizing complex and hence S states was evident from the observations that the band was sensitive to mild heating (Sane et al., 1977) and that it was lost upon treatment of the thylakoids by Tris-washing or tetranitromethane that destroy the S states (Sane et al., 1983b). The involvement of S_2 was most clearly demonstrated by Rutherford et al. (1982) who showed that this peak appears in DCMU-treated thylakoids upon excitation by a single flash at −15°C. In the presence of some of the herbicides there is a shift in the temperature (T_M) of its peak. Droppa et al. (1981) observed that depending upon the type of herbicide used the Q band appeared at three different temperatures namely +5°C, 0°C or –14°C. They attributed this variation to the various redox states of the quinone acceptors due to the binding of the herbicides.

e. The B Bands

The most investigated bands that have provided valuable information on the function of PS II are the B bands arising in $S_2/S_3Q_B^-$. These bands are lost in the presence of inhibitors such as DCMU that block electron transport between the primary (Q_A) and secondary (Q_B) quinone acceptors (Arnold and Azzi, 1968; Rubin and Venediktov, 1969; Desai et al., 1975; Ichikawa et al., 1975). The band appearing around 30°C is also lost if the water-oxidizing complex is either destroyed or absent (Inoue, 1976; Rosza and Demeter, 1982; Sane et al., 1983a). Furthermore, this band was shown to oscillate with a periodicity of four (Inoue and Shibata, 1977a, b). These observations were clear indications that the B bands arise in a recombination reaction between the oxidized S states and Q_B^-. The involvement of Q_B^- has been confirmed by several workers (see reviews by Sane and Rutherford 1986; Inoue, 1996; and Vass and Govindjee, 1996; Rappaport and Lavergne, 2009). Although the bands appearing around 30°C were earlier assigned to $S_2/S_3Q_B^-$ it became clear that there are two bands, one arising in $S_2Q_B^-$ and another in $S_3Q_B^-$. Inoue (1981) showed that at pH values lower than 6 the two bands appear as distinct TL glow peaks at 20°C and 35°C respectively, while at higher pH values the bands appear as one peak at 35°C. The $S_3Q_B^-$ peak has been designated as the B1 band while the $S_2Q_B^-$ peak has been designated B2. The B1 band appears at lower temperatures (8–10°C lower than the $S_2Q_B^-$ band). If a dark-relaxed leaf is excited by one short saturating (single turnover) flash, a major band related to $S_2Q_B^-$ is seen, while with two flashes a composite B band ($S_2Q_B^- + S_3Q_B^-$) is seen. The emission intensity of the $S_3Q_B^-$ recombination has been shown to be 1.7–2 times higher than that arising from $S_2Q_B^-$ for reasons still not well understood (Demeter et al., 1985a, b; Rutherford et al., 1985). Since these two bands involve the participation of the S_2 and S_3 states and the two-electron acceptor quinone (Q_B), they provide information on important changes in the PS II reaction centers. Thus, numerous studies on photoinhibition and stress responses have monitored the changes in the intensities and shifts in the T_M or peak emission temperatures of these two bands to understand and probe the effects on PS II and electron transport

between the two photosystems. These aspects will be discussed later.

Although the usual temperature activating recombination of the $S_2Q_B^-$ state is around 30°C, the characteristic T_M of this band is highly variable and depends upon the growth conditions, species, and abiotic or biotic stress (see: Tables 19.2 and 19.3). For example, in *Synechosystis* sp. PCC 6803 grown at 30°C the $S_2Q_B^-$ band was reported at 36°C (Minagawa et al., 1999), while for the same strain also grown at 30°C but in another laboratory the $S_2Q_B^-$ signal appeared at 24–26°C (Shen et al., 1998). These variations are due to the use of different equipment, changes in growth conditions other than temperature and different heating rates. Similar variations can be seen in other bands also. Govindjee et al. (1985) have shown that in thermophilic cyanobacteria the Q and B bands appear at much higher temperatures of 35°C and 50–55°C, respectively. Therefore, the T_M of the peak alone does not reflect the charge pair responsible for its emission. One must provide additional evidence to ascertain the charge pair responsible for the band under consideration.

f. The C Band

In spinach leaves and cells from a species belonging to the genus *Euglena*, Desai et al. (1975) observed a prominent band appearing around 45–50°C that could be excited by far-red light and enhanced by DCMU (Ichikawa et al., 1975). The band was suggested to arise in PS I in view of its excitation by far-red light, its enrichment in a PS I fraction and its insensitivity to tetranitromethane and Tris treatment (Desai et al., 1975; Sane et al., 1977; Inoue et al., 1977) that destroy the water-oxidizing complex and hence no S-states are detected.

While studying the flash excitation of this band appearing at 50°C in maize inside-out thylakoid membranes, Demeter et al. (1984) suggested that the C band may arise in a recombination between $S_0Q_A^-$ or $S_1Q_A^-$. The involvement of Q_A^- was inferred because of the enhancement of the band in the presence of DCMU. Neither the S_0 nor the S_1 states are known to carry a positive charge and hence they cannot be involved in a recombination reaction. In a subsequent study, Demeter et al. (1993) compared the TL and Electron Paramagnetic Resonance (EPR) measurements in DCMU-treated PS II particles. They concluded that the C band seemed to arise in a recombination of the g=1.82 form of $Q_A^-Fe^{2+}$ and the oxidized tyrosine Y_D^+ (of D_2) responsible for the EPR signal II. Krieger et al. (1993) showed that low pH treatment of PS II particles caused a decrease in the $S_2Q_A^-$ signal with a concomitant increase in the intensity of the C band. The interconversion of the $S_2Q_A^-$ band and C band was more pronounced in the presence of the Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA). The $S_2Q_A^-$ band was restored upon the addition of Ca^{2+} with a decrease in the C band. Based on redox titration of the fluorescence yield they suggested that the C band was due to the low pH-induced high midpoint redox potential form of Q_A. The origin of the C band was further investigated by Johnson et al. (1994) using thylakoid membranes. The decay of the C band, enhanced by DCMU and illumination at 77 K, paralleled the disappearance of the EPR signal attributable to Q_A^- and the decay of the free radical Y_D^+. It therefore appears that the C band arises in a recombination of the $Q_A^-Y_D^+$ pair. These workers also investigated low pH Ca^{2+}-depleted PS II preparations but did not find a change in the redox potential of Q_A. Ducruet (1999) suggested that a back transfer of an electron from the secondary quinone acceptor (i.e., Q_B^-) to the primary acceptor Q_A of PS II was responsible for the luminescence-emitting recombination with Y_D^+. This band has been suggested to arise in PS II Q_B – non-reducing centers by Andree et al. (1998).

g. The A_G Band

A detailed investigation on the far-red light-induced band arising around 40–50°C (Desai et al., 1983) was made by Miranda and Ducruet (1995b). Several features of this band have been brought out by their studies. These authors suggested that although the band arises in $S_2/S_3Q_B^-$ recombination it is distinct from the B1/B2 bands since the Q_B^- is created as a result of a back transfer of an electron from the PQ pool in the dark during warming. The stable S_2/S_3 states created as a result of far-red illumination participate in the recombination reaction. Miranda and Ducruet (1995b) argued that far-red excitation oxidizes the PQ pool and randomizes the S_2/S_3 states. This produces stable S_2 and S_3 in the dark. The absence of Q_B^- resulting from oxidation of Q_B^- by far-red

light does not allow S_2/S_3 Q_A^-/Q_B^- recombination thus preventing deactivation of S_2/S_3 states. Far-red excitation could also result in cyclic electron flow around PS I and ATP production that may contribute to the back transfer of electrons from the PQ pool to Q_B, thus generating Q_B^- in the dark. This Q_B^- recombines with the stable S_2/S_3 states to yield an A_G (afterglow) band. This band is the expression of the far-red light-induced after glow and shares common properties with it. Since cyclic ATP synthesis participates in the emission of this band, the band is indicative of the metabolic state of the leaf/sample.

A band appearing at 46°C in a *Mesembryanthemum crystallinum* exhibiting facultative crassulacean acid metabolism (CAM) was induced by salt (Krieger et al., 1998). This band could be excited by a single turnover flash, oscillated with a periodicity of four and its intensity was related to the changes in the ratio of dihydroxyacetone phosphate to phosphoglyceric acid – an indicator of the energy status of the chloroplasts. Krieger et al. (1998) suggested that the band arose in the oxidized S_2/S_3Q_B condition in which Q_B becomes reduced by a reversed electron flow or by electron flow from NAD(P)H/PQ oxidoreductase. Soon thereafter, Janda et al. (1999) suggested that the far-red excited band peaking at 45°C is an indicator of abiotic stress in plants. Roman and Ducruet (2000) used this band as an indicator of the NADPH+ATP energetic potential in plants. Ducruet et al. (2005) have used the A_G band to monitor the cyclic electron flow around PS I. These latest studies indicated the presence of a 45°C peak designated as the A_G band in the stroma lamellae fraction. Its excitation by far-red light, and its association with cyclic electron flow around PS I, seem to confirm the earlier observations (Sane et al., 1977), but they equally demonstrate that it does not originate in PS I, although ATP generated by PS I cyclic electron transport may contribute to its appearance. We point out that the C band and A_G band are two different bands that could occur in the same temperature range but are distinguishable by their different characteristics.

3. High Temperature Bands

Arnold and Sherwood (1957) in their earlier experiments had shown light emission peaking at 125°C in irradiated dried thylakoids. However, its origin was not discussed. A TL band that appears at a temperature of about 73°C was described by Desai et al. (1982b). On the basis of its excitation and emission characteristics the origin of this band was assigned to chemiluminescence from chlorophyll upon the destruction of the membrane. The band was not related to functional electron transport. Kafarov et al. (1988) demonstrated that the intensity of this high temperature peak is correlated with the concentration of malondialdehyde (MDA), a product of lipid peroxidation indicating that this band is related to lipid peroxidation. Bohne et al. (1986) showed that chlorophylls in chloroplasts or micelles can efficiently detect electronically excited species generated in enzyme reactions through red emission.

In a study of photoinhibition in natural phytoplankton of the Black Sea, Matorin et al. (1992) observed peroxidation of the thylakoid membrane lipids associated with the appearance of a high temperature chlorophyll TL; the authors suggested that this high temperature TL band could be used to assess the extent of irreversible cell damage during photoinhibition. Further elucidation of this high temperature TL peak has come from the work of Hideg and Vass (1993) who suggested that this band arises in temperature enhanced interaction of molecular oxygen and the thylakoid membrane probably involving lipid peroxidation.

Solntsev (1995) demonstrated that the band appearing at 50–70°C is not affected by the spectral composition of irradiation. The variation in the T_M of this peak reported by different research groups has been explained by Stallaert et al. (1995) who showed that the T_M depends upon the rate of heating during TL measurement; this band showed a temperature maximum of 90°C if the rate of heating was 30°C min^{-1}, while the T_M appeared at 70°C if the rate of heating was 3°C min^{-1} The fungal elicitor cryptogein that induces lipid peroxidation also results in the increased high temperature TL band (Stallaert et al., 1995). Further elucidation and characterization of this band was done by Vavilin and Ducruet (1998) who studied this high temperature TL band in samples that were allowed to dry. Existence of three bands appearing at 62–75°C, 114–128°C and 151–158°C was observed. Treatments causing oxidative damage to membrane lipids produced a

small 62–75°C band but a significant rise in the 114–128°C peaks. Since this band did not change upon treatment with quenchers of active oxygen species or scavengers of free radicals or replacement of oxygen by argon, Vavilin and Ducruet (1998) proposed that the 114–128°C band arises as a result of thermal decomposition of lipid cyclic peroxides. In contrast, the 151–158°C bands were not related to thermolysis of lipid peroxidation products. The stress-induced appearance of this band has been also confirmed (Vavilin et al., 1998; Marder et al., 1998; Skotnica et al., 1999). Havaux (2003) has reviewed the studies on the use of high temperature TL for quantifying oxidative stress in plants. He has suggested that the TL bands appearing in the range of 80–150°C could be used for detecting and quantifying both lipid peroxidative damage and oxidative stress in plants.

III. Mechanism and Parameters of Light Emission

A. Mechanism

The emission spectra of the TL bands are similar to the fluorescence spectra from chloroplasts and therefore the light emission must occur from the PS II antenna system, as it does for fluorescence (Sonoike et al., 1991; Papageorgiou and Govindjee, 2004). When the light is absorbed by the chlorophylls of PS II, charge separation in the reaction center produces the excited singlet state ^{1}P680*. The electron from this excited singlet is transferred to pheophytin (Pheo) generating the charge separated state $P680^+Pheo^-$. The energy gap between $P680^+$ and $Pheo^-$ is about 1.8 V. The separated charge pair is stabilized by the production of an oxidized donor D^+ and a reduced acceptor A^-. The recombination of the oxidized donor and reduced acceptor is prevented because part of the energy absorbed is lost during the charge stabilization process. In order to produce delayed fluorescence or TL, it is necessary that the singlet excited P680* is regenerated. This requires generation of the singlet radical pair $^1(P680^+Pheo^-)$. The reducing equivalents required for generating the radical pair seem to arise from the reductants (Q_A^- and/or Q_B^-) and the oxidizing equivalents are apparently contributed by the S states. Since only the S_2 and S_3 states possess positive charges (because of the 1:0:1:2 proton release pattern during sequential oxidation of the Mn complex) they are the only logical donors of positive charges (see Fig. 19.1).

The recombination of the positive charges on the electron donors and the negative charges on the electron acceptors can occur through a series of back reactions culminating in the production of the $P680^+Pheo^-$ radical pair. A schematic representation of generation of the positively and negatively charged pairs as a result of photosynthetic electron transport in PS II leading to TL emission upon recombination is depicted in Fig. 19.1. In fact, many studies have already identified the reducing and the oxidizing species that participate in the recombination reaction leading to TL (see e.g., Vass and Govindjee, 1996; Inoue, 1996). In some cases besides the S states, other oxidized donors such as His^+ or Y_D^+ could also participate in the recombination reaction. These two donors are involved in charge recombination when the water-oxidizing complex is unable to reduce these donors for one reason or the other.

A modified version of an earlier energetic scheme of photosynthetic TL emission arising from charge recombinations in PS II (Vass and Govindjee, 1996; Inoue, 1996) is presented in Fig. 19.2. (For a review of PS II, see Govindjee et al., 2010.) Light absorption by the photosynthetic pigments results in charge separation produced upon charge stabilization, reduced acceptors Q_A^-/Q_B^- and the oxidized donors S_2 and S_3 (Fig. 19.1). The primary electron donor P680 accepts excitation energy and produces charge separation via the first singlet excited state (for a discussion of excitation energy transfer, see Clegg et al., 2010). Excitation of P680 results in the formation of the radical pair $P680^+Pheo^-$ (P^+Pheo^-). This is followed by the electron transfer from $Pheo^-$ to the first stable electron acceptor A (Q_A). The donors (D) and acceptors (A) of PS II generate stabilized charge pairs in generic terms as normal ($[D^+A^-]n$), shallower ($[D^+A^-]s$) and or deeper ($[D^+A^-]d$) traps, respectively and the TL peak position is determined by the free activation energy (ΔG^*) for the respective radiative charge pair. Various modifications of the molecular and electrostatic environment of the charge trapping species in both the donor (denoted as the water-oxidizing complex – WOC in Fig. 19.2) and acceptor (primary and secondary quinone acceptors – Q_A and Q_B) side of PS II can induce deeper or shallower stabilization of the charge pair accompanied by

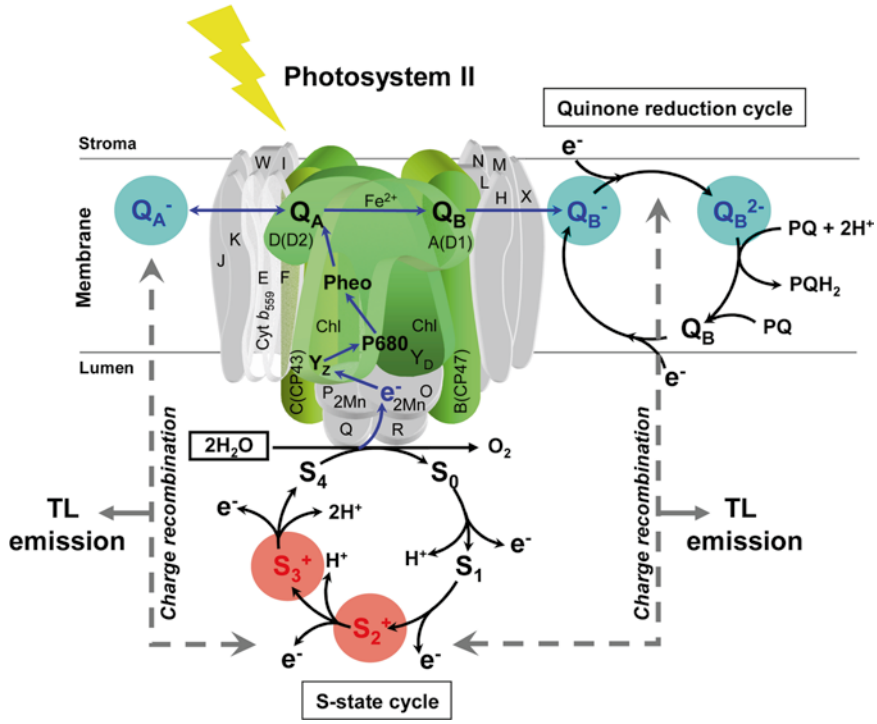

Fig. 19.1. Diagram of photosystem II (PS II) representing the polypeptide composition, electron transport carriers and light-driven photosynthetic electron transport reactions (*blue solid arrows*) from Mn to the secondary quinone electron acceptor (Q_B) in PS II resulting in water oxidation by the Mn cluster at the donor side of PS II. Electron transport in PS II generates light-dependent turnover of positive charges via the S-state cycle (Kok's cycle) by withdrawing e^- and H^+ from H_2O. The electron transport also generates positively charged $P680^+$, Y_Z^+ and Y_D^+, thus reducing the primary quinone electron acceptor (Q_A) to Q_A^-. Consequently, Q_A^- donates an electron to Q_B and the negative charges at the acceptor side of PS II are generated by the quinone reduction cycle forming semiquinol or quinol molecules. Charge recombinations (*grey dashed lines*) between different combinations of negatively and positively charged species result in thermoluminescence (TL) emission which is characteristic for each redox pair.

higher or lower ΔG* resulting in up-shifted or down-shifted TL emission peaks, respectively.

The energy barrier can be overcome by a supply of thermal energy enabling the charge pairs to recombine generating the $P680^+Pheo^-$ singlet radical pair and finally P680*. When P680* returns to the ground state, it can emit light as TL (the probability of which is very low due to its low abundance), but most of the energy is transferred to the more abundant antenna chlorophyll molecules that emit light as TL. Since there are four distinct charge pairs ($S_2Q_A^-$, $S_3Q_A^-$, $S_2Q_B^-$ and $S_3Q_B^-$) generated within PS II, the detection of four TL bands appearing at characteristic peak temperatures (T_M) is to be expected. Furthermore, the T_M of each of these peaks strongly depends on the redox potential difference between the recombining charge species. Therefore, if there is a change of the redox potential in any of the participating components this will be reflected in a shift in the T_M of the peak generated by the recombining charge pair. Thus, the same recombining species can have an up-shifted or a down-shifted peak. Extending this explanation further we can argue that a back flow of electrons from another component to the oxidized acceptor, e.g., Q_A/Q_B could give rise to a reduced acceptor that was not present earlier and if the oxidized donor such as S_2 or S_3 was present this will permit recombination of the charge pairs. However, in this case the energy required for overcoming the energy barrier will depend upon the energy needed for the back transfer of the electron from a component such as reduced plastoquinone. The resultant peak in this case will have a different T_M although it is still generated by the same charge pair.

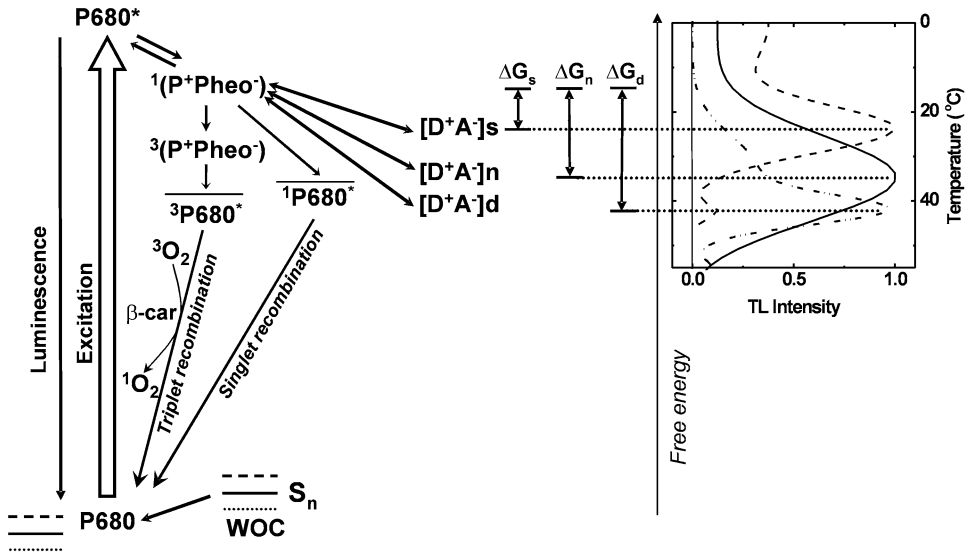

Fig. 19.2. Energetic scheme of photosynthetic TL emission arising from charge recombinations in PS II. The primary electron donor P680 accepts light excitation energy and the charge separation via the first singlet excited state results in the radical pair $P680^+Pheo^-$ (P^+Pheo^-) followed by the electron transfer from $Pheo^-$ to the first stable electron acceptor A (Q_A). *D* and *A* represent the donors and acceptors of PS II and $[D^+A^-]n$, $[D^+A^-]s$ and $[D^+A^-]d$ indicate the stabilized charge pairs with normal (*n*) shallower (*s*) and deeper (*d*) traps, respectively. The free activation energy for the radiative charge pair (ΔG*) determines the *TL* peak position. Various modifications of the molecular and electrostatic environment of the charge trapping species in both donor (water-oxidizing complex - WOC) and acceptor (primary and secondary quinone acceptors – Q_A and Q_B) side of PS II can induce deeper or shallower stabilization of the charge pair accompanied by higher or lower ΔG* resulting in up-shifted or down-shifted TL emission peaks, respectively. In some cases, back electron transfer from Q_A^- to Pheo may result in a singlet $^1(P680^+Pheo^-)$, or by spin de-phasing a triplet $^3(P680^+Pheo^-)$, radical pair. Non-radiative charge recombination pathways via singlet recombination $[^1(P680^+Pheo^-)]$ to the ground state P680 or the first singlet excited state $^1P680^*$ and via triplet recombination $[^3(P680^+Pheo^-)]$ resulting in the triplet excited state $^3P680^*$, which may be quenched by β-carotene via singlet oxygen, are shown.

The reductants produced during electron transport are the reduced forms of the two plastoquinone electron acceptors of PS II. The Q_A species is a single electron acceptor and is reduced by $Pheo^-$. It immediately transfers this electron to the two electron acceptor Q_B to first produce Q_B^-. If Q_B^- was already there then the transfer of electron to Q_B^- from Q_A^- produces Q_B^{2-}, which is immediately protonated. The plastoquinol (Q_BH_2) thus formed, leaves the PQ-binding site and is replaced by another plastoquinone molecule from the PQ pool. Thus, when the PQ pool is in an oxidized state, Q_A^- is not expected to accumulate and hence it cannot contribute to the reducing equivalents for a recombination reaction. However, if for some reason the electron transport from Q_A to Q_B is restricted, then Q_A^- would accumulate and provide reducing equivalents for TL. Another situation that could result in substantial accumulation of Q_A^- is if the PQ pool is fully reduced and Q_B^- cannot be oxidized. In this situation Q_A^- accumulation could also take place. Such a situation does occur in nature when the PQ pool is kept reduced by chlororespiration under certain environmental conditions.

Under normal conditions the redox potential difference between Q_A and Q_B is such that the equilibrium constant is vastly in favour of Q_A^- oxidation. However, if the redox potential of Q_A or Q_B changes such that the redox potential difference between the two is considerably reduced then a large proportion of Q_A could remain in a reduced condition under a steady state situation. There are several examples where the redox potential difference between these two plastoquinone acceptors is reduced and the peaks arising from recombination involving Q_A^- or Q_B^- appear at almost the same temperature. In a later section these aspects are discussed in more detail with specific examples.

B. Oscillations of the B Band(s)

Most TL studies are currently being carried out using flash excitation of dark-adapted samples. As noted above, the positively charged oxidizing equivalents arise primarily in S_2 and S_3 states of the water-oxidizing complex. In dark-adapted samples, only S_1 and S_0 are present and these advance to higher oxidized states under flash illumination. Dark-adapted samples contain about 25% S_0 and 75% S_1 and the distribution of Q_B and Q_B^- has been shown to be 50 : 50 (see, e.g., the review by Inoue, 1996). In general, the Q_A reduced by flash excitation will immediately reduce Q_B within ~200 μs. Assuming the above distribution of the $Q_B : Q_B^-$ and $S_0 : S_1$ in a dark-adapted sample, we can predict the intensity of the TL bands as a function of flash numbers. The distribution of different charge pairs (S-states and Q_B) and the predicted TL emission following consecutive flash excitation is depicted in Fig. 19.3. In a dark-adapted sample the proportion of the charge pairs is expected to be as follows (Fig. 19.3a):

S_0Q_B	:	$S_0Q_B^-$	:	S_1Q_B	:	$S_1Q_B^-$
1	:	1	:	3	:	3

None of the S-states in this situation carry a positive charge and hence there is no possibility of any TL emission although 50% of Q_B is in a reduced state. The actual glow curve shows that there is a general rise above 40°C that, in fact, yields a TL peak unrelated to PS II (Fig. 19.3a). However, there is no TL arising in PS II.

Excitation of the sample by a single saturating turnover flash will advance S_0 to S_1 and S_1 to S_2, respectively and simultaneously convert Q_B to Q_B^- and Q_B^- to Q_B^{2-}. The deposition of the second charge on Q_B^- will cause protonation of the Q_B^{2-} and it will be replaced by a new plastoquinone molecule Q_B. Under these conditions the expected combination of S-states and Q_B is (Fig. 19.3b):

$S_1Q_B^-$	:	S_1Q_B	:	$S_2Q_B^-$	:	S_2Q_B
1	:	1	:	3	:	3

Only $S_2Q_B^-$ will produce TL in proportion to its presence. The accompanying TL trace in Fig. 19.3b shows the expected $S_2Q_B^-$ peak appearing at 40°C in Scots pine (A.G. Ivanov, P.V. Sane, N.P.A. Hüner and G. Őquist, unpublished results).

If the dark-adapted sample was excited by two successive flashes the second flash will convert S_1 to S_2, S_2 to S_3 and Q_B^- to Q_B and Q_B to Q_B^- and the distribution of the S-states and Q_B would be as follows (Fig. 19.3c):

S_2Q_B	:	$S_2Q_B^-$	:	S_3Q_B	:	$S_3Q_B^-$
1	:	1	:	3	:	3

Such a distribution of the S-states and Q_B will result in producing 3 arbitrary units of TL intensity from $S_3Q_B^-$ plus one arbitrary unit of TL intensity from $S_2Q_B^-$ (Fig. 19.3c). If one presumes that $S_3Q_B^-$ is 1.8 times more luminescent than $S_2Q_B^-$ (Rutherford et al., 1985), the expected total luminescence (TL intensity) will be about 6.4 units. It should be noted that the major TL in this case will be emitted due to $S_3Q_B^-$ charge recombination. The characteristic peak (T_M) of $S_3Q_B^-$ appears at lower temperatures than $S_2Q_B^-$ and in fact, the TL peak appeared at 35°C after two flashes in the experiment shown (Fig. 19.3c). Apparently, there was some TL emission peaking around 0°C arising from $S_2Q_A^-$ indicating that not all of the Q_A^- produced was oxidized by Q_B. Furthermore, the total luminescence was not twice that produced after one flash, indicating that theoretically predicted values are not always seen in the experiment. However, the trends are as predicted.

If the relaxed sample was excited by three successive flashes the distribution of the S states and Q_B would be as follows (Fig. 19.3d):

$S_3Q_B^-$	:	S_3Q_B	:	$S_0Q_B^-$	:	S_0Q_B
1	:	1	:	3	:	3

Only the $S_3Q_B^-$ will produce TL and its intensity will be about 1.8 TL units (Rutherford et al., 1985). The accompanying TL trace (Fig. 19.3d) shows a smaller peak appearing at about 35°C characteristic of $S_3Q_B^-$ recombination, e.g., in Scots pine. Thus, the theoretical prediction seems to match the experimental observations. The flash-induced predicted changes in the charge pair generation do not make allowance for the retention of some charge on Q_A. However, as seen in Fig. 19.3 the peak representing $S_2Q_A^-$ is seen around 0°C and this happens because the samples were illuminated at a temperature that slows down the movement of charge from Q_A^- to Q_B.

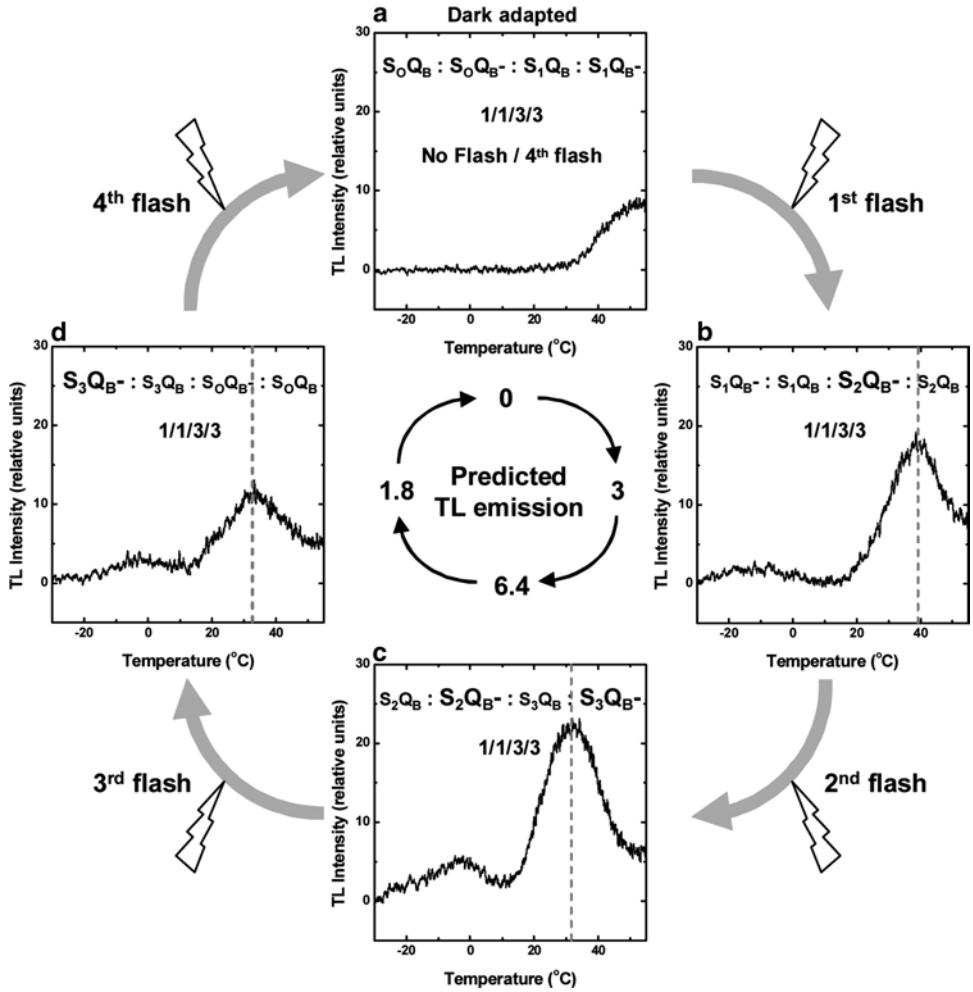

Fig. 19.3. Predicted distribution of S states and TL emission intensity after excitation with successive single turnover saturating flashes of white light. (**a**) Considering that TL emission is generated only in $S_2Q_B^-$ and $S_3Q_B^-$ recombinations in dark-adapted photosynthetic membranes (cells, leaf tissue, chloroplasts) TL is not observed. Under such a dark-adapted condition the only S states observed are S_0 and S_1. (**b**) After one single saturating flash the distribution of S states will change and the appearance of three $S_2Q_B^-$ charge recombinations will result in TL emission of three relative units. (**c**) If the dark relaxed sample is exposed to two successive single saturating flashes the distribution of S states will change again and the four luminescent charge pairs (one $S_2Q_B^-$ and three $S_3Q_B^-$) will increase the TL emission to 6.4 relative units. (**d**) After three successive saturating flashes only one luminescent charge pair is produced ($S_3Q_B^-$) and the relative TL emission drops to 1.8 relative units. After four flashes the distribution of S states will be the same as existed in the dark-adapted sample and no TL emission will be produced (TL glow curve not shown). The representative TL glow curves were recorded in intact Scotch pine (*Pinus sylvestris* L) needles. Note that when the emission is primarily from $S_3Q_B^-$ the T_M is lower by 8–10°C compared to when it is primarily from $S_2Q_B^-$.

The excitation of the sample by four successive flashes should result in the production and distribution of charge pairs identical to those in the dark-adapted sample with zero TL emission. However, numerous experimental studies of S_2Q_B oscillations have demonstrated that TL emission is rarely zero after four successive flashes and this happens because of double hits, misses and other reasons such as heterogeneity in PS II centers, as well as recombination and decay of some of the charge pairs. The predictions suggest that $S_2Q_B^-$ recombination alone could be observed only after one flash, while application of two consecutive flashes will result in a composite peak consisting primarily of $S_3Q_B^-$ TL emission. The assumption that in the dark-adapted samples $Q_B : Q_B^-$ will be present in 50:50 proportion may also not be always true (see Inoue, 1996). However, even if

the proportion of $Q_B : Q_B^-$ in the dark is shifted to 75:25, one flash will still produce TL primarily from $S_2Q_B^-$. Thus, irrespective of the dark-adapted state the TL emission following the first flash will always be due to $S_2Q_B^-$.

Explanations for flash-induced TL bands that are induced by illumination at different temperatures (even after freezing the samples to liquid nitrogen temperatures) have been offered.

For example, if the reduced PQ pool reduces Q_B in S_2Q_B or S_3Q_B they would contribute to TL. Krieger et al. (1998) have used such considerations to explain the occurrence of a 46°C band in CAM plants, suggesting that it arises in S_2/S_3Q_B upon reduction of Q_B in the dark through reversed electron flow from PQ or the reduction of PQ pool by NAD(P)H/PQ oxidoreductase.

After flash excitation at low temperatures (77 K) the following event takes place: Cyt b_{559} is oxidized and Q_A is reduced, and subsequently at an appropriate temperature Q_B is reduced forming $S_2Q_B^-$ and $S_3Q_B^-$ leading to TL (Rutherford et al., 1984). If $S_2Q_B^-$ or $S_3Q_B^-$ were generated after the flash excitation, the illumination at 77 K would make them non-luminescent by introducing one more electron on Q_B to produce Q_B^{2-} that would get protonated and replaced by oxidized Q_B. We know that low temperature illumination does not change the distribution of the S states but merely changes the redox state of Q_B. Thus, shuffling the charge pairs can be manipulated by changing the illumination regime to which the sample is subjected (Rutherford et al., 1984).

If the electron transport in the sample has been blocked by DCMU (or a similar compound) then the charge by illumination at low temperature will be located on Q_A rather than Q_B. With such manipulations it is possible to observe oscillations even of $S_2/S_3Q_A^-$ (Koike et al., 1986).

C. Biophysical Parameters

The phenomenon of TL occurring in photosynthetic membranes is somewhat similar to the one that occurs in semiconductors/inorganic crystals. The electrons trapped in metastable states during exposure to electromagnetic radiation get released during subsequent warming of the sample and migrate to a stable location emitting energy as luminescence. The theoretical framework for calculating different physical parameters from TL curves, developed by Randall and Wilkins (1945), had assumed that the de-trapping process involved a single temperature dependent rate constant with Arrhenius behavior. Constants such as activation energy, frequency factor, and lifetime of the electron in the trapped state, could be calculated using the Randall-Wilkins theory. Using this theory of a single step de-trapping mechanism, the photosynthetic glow curves were analyzed in the late 1960s (Arnold and Azzi, 1968 and Shuvalov and Litvin, 1969). In these early calculations an arbitrary frequency factor was assumed. Subsequently, the activation energies were first calculated by using the initial rise method, and then, using the calculated values of the activation energies, other physical parameters of the glow curves were determined (Tatake et al., 1981). The initial rise method requires that each of the glow peaks should be without any overlap. Tatake et al. (1981) obtained well-separated glow peaks and discovered that the values obtained for frequency factors were too large and concluded that Randall-Wilkins theory as such was not applicable in analyzing the photosynthetic glow curves. At the same time Vass et al. (1981) used computer-assisted programs to resolve the glow peaks and calculated the physical parameters using Randall-Wilkins theory after introducing a small modification to accommodate absolute rate theory. Vass et al. (1981) further noted that even after the modifications that they introduced, the activation energies as well as the lifetime of the trapped states were unrealistically larger than expected. They suggested that in photosynthetic TL the recombination involves back reactions or reversal of the electron transport steps.

To explain the discrepancies between the observed results and the predictions of the existing theory, DeVault et al. (1983) modified the Randall-Wilkins theory by postulating temperature dependent equilibria between two or more electron carriers acting as traps for electrons or holes. By this modification the abnormally large apparent activation energies and frequency factors could be partly explained; they suggested that the rate-limiting back reaction step is not the only one that determines the rate of recombination but earlier steps that affect the concentration of charges are also important and need to be factored in. The newer concepts were further extended by DeVault and Govindjee (1990) who related

the free energies with the redox potentials of the intermediates involved in TL emission. Earlier work of Demeter et al. (1985b) reporting shifts of T_M of the B band in triazine-resistant biotypes of *Erigeron canadensis* had suggested that the shifts of the peak temperatures, T_M, were proportional to the changes in the midpoint potentials of Q_B. The studies of DeVault and Govindjee (1990) demonstrated that changes in T_M are proportional to changes in free energy and hence changes in midpoint potentials. This study provided strong theoretical support for inferring redox midpoint potential changes from shifts in peak temperature, T_M. Several research groups including the authors of this chapter have extensively used the T_M shifts to indicate changes in redox potentials of charge pairs involved in recombination leading to TL. Krieger et al. (1993) have provided confirmation of such a relationship in their studies which determined the redox potential of Q_A by redox titrations and related it to the shift of the T_M of the band involving Q_A.

Vidyasagar et al. (1993) argued that the methods used above have not considered the probability of re-trapping during the process of de-excitation while there was evidence for a re-trapping possibility in the reversal of electron transport during photosynthetic TL. They applied the general order kinetic model and calculated the physical parameters. These calculations certainly showed lower values of activation energies for the high temperature peaks but they did not improve the values of activation energies for low temperature peaks. However, this method provided much smaller and acceptable values of frequency factors for the high temperature peaks.

Rappaport et al. (2005) considered the $S_2Q_A^-$ recombination in PS II with a more complete theory that took into account the various competing recombination routes. However, they realized that such a more complete theory could not account for the location of the TL bands that occurs at much higher temperatures than predicted from the overall rate and temperature dependence of the $S_2Q_A^-$ recombination. They suggested that this happens due to the presence of kinetic heterogeneity of PS II recombination reactions. Using mutants of *Chlamydomonas reinhardtii* the role of membrane potential on the T_M and the intensity of the $S_2Q_A^-$ TL band was elaborated (Rappaport et al. 2005). The E130L mutant in which residue 130 of the D1 subunit was changed from glutamate to leucine was specifically chosen for this purpose because it has an enhanced $S_2Q_A^-$ band that appears at much higher temperature. This allowed Rappaport et al. (2005) to resolve the entire TL band without going below −10°C. In the absence of an uncoupler, the $S_2Q_A^-$ TL band appeared at a much lower temperature (around 20°C in the E130L mutant) as compared to its appearance at almost 30°C in the presence of the uncouplers nonactin and nigericin. The shift to higher temperature was accompanied by a decrease in its amplitude. They argued that while the modification of the energy barrier for recombination should shift the TL band along the temperature scale in the Randall-Wilkins scheme, the integral of the band should not have changed since the recombination reaction coincides with the radiative pathway. Their data, they argued, 'falsifies' this prediction of Randall-Wilkins theory. (for a review on the theory of TL, see Rappaport and Lavergne, 2009.)

Rantamäki and Tyystjärvi (2011) applied Arrhenius, Eyring and Marcus theories to analyze $S_2Q_A^-$ charge recombination measured as Q band and the decay of chlorophyll fluorescence yield after a single turnover flash at different temperatures in the presence of DCMU. They observed that all of the three theories gave the correct Q band position. However, Marcus theory gave a better fit for the rising part of the curve while both Eyring and Marcus theories gave good fits for the decreasing part of the TL curve. This paper discusses these theories in detail and provides information on their applicability in analyzing TL and fluorescence resulting from more than one competing route. In a previous paper these authors (Tyystjärvi and Rantamäki, 2009) have discussed retrapping in photosynthetic TL.

The effects of transmembrane electrochemical potential on TL bands were addressed by Farineau (1996) and it was shown that its presence reduced the activation energies of B bands. Upon addition of uncouplers of phosphorylation the bands revert to their normal T_M values and hence have increased activation energies. It is now obvious that the Randall-Wilkins theory for the calculation of the different physical constants from TL in phototsynthetic membranes as such cannot be applied and modifications, as initially suggested by DeVault et al. (1983) and subsequently elaborated upon

by others, must not be ignored. Rose et al. (2008) have combined the ideas of Rappaport et al. (2005) and of DeVault and Govindjee (1990) in explaining changes in the T_Ms, and thus in the redox potentials of Q_B/Q_B^- bands, in D1-Arg275 mutants in *Chlamydomonas reinhardtii*. Further research is needed to establish a final definitive model.

IV. Thermoluminescence and Delayed Light Emission

Both the TL and delayed light emission (DLE; also called delayed fluorescence; discovered by Strehler and Arnold, 1951) of photosynthetic membranes are related phenomena. Shuvalov and Litvin (1969) associated the TL bands they observed with different phases of delayed fluorescence. Indications that the delayed light and TL may be related to each other were also apparent from the temperature jump experiments of Mar and Govindjee (1971), Jursinic and Govindjee (1972) and Malkin and Hardt (1973). The fact that TL bands and components of DLE not only originate in light-induced electron transport but share many other common properties such as oscillations and inhibition by inhibitors of electron transport suggested that DLE and TL may be related to each other. Furthermore, the analysis of different TL bands by the application of Randall and Wilkins theory suggested that the lifetimes of the electrons in the trapped states at their respective glow peak temperatures were in a time scale of seconds (Tatake et al., 1981) indicating that DLE components that decay on a time scale of seconds may be related to the TL bands. A detailed study of the possibility that the two are identical was investigated by Desai et al. (1982a) who elegantly showed that a slow component of DLE mimics glow peaks and that the DLE and TL are quantitatively related and represent the expression of the same phenomenon. In a subsequent study, Rane and Sane (1985) identified specific components of DLE with four major TL peaks associated with photosynthetic electron transport. The relationship between TL and DLE was also shown in the studies of Rutherford et al. (1984) and Hideg and Demeter (1985). Several subsequent studies have strengthened the relationship between TL bands and the components of DLE (Vass et al., 1988). While each TL band is associated with one or the other DLE component there are some DLE components that do not have a corresponding TL band. However, there are advantages in using TL rather than DLE under some conditions as the components of DLE are much better resolved by TL as each separate band corresponds with one or the other DLE component.

V. Thermoluminescence and Photoinhibition

Photoinhibition has been extensively studied by TL in cyanobacteria, algal cells, higher plants and isolated thylakoids (Ohad et al., 1988, 1990; Briantais et al., 1992; Mäenpää et al., 1995; Ono et al., 1995; Andree et al., 1998). In all the cases a loss in the intensity of the $S_2Q_B^-$ peak has been seen as indicative of the degradation of the D1 polypeptide. Most studies have indicated that the rate of electron transfer from the reduced Q_A to Q_B is decreased. Besides these expected results TL studies have indicated shifts of $S_2Q_B^-$ and $S_2Q_A^-$ recombination that cannot be attributed to the shift of redox change in the donor components that contribute oxidizing equivalents, specifically the S states. The obvious conclusion drawn from these studies is that the redox species on the acceptor side (Q_A^- and Q_B^-) contributing to the charge recombination seem to undergo changes. The redox shifts are such that the redox gap between Q_A and Q_B is considerably reduced. Another interesting observation made is that these shifts occur only in intact systems and not in isolated thylakoids suggesting that the shifts are associated with the dynamic nature of the cell components other than membranes themselves. An additional observation made is the intensification of the high temperature peak related to the lipid peroxidation that invariably occurs under many stress conditions including light stress (Havaux, 2003).

Studies on *Chlamydomonas reinhardtii* cells by Ohad et al. (1988) suggested a shift of $S_2Q_B^-$ to lower temperature by about 15°C that was associated with an increase in the minimal (F_o) fluorescence intensity, when the cells were photoinhibited at intensities of 300–1,000 W m^{-2}. Concurrently, $S_2Q_A^-$ intensity decreased to the extent of 30–40% along with a decrease in maximal variable

chlorophyll *a* fluorescence ($F_m - F_o$) in the presence of DCMU. These changes were attributed to a light-dependent turnover of D1 protein. In a subsequent study, Ohad et al. (1990) showed a decrease in the activation energy of $S_2/S_3Q_B^-$ in the cells and also in isolated thylakoids. In *Synechocystis* sp. PCC 6803 cells in which the D1 polypeptide was modified by substitution of glutamine 241 to histidine (the CA1 mutant), Mäenpää et al. (1995) observed that photoinhibition resulted in the shift of $S_2/S_3Q_A^-$ recombination to a higher temperature by 10°C. Expectedly there was an increase in the $t_{1/2}$ of chlorophyll fluorescence decay in the presence of DCMU. Mäenpää et al. (1995) suggested that a modification of the redox couple Q_A/Q_A^- had occurred and this rendered the reoxidation of Q_A^- more difficult. Similar shifts in $S_2Q_B^-$ have been observed in *Synechococcus* sp. PCC 7942 cells by Sane et al. (2002) when the cells growing at 36°C were shifted to 25°C. The shifts were reversed when the cells were shifted back to 36°C. The reduction in growth temperature results in increased excitation pressure causing conditions similar to photoinhibition. The cells responded by substitution of D1:1 by D1:2 – a characteristic response of these cells to various stress conditions. Upon shifting to low temperature the $S_2Q_B^-$ recombination occurred at a temperature similar to that of $S_2Q_A^-$. This had reduced the gap between the redox potentials of Q_A and Q_B to almost zero. The redox state of the donors (S states) was not changed.

In higher plants, the $S_2Q_B^-$ band has also been observed to shift to low temperature during photoinhibition, demonstrating a more negative redox potential of Q_B, closer to that of Q_A (Briantais et al., 1992). It appears that the S states had not undergone a change in their redox potential since the $S_2Q_A^-$ band had not shifted. Sane et al. (2003) studied responses of cold acclimated *Arabidopsis thaliana* plants to high light using TL and chlorophyll fluorescence measurements. They observed that the $S_2Q_A^-$ band was shifted to a higher temperature while the $S_2Q_B^-$ band was shifted to a lower temperature in cold acclimated plants. Sane et al. (2003) suggested that the narrowing of the gap in the T_Ms of these two bands was related to the increased redox potential of Q_A and decreased redox potential of Q_B in cold acclimated plants: these effects resulted in an increased reaction center quenching, i.e., enhanced probability for non-radiative $P680^+Q_A^-$ radical pair recombination facilitated by an increased population of reduced primary electron-accepting quinone (Q_A) in PS II. The expected enhanced reaction center quenching was confirmed by in vivo chlorophyll *a* fluorescence quenching analysis. The enhanced dissipation of excess light within the reaction center of PS II (reaction center quenching) must have been the reason for resistance of cold acclimated plants to photoinhibition. Ivanov et al. (2003) and Huner et al. (2006) have suggested how redox potential changes of the two plastoquinone acceptors could explain the increased reaction center quenching and hence improved tolerance to photoinhibition. Furthermore, Ivanov et al. (2006) have shown similar shifts resulting in the narrowing of the gap in the redox potential of Q_A and Q_B in barley and rye grown under high excitation pressure. The reaction center quenching concept proposed by Sane et al. (2003) could explain, in part, the resistance or decreased photoinhibition in high light acclimated or low temperature acclimated plants. Probably the same explanation can be extended to understand the stress response of the plants to various stresses as explained later.

Interestingly the photoinhibition of isolated thylakoids/membranes demonstrate somewhat different results than observed in intact leaves or cells. Vass et al. (1988) working with isolated spinach thylakoids did not observe any shifts either in the Q or the B bands or in the half times of related delayed fluorescence. Similarly Andree et al. (1998) using grana, stroma and margin fractions did not observe any shifts of Q or B bands although the TL intensities decreased. Photoinhibition under aerobic conditions using oxygen-evolving membrane preparations from spinach showed an expected decrease in the intensity of the $S_2Q_B^-$ peak but there was no temperature shift (Ono et al., 1995).

Photoinhibition seems to result in oxidative damage and this can be monitored by TL. Matorin et al. (1992) observed an increased high temperature band indicative of lipid peroxidation when microalgal cells of Black Sea phytoplankton were exposed to high light. Thus, this high temperature band could be used to assess the extent of irreversible damage of cells following photoinhibition. Havaux and Niyogi (1999) studying the *npq1* mutant of *Arabidopsis thaliana* lacking violaxanthin

Table 19.2. Shifts of $S_{2/3}Q_B^-$ and $S_2Q_A^-$ bands in response to various environmental stress conditions

Stress	Plant material	$S_{2/3}Q_B^-$ T_M	$S_2Q_A^-$ T_M	References
High light	*Chamydomonas reinhardtii* cells	Downshift	NC	Ohad et al., 1988
	Chlamydomonas reinhardtii cells	Downshift	NC	Ohad et al., 1990
	Pea leaves	Downshift	NC	Janda et al., 1992
	PS II membranes	Downshift	NM	Ono et al., 1995
	Barley leaves	Downshift	NC	Walters and Johnson, 1997
Cold stress/ Acclimation	Spinach leaves	Downshift	NM	Briantais et al., 1992
	Maize leaves	Downshift	NM	Janda et al., 2000
	Synechococcus sp. PCC 7942 cells	Downshift	NC	Sane et al., 2002
	Scots pine needles	Downshift	NC	Ivanov et al., 2002
	Arabidopsis thaliana leaves	Downshift	Upshift	Sane et al., 2003
	Barley and Rye leaves	Downshift	Upshift	Ivanov et al., 2006
Water stress	Bean leaves	Downshift	NM	Metwally et al., 1997
Desiccation	Barley leaves	Downshift	NM	Skotnica et al., 2000
	Spinach leaves	Downshift	Upshift	Peeva and Maslinkova, 2004
CO_2 depletion	*Chlamydobotrys stellata* cells	Downshift	Upshift	Wiessner et al., 1992
	Chlamydomonas reinhardtii cells	Downshift	NC	Wiessner et al., 1992
	Chlamydobotrys stellata cells	Downshift	Downshift	Demeter et al., 1995
	Synechococcus sp. PCC 7942 N5 mutant	Downshift	NM	Marco et al., 1993
Copper stress	Spinach thylakoids	Downshift	Upshift	Horváth et al., 1998
Mercury stress	*Chlorella kessleri* cells	Downshift	NC	El-Seekh, 1999
UV-B stress	Barley leaves	Downshift	Upshift	Gilbert et al., 2004a
Ozone stress	Barley leaves	Downshift	NM	Skotnica et al., 2003
	Barley leaves	Downshift	NM	Skotnica et al., 2005

NC no change, *NM* not measured

de-epoxidase (and which therefore cannot form zeaxanthin in strong light) observed an increased high temperature band reflecting lipid peroxidation. The use of high temperature TL bands to study and assess the extent of lipid peroxidation under in vivo conditions is another use of the TL technique. Havaux (2003) has discussed methods and quantification of the oxidative damage using high temperature TL bands.

VI. Thermoluminescence and Stress

Both biotic and abiotic stresses alter the photosynthetic characteristics and metabolic status of photosynthetic photoautotrophs, which may be reflected in specific modifications of PS II. TL patterns have been used as a probe for stress responses in intact organisms. Among the environmental factors temperature, light, salt, and drought are some of the important stresses that plants experience under natural conditions and many of these have been studied by the TL technique (see reviews by Misra et al., 2001 and Ducruet, 2003). Since PS II activity is usually reduced under severe stress conditions a decrease in all the TL bands that arise in PS II is expected, as has been reported (Ivanov et al., 2001; Sane et al., 2002). However, when the stress is relatively less severe, the band related to $S_2Q_B^-$ has been shown to shift to lower temperatures under numerous stress conditions (Table 19.2 and the references cited therein). Further, induction of a TL band appearing at a higher temperature in response to high temperature stress that is associated with lipid peroxidation has been demonstrated. Some studies have also demonstrated changes in the A_G band under photoinhibitory conditions. The most significant feature of the stress, irrespective of its kind, seems to be the decrease in the emission temperature of the $S_2Q_B^-/S_3Q_B^-$ band. This shift is also commonly seen during low temperature acclimation as well as acclimation to high light intensity.

The responses to high light causing photoinihibition have been discussed above.

A. Temperature

Both high and low temperature stress have been studied using TL. Down-shift of the B band ($S_2Q_B^-$) has been reported in cold acclimated spinach leaves (Briantais et al., 1992). Janda et al. (2000) have reported a down-shift in the T_M of the B band in low temperature grown maize. Similar observations have been made by Sane et al. (2002) in cold-stressed *Synechococcus* sp. PCC 7942 cells, and in cold acclimated *Arabidopsis thaliana* (Sane et al., 2003). There are also similar data available on barley and rye leaves grown at low temperature (5°C) (Ivanov et al., 2006) and on cold acclimated Scots pine needles (Ivanov et al., 2002; Sveshnikov et al., 2006). In maize subjected to low temperature (0°C) for 4 h, the shift of the B band to low temperature was associated with induction of the A_G band (Janda et al., 2000). In all the studies mentioned above, besides the down-shift of $S_2Q_B^-$ peak temperature, the overall reduction of the B band TL emission was also observed.

Exposure of barley plants to high temperature induced a high temperature TL band appearing between 70°C and 120°C (Havaux, 1998). Havaux and Niyogi (1999) observed an increase in high temperature TL in the *npq1* mutant of *Arabidopsis thaliana* that lacks the violaxanthin de-epoxidase enzyme.

B. Salt Stress

Salt stress has been found to reduce the intensity of $S_2Q_B^-$ band more than that of $S_2Q_A^-$ band (see Misra et al., 1999), but there were no shifts in the peak positions. In *Chlorella vulgaris,* El-Seekh (2004) showed that the S_2 to S_3 transition of the water-oxidizing system was inhibited by NaCl; in addition the intensities of both Q and B bands were reduced.

C. Heavy Metals

Studies on intact thylakoids that were treated with a 500 μM concentration of copper showed a down-shift of $S_2Q_B^-$ band to 15°C (Horváth et al., 1998). These results were explained as donor side effects. However, the A band ($S_3Q_A^-$) had shifted to a higher temperature. Mercury at 100 μM did not affect the $S_2Q_A^-$ charge recombination in *Chlorella kessleri*, while $S_2Q_B^-$ recombination was inhibited and shifted to lower temperature (El-Seekh, 1999)

D. Desiccation and Water Stress

In the initial stages of leaf desiccation both $S_2Q_B^-$ and $S_3Q_B^-$ bands have been observed to shift to lower temperatures, while an up-shift of the $S_2Q_A^-$ band was observed under the same conditions (Skotnica et al., 2000). The shifts of Q_A and Q_B related bands in opposite directions suggest that the electron donor side had not undergone a change in redox characteristics, rather the electron acceptors Q_A and Q_B came closer to each other in their redox potential values. Although the authors did not argue in terms of the increased possibility for reaction center quenching during the desiccation as a response to increased radiation stress we suggest that the redox properties of the photosynthetic apparatus were modified to accommodate the high excitation at least partially through the reaction center quenching mechanism as proposed by Sane et al. (2003) and Ivanov et al. (2003). The shift of the $S_2Q_B^-$ band to lower temperature from 32°C to 25°C has also been observed in water stressed bean plants (Metwally et al., 1997). Interestingly, the down-shift of the $S_2Q_B^-$ band was not seen in a desiccation tolerant plant (*Haberlea rhodopensis*) while in spinach at 10% relative water content the shift of $S_2Q_B^-$ was as much as 10°C in the direction of a lower temperature (Peeva and Maslinkova 2004).

E. Ozone and UV

Exposure of tomato and barley plants to ozone resulted in a concentration dependent down-shift of the $S_2Q_B^-$ band from 23°C to 15°C (Skotnica et al., 2003). Surprisingly, the chlorophyll fluorescence parameters F_v (variable fluorescence, F_m minus F_o), F_m (maximal fluorescence) and F_o (minimal fluorescence) did not change under the same conditions, thus indicating that PS II photochemistry in these leaves was still as efficient as in leaves not exposed to ozone. This could be explained by the possibility that the $S_2Q_B^-$ shift to lower temperature protected the reaction centers through the reaction center quenching mechanism

Table 19.3. Shifts of $S_2Q_A^-$ and $S_{2/3}Q_B^-$ bands in response to various mutations and treatments

Plant material	Mutations/Treatment	$S_2Q_A^-$ (T_M)	$S_{2/3}Q_B^-$ (T_M)	References
Synechocystis sp. PCC 6803 E189Q mutant cells	Replacement of D1-Glu189 with Gln	Upshift to 24°C	NM	Kimura et al., 2005
Synechocystis sp. PCC 6803 A1K mutant cells	Replacement of D1 with D1'	Upshift to 28°C	Upshift to 33°C	Sicora et al., 2004
Synechocystis sp. PCC 6803 D1-H332D mutant cells	Replacement of His332 in D1	Upshift to 24°C	Upshift to 47°C	Allahverdieva et al., 2004
Synechocystis sp. PCC 6803 D1-A244G mutant cells	Replacement of D1-Ala244 with Gly	Upshift by 5°C	Upshift by 5°C	Mizusawa et al., 2004
Synechocystis sp. PCC 6803 Y112L mutant cells	Replacement of D1Tyr112 with Leu	NS	Downshift to 20°C	Tal et al., 1999
Synechocystis sp. PCC 6803 NDSF mutant cells	Mutations in the PEST region of D1	NC	Downshift to 28°C	Minagawa et al., 1999
Synechocystis sp. PCC 6803 I6 mutant cells	Mutations in the C-terminal of D1	Upshift to 16°C	NC	Minagawa et al., 1999
Synechocystis sp. PCC 6803 CA1 mutant cells	Mutations in the D-de loop of D1	Upshift by 10°C	Upshift by 7°C	Mäenpää et al., 1995
Chlamydomonas reinhardtii D1 mutants	D1-arginine257 mutants	NC	Downshift by 10°C	Rose et al., 2008
Synechocystis sp. PCC 6803 C7-3 mutant cells	Mutations in the CD-loop of D2	Upshift by 9°C	NM	Vavilin and Vermaas, 2000
Synechocystis sp. PCC 6803 IC7 mutant cells	*psbH* null mutant	NC	Downshift to 27°C	Mayers et al., 1993
Tobacco Δ*psbJ* mutant cells	*psbJ* deletion mutant	Upshift by 10°C	Downshift by 4°C	Regel et al., 2001
Costata-2/133 mutant of pea	Reduction of LHCII oligomerization	Upshift by 5°C	Downshift by 4°C	Ivanov et al., 2005
Spinach PS II membranes	Removal of 16, 23 and 33 kDa proteins	Upshift by 20°C	Downshift by 5°C	Vass et al., 1987
Spinach PS II membranes	Removal of 33 kDa protein	Upshift by 20°C	NC	Vass et al., 1987
Synechocystis sp. PCC 6803 *ΔpsbO* mutant	*psbO deletion mutant*	Upshift to 26°C	Upshift to 39°C	Burnap et al., 1992
Spinach thylakoids	High pH	Upshift	Downshift	Vass and Inoue, 1986
Spinach thylakoids	Dark ΔpH	Downshift	Downshift	Miranda and Ducruet, 1995a
PS II membranes	Ca^{2+} depletion	Upshift	NM	Homann and Madabusi, 1993
Euglena cells	Diuron resistant mutant	NM	Downshift	Farineau and Laval-Martin, 1995
Synechocystis sp. PCC 6803A263P/mutants	Herbicide resistant mutants	NC	Downshift	Dalla Chiesa et al., 1997

NC no change, *NM* not measured, *NS* not shown

as proposed by Sane and co-workers (Sane et al., 2003; Ivanov et al., 2003). These results also indicate that in some cases TL can be a far more sensitive technique than fluorescence in monitoring environmental effects. The shift was reversed within 24 h upon shifting back the plants to an ozone free atmosphere. A similar, but smaller shift in the $S_2Q_B^-$ was observed in tomato and barley plants exposed to UV and this effect was associated with changes in high temperature peaks (Gilbert et al., 2004a).

F. Shifts of Q and B Bands

Table 19.2 summarizes reports that have shown shifts of $S_2/S_3Q_B^-$ and $S_2Q_A^-$ under different stress conditions. Shifts of the B and Q bands have also been documented by several research groups in response to various mutations and treatments (Table 19.3). It is apparent from Table 19.2 that the shift of the characteristic T_M of the B bands towards lower temperature is a general response of the photosynthetic apparatus to most of the

stress conditions studied. An up-shift of the $S_2Q_A^-$ band has been documented in some of the cases although this response is not as general as the shift of the B bands. In a series of studies, Ivanov et al. (2002, 2005, 2006); Sane et al. (2002, 2003) and Pocock et al. (2007) have demonstrated the down-shift of the B band and in some cases an up-shift of the Q band. These shifts were in the opposite directions and hence could not be attributed to the changes in the redox state of the electron donor side of PS II. Therefore, Ivanov et al. (2002) and Sane et al. (2003) suggested that the shifts resulted in the reduction of the redox gap between the two plastoquinone acceptors (Ivanov et al., 2002; Sane et al., 2003). This should result in the modification of the rate constants in the electron flow between Q_A and Q_B such that the electron will have a higher probability of being retained on Q_A.

What is the extent of the redox shifts when the temperature gap between $S_2Q_A^-$ and $S_2Q_B^-$ peaks decreases by say 10°C in the 15–30°C range? In *Synechocystis* sp. PCC 6803, Minagawa et al. (1999) estimated an equilibrium constant K_{AB} of 26 in control cells with a gap in the T_M of Q and B bands equivalent to 26°C. In *Synechocystis* sp. PCC 6803 site-directed mutants with modified PS II, the gap in the T_M was reduced to about 20°C and the K_{AB} was reduced to 8–9. Similarly, narrowing the T_M gap between Q and B bands by 6–8°C resulted in a calculated decrease of the redox potential difference of 22 mV between the two quinone acceptors. Rose et al. (2008) using D1 mutations in *Chlamydomonas reinhardtii* analyzed the TL data theoretically, based on the recombination model of Rappaport et al. (2005). They concluded that the observed shift of the B band to lower temperature by 8–10°C due to mutations in D1 corresponded with the reduction in the free energy difference by 20–40 mV for the recombination of S_2 with Q_B^-. This difference was interpreted to be due to a lowering of redox potential of Q_B/Q_B^-. The apparent equilibrium constant (K'_{app}) values were almost one-third in the mutants as compared to the wild type.

We suggest that under stress conditions, changes in equilibrium constant and redox potential difference of similar magnitude, if not more, occurs as the T_M gap has been reduced in the same or even greater temperature range. Such a condition will favor and increase the probability for non-radiative recombination of $P680^+Q_A^-$ that will not generate the triplet chlorophylls and the accompanying production of reactive oxygen species responsible for the destruction of PS II. One expects a reduction in the radiative pathway that should be reflected in the decreased luminescence as TL. In fact, a reduction in the TL yield to 40% was observed when the T_M gap was reduced in cold-stressed *Synechococcus* sp. PCC 7942 cells and cold acclimated *Arabidopsis thaliana* leaves (Sane et al., 2002, 2003). The increased difference in the redox potential of Q_A and Pheo has been suggested to result in a lower probability of $P680^+Pheo^-$ recombination that yields chlorophyll triplets (Johnson et al., 1995; Krieger and Rutherford, 1997; Krieger-Liszkay and Rutherford, 1998).

Based on the above studies, we have advanced a proposal for reaction center quenching (non-photochemical) that explains in part the resistance of the plant/leaf to increased excitation pressure including photoinhibition (Sane et al., 2003; Ivanov et al., 2003, 2006; Huner et al., 2006).

A schematic model for explaining the reaction center quenching mechanism associated with the shifts in the T_M of $S_2Q_B^-$ and $S_2Q_A^-$ bands is presented in Fig. 19.4. Under unstressed conditions the T_M gap between the acceptors Q_A and Q_B is higher and this encourages increased luminescence yield and lower non-radiative dissipation of energy. Under stress conditions the T_M gap decreases due to the shifts of $S_2Q_B^-$ and $S_2Q_A^-$ bands towards each other. This increases the k_2 (see Fig. 19.4) retaining the electron on Q_A. The redox potential difference between Q_A and P680* is increased restricting the back transfer of the electron to $P680^+$ to regenerate P680*. Instead the Q_A^- recombines with $P680^+$ through a non-radiative pathway. The TL yield is therefore lowered. This increases the probability for non-radiative dissipation of excitation energy within the PS II reaction center (reaction center quenching) with reduced destruction of PS II (Fig. 19.4b).

The use of the TL technique has brought forth these explanations and it appears that any stress that plants experience results in increased excitation pressure. The reduction in the redox potential gap of the acceptors is a response that helps plants to overcome the excitation stress. The response appears to be universal: it is present in cyanobacteria, algae, higher plants and

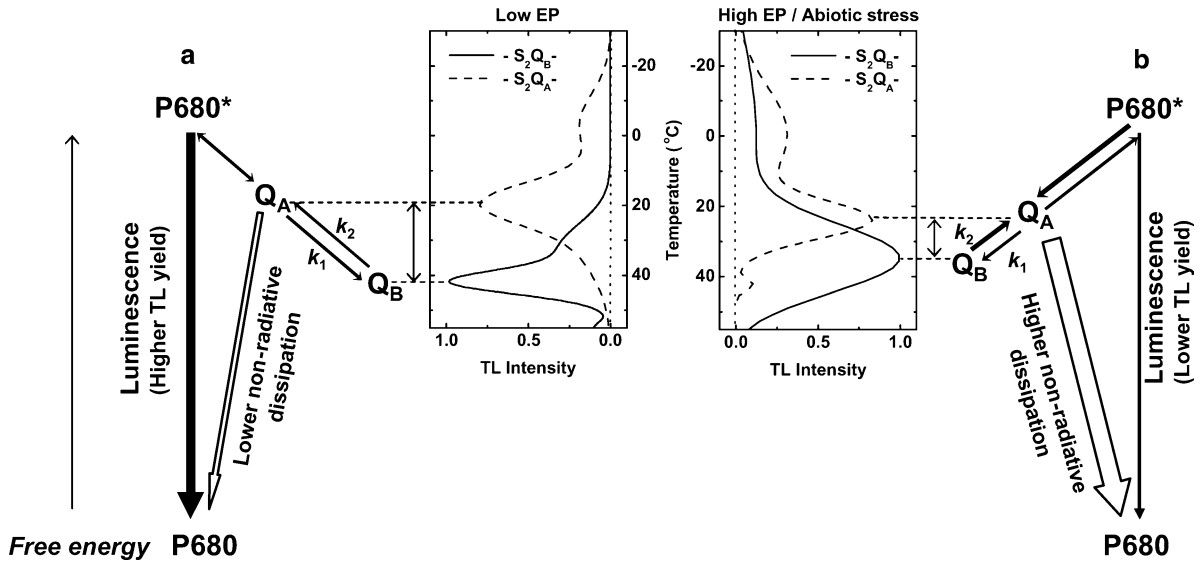

Fig. 19.4. Schematic diagram of the free energy levels explaining the differences in radiative vs non-radiative energy dissipation pathways in unstressed plants acclimated to low excitation pressure (EP) (**a**) and plants acclimated to high EP/stress conditions (**b**). (**a**) In unstressed plants (low EP) the free energy gap between $P680^+$ and Q_A^- would predominantly favor the radiative energy dissipation pathway characterized by higher TL yield (*thick black arrow*). When P680* returns to the ground state the energy is transferred to the antenna chlorophylls that emit light as TL. (**b**) In plants acclimated to high EP/stress conditions the increased free energy gap between $P680^+$ and Q_A^- determined by the higher TL peak emission temperature of $S_2Q_A^-$ charge recombination (see the TL glow curve of $S_2Q_A^-$ charge recombination under high EP conditions) would decrease the probability for a charge recombination pathway involving $P680^+Pheo^-$ and will cause stabilization of the $S_2Q_A^-$ pair. In addition, shifting the redox potential of Q_B toward Q_A determined by the lower TL peak emission temperature of $S_2Q_B^-$ charge recombination (see the TL glow curve of $S_2Q_B^-$ charge recombination under high EP conditions) favours the k_2 rate constant and also results in an increased steady state proportion of reduced Q_A. It is proposed that this will increase the probability for direct non-radiative recombination (*empty arrow*) of Q_A^- with $P680^+$ via the non-radiative pathway resulting in low TL yield without generating the chlorophyll triplet. In both types of plants the radiative charge recombination occurs, but is proportionally less in plants acclimated to high EP. The model is based on the assumption that redox properties of the donor side were not modified. The representative TL glow curves for $S_2Q_A^-$ (*dashed lines*) and $S_2Q_B^-$ (*solid lines*) were recorded in intact leaves of rye (*Secale cereale* L.) plants grown under low (20°C/50 μmol photons m^2 s^{-1}) and high (20°C/800 μmol photons m^2 s^{-1}) EP conditions.

even perennial trees and may be a complementary mechanism to the antenna quenching but more fundamental as it is present in even those organisms that have no antenna quenching possibilities (Ivanov et al., 2008). While the reduction in redox gap of acceptors in some cyanobacteria occurs through the replacement of D1:1 by D1:2 (as for example in *Synechococcus* sp. PCC 7942*)*; this D1 exchange mechanism is not possible in eukaryotes since they possess one *psbA* gene. However, we suggest that modifications in the lipid components of the chloroplast thylakoid membrane closely associated with PS II may regulate the redox gap between Q_A and Q_B. This may, in part, explain why isolated thylakoids do not show the $S_2Q_B^-$ shift while the intact tissue does.

VII. Concluding Remarks

Although the non-invasive TL technique is an attractive, simple technique to study the functioning of different aspects of PS II including studies of S-state turnover, modifications of D1 and D2, herbicide binding, function of the extrinsic proteins and cofactors related to the water-oxidizing complex and acclimation to various environmental conditions and stresses, the technique by itself is inadequate. It is often necessary to complement the data obtained using the TL technique with other biophysical and biochemical analyses. However, it does provide valuable and quick information on the relative redox state of important charge pairs that no other simple technique can. The TL peaks are indicative of a back reaction involving a

set of charged pairs but the confirmation of which charge pairs are involved must come from the use of other known perturbations.

DeVault and Govindjee (1990) provided a basis for relating the change in total free energy arising from the shifts in the T_Ms of the glow peaks to the changes in the redox potentials of the participating charge pairs. However, this relationship allows one to infer changes in the mid-point potentials of the redox carriers but not their actual redox potentials. These have to be determined by some other procedures such as redox titrations as has been done in some laboratories. Thus, the T_M shifts provide a basis for quickly assessing even the subtle changes taking place in the functional modifications of both the donor side and the acceptor side of PS II. In addition, it is now clear that TL can throw light on cyclic phosphorylation by PS I and the lipid peroxidation associated with stress-related responses. However, other procedures need to be used to understand the precise changes that have taken place in the energy transducing photosynthetic membranes.

Several laboratories have reported changes in TL characteristics following pretreatments of the sample material. The effect of freezing of the sample following the excitation was investigated by Homann (1999) and it was suggested that depending on the type of photosynthetic material the modifications in the TL characteristics could be interpreted as artefacts. Although this has to be kept in mind while conducting experiments, it is possible to determine whether such artefacts are occurring in one's experimental procedures. In most TL studies the data are used to compare two types of treatments and recombining species are identified using inhibitors or some other experimental treatments and not just by the temperature of their emission. Furthermore, the temperature range of recombining species is now well documented. In addition, the shifts in T_M are reversible. Thus, the generation of artefacts can be easily ascertained and overcome. However, we must be aware that potential artefacts could arise and those could lead to unwarranted interpretations.

The characteristics of different TL bands are very sensitive to the metabolic state of the cells that results in poising the redox state of components participating in the recombination reaction. During dark adaptation of the sample, depending upon the reduction status of the PQ pool and the possibility of its reduction through stromal enzymatic activities, the electron transport components such as Q_A and Q_B may become reduced and influence the TL patterns obtained in subsequent flash-induced studies. Moreover, the transmembrane electrochemical potential could also be influenced by the dark metabolic activity of the sample. As discussed in this chapter, the presence of a membrane potential down-shifts the T_M of some of the bands. In our opinion, precise control of growth conditions, developmental stage and the metabolic status of the organism is critical for the proper assessment and interpretation of changes in TL emission patterns.

The information now available about the mechanism and characteristics of different glow peaks has made the TL technique popular to monitor not just PS II function, but also to obtain information on energy charge, oxidative stress and several other responses of the photosynthetic apparatus. In some respects it is preferable to DLE as the components are better resolved in this TL method. We expect that the TL technique will be used as a routine tool in understanding of subtle but important changes that PS II may undergo during acclimation to an ever-changing environment.

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References

Allahverdieva Y, Deak S, Szilard A, Diner BA, Nixon PJ and Vass I (2004) The function of D1-H332 in photosystem II electron transport studied by thermoluminescence and chlorophyll fluorescence in site-directed mutants of *Synechocystis* 6803. Eur J Biochem 271: 3523–3532

Andree S, Weis E and Krieger A (1998) Heterogeneity and photoinhibition of photosystem II studied with thermoluminescence. Plant Physiol 116: 1053–1061

Arnold W (1965) An electron-hole picture of photosynthesis. J Phys Chem 69: 188–791

Arnold W (1966) Light reaction in green plant photosynthesis: A method of study. Science 154: 1046–1049

Arnold WA and Azzi JR (1968) Chlorophyll energy levels and electron flow in photosynthesis. Proc Natl Acad Sci USA 61: 29–35

Arnold WA and Sherwood HK (1957) Are chloroplasts semiconductors? Proc Natl Acad Sci USA 43: 105–114

Bhatnagar R, Saxena P, Vora HS, Dubey VK, Sarangpani KK, Shirke ND and Bhattacharjee SK (2002) Design and performance of a versatile, computer controlled instrument for studying low temperature thermoluminescence from biological samples. Meas Sci Technol 13: 2017–2026

Bohne C, Campa A, Cilento G, Nassi L and Villablanca M (1986) Chlorophyll: an efficient detector of electronically excited species in biochemical systems. Anal Biochem 155: 1–9

Briantais JM, Ducruet JM, Hodges M and Krause GH (1992) The effects of low temperature acclimation and photoinhibitory treatments on photosystem 2 studied by thermoluminescence and fluorescence decay kinetics. Photosynth Res 31: 1–10

Burnap RL, Shen J-R, Jursinic PA, Inoue Y and Sherman LA (1992) Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of photosystem II. Biochemistry 31: 7404–7410

Chapman DJ, Vass I and Barber J (1991) Secondary electron transfer reactions of the isolated photosystem II reaction centre after reconstitution with plastoquinone-9 and diacylglycerolipids. Biochim Biophys Acta 1057: 391–398

Clegg RM, Sener M and Govindjee (2010) From Förster Resonance Energy Transfer (FRET) to Coherent Resonance Energy Transfer (CRET) and Back --A wheen o' mickles mak's a muckle. In: Alfano RA (ed) Optical Biopsy VII, Proceedings of SPIE, Vol. 7561. SPIE, Bellingham (paper number: 7561–12; article CID number: 75610 C, 21)

Dalla Chiesa M, Friso G, Deak Z, Vass I, Barber J and Nixon PJ (1997) Reduced turnover of the D1 polypeptide and photoactivation of electron transfer in novel herbicide resistant mutants of *Synechocystis* sp. PCC 6803. Eur J Biochem 248: 731–740

Demeter S and Govindjee (1989) Thermoluminescence in plants. Physiol Plant 75: 121–130

Demeter S, Herczeg T, Droppa M and Horvath G (1979) Thermoluminescence characteristics of granal and agranal chloroplasts of maize. FEBS Lett 100: 321–324

Demeter S, Vass I, Horvath G and Laufer A (1984) Charge accumulation and recombination in photosystem II studied by thermoluminescence. II: Oscillation of the C band by flash excitation. Biochim Biophys Acta 764: 33–39

Demeter S, Rosza Z, Vass I and Hideg E (1985a) Thermoluminescence study of charge recombination in photosystem II at low temperature II: Oscillatory properties of the Z and A thermoluminescence bands in chloroplasts dark-adapted for various time periods. Biochim Biophys Acta 809: 379–387

Demeter S, Vass I, Hideg E and Sallai A (1985b) Comparative thermoluminescence study of triazine-resistant and triazine susceptible biotypes of *Erigeron canadensis*. Biochim Biophys Acta 806: 16–24

Demeter S, Goussias C, Bernet G, Kovacs L and Petrouleas V (1993) Participation of the g = 1.9 and g = 1.82 EPR forms of the semi-quinone-iron complex, $Q_A^-Fe^{2+}$ of photosystem II in the generation of the Q and C thermoluminescence bands respectively. FEBS Lett 336: 352–356

Demeter S, Janda T, Kovacs L, Mende D and Wiessner W (1995) Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1229: 166–174

Desai TS, Sane PV and Tatake VG (1975) Thermoluminescence studies on spinach leaves and *Euglena*. Photochem Photobiol 21: 345–350

Desai TS, Tatake VG and Sane PV (1977) Characterization of the low temperature thermoluminescence band Z_v in leaf: An explanation for its variable nature. Biochim Biophys Acta 462: 775–780

Desai TS, Tatake VG and Sane PV (1982a) A slow component of delayed light emission as a function of temperature mimics glow peaks in photosynthetic membranes. Evidence for identity. Biochim Biophys Acta 681: 383–386

Desai TS, Tatake VG and Sane PV (1982b) High temperature peak on glow curve of photosynthetic membrane. Photosynthetica 16: 129–133

Desai TS, Rane SS, Tatake VG and Sane PV (1983) Identification of far-red-induced relative increase in the decay of delayed light-emission from phototsynthetic membranes with thermoluminescence peak-V at 321 K. Biochim Biophys Acta 724: 485–489

DeVault D and Govindjee (1990) Photosynthetic glow peaks and their relationship with the free energy changes. Photosynth Res 24: 175–181

DeVault D, Govindjee and Arnold W (1983) Energetics of photosynthetic glow peaks. Proc Natl Acad Sci USA 80: 983–987

Droppa M, Horvath G, Vass I and Demeter S (1981) Mode of action of photosystem II herbicides studied by thermoluminescence. Biochim Biophys Acta 638: 210–216

Ducruet J-M (1999) Relation between the heat-induced increase of F_o fluorescence and a shift in the electronic equilibrium at the acceptor side of photosystem 2. Photosynthetica 37: 335–338

Ducruet J-M (2003) Chlorophyll thermoluminescence of leaf discs: simple instruments and progress in signal interpretation open the way to new ecophysiological indicators. J Exp Bot 54: 2419–2430

Ducruet J-M and Miranda T (1992) Graphical and numerical analysis of thermoluminescence and fluorescence F_o emission in photosynthetic material. Photosynth Res 33: 15–27

Ducruet J-M and Vass I (2009) Thermoluminescence: experimental. Photosynth Res 101: 195–204

Ducruet J-M, Roman M, Havaux M, Janda T and Gallais A (2005) Cyclic electron flow around PS I monitored by afterglow luminescence in leaves of maize inbred lines (*Zea mays* L): correlation with chilling tolerance. Planta 221: 567–579

El-Seekh MM (1999) Inhibition of the photosynthetic electron transport in the unicellular green alga *Chlorella kessleri* by mercury at multiple sites. Cytobios 98: 25–37

El-Seekh MM (2004) Inhibition of the water splitting system by sodium chloride stress in the green alga *Chlorella vulgaris*. Braz J Plant Phsiol 16: 25–29

Farineau J (1996) The role of transmembrane electrochemical potential and phosphorylation of PS II proteins in temperature induced light emission from ATP-treated lettuce thylakoids. Photosynth Res 47: 219–230

Farineau J and Laval-Martin D (1995) Characteristics of thermoluminescence bands of *Euglena* cells belonging to 2 lines presenting different degrees of diuron-resistance. Z Naturforsch 50c: 86–92

Gilbert M, Skotnica J, Weingart I and Wilhelm C (2004a) Effects of UV irradiation on barley and tomato leaves: thermoluminescence as a method to screen the impact of UV radiation on crop plants. Funct Plant Biol 31: 825–845

Gilbert M, Wagner H, Weingart I, Skotnica J, Nieber K, Tauer G, Bergmann F, Fischer H and Wilhelm C (2004b) A new type of thermoluminometer: A highly sensitive tool in applied photosynthesis research and plant stress physiology. J Plant Physiol 161: 641–651

Govindjee, Kern JF, Messinger J and Whitmarsh J (2010) Photosystem II. In: Encyclopedia of Life Sciences (ELS), John Wiley & Sons, Chichester; doi: 10.1002/9780470015902.a0000669.pub2

Govindjee, Koike H and Inoue Y (1985) Thermoluminiscence and oxygen evolution from a thermophilic blue-green alga obtained after single-turnover light flashes. Photochem Photobiol 42: 579–585

Havaux M (1998) Probing electron transport through and around photosystem II *in vivo* by the combined use of photoacoustic spectroscopy and chlorophyll fluorometry. Israel J Chem 38: 247–256

Havaux M (2003) Spontaneous and thermoinduced photon emission: New methods to detect and quantify oxidative stress in plants. Trends Plant Sci 8: 409–413

Havaux M and Niyogi KK (1999) The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. Proc Natl Acad Sci USA 96: 8762–8767

Hideg E and Demeter S (1985) Binary oscillation of delayed luminescence – Evidence of the participation of Q_B^- in the charge recombination. Z Naturforsch 40c: 827–831

Hideg E and Vass I (1993) The 75°C thermoluminescence band of green tissues: Chemiluminescence from membrane-chlorophyll interaction. Photochem Photobiol 58: 280–283

Homann PH (1999) Reliability of Photosystem II thermoluminescence measurements after sample freezing: Few artefacts with Photosystem II membranes but gross distortions with certain leaves. Photosynth Res 62: 219–229

Homann PH and Madabusi LV (1993) Modification of the thermoluminescence properties of Ca^{2+} depleted photosystem II membranes by the 23 kDa extrinsic polypeptide and by oligocarboxylic acids. Photosynth Res 35: 29–39

Horváth G, Arellano JB, Droppa M and Baron M (1998) Alterations in photosystem II electron transport as revealed by thermoluminescence of Cu-poisoned chloroplasts. Photosynth Res 57: 175–182

Huner NPA, Ivanov AG, Sane PV, Pocock T, Krol M, Balseris A, Rosso D, Savitch LV, Hurry VM and Öquist G (2006) Photoprotection of photosystem II: Reaction center quenching versus antenna quenching. In: Demming-Adams B, Adams WWIII, Mattoo AK (eds) Photoprotection, Photoinhibition, Gene Regulation and Environment, Advances in Photosynthesis and Respiration, Vol 21, pp 155–173. Springer, Dordrecht

Ichikawa T, Inoue Y and Shibata K (1975) Characteristics of thermoluminescence bands in intact leaves and isolated chloroplasts in relation to the water-splitting activity in photosynthesis. Biochim Biophys Acta 408: 228–239

Inoue Y (1976) Manganese catalyst as a possible cation carrier in thermoluminescence. FEBS Lett 72: 279–282

Inoue Y (1981) Charging of the A band thermoluminescence dependent on S_3-state in isolated chloroplasts. Biochim Biophys Acta 634: 309–320

Inoue Y (1996) Photosynthetic thermoluminescence as a simple probe of photosystem II electron transport. In: Amesz J and Hoff AJ (eds) Biophysical Techniques in Photosynthesis, Advances in Photosynthesis, Vol 3, pp 93–107. Kluwer Academic Publishers, Dordrecht

Inoue Y and Shibata K (1977a) Oscillation of thermoluminescence at medium low temperature. FEBS Lett 85: 192–197

Inoue Y and Shibata K (1977b) Thermoluminescence bands of chloroplasts as characterized by flash excitation. In: Hall DO, Coombs J and Goodwin TW (eds) Proceedings of the Fourth International Congress on Photosynthesis, pp 211–221. The Biochemical Society, London

Inoue Y and Shibata K (1982) Thermoluminescence from photosynthetic apparatus. In: Govindjee (ed) Photosynthesis: Energy Conversion by Plants and Bacteria, pp 507–533. Academic Press, New York

Inoue Y, Yamasita T, Kobayashi Y and Shibata K (1977) Thermoluminescence changes during inactivation and reactivation of the oxygen-evolving system of isolated chloroplasts. FEBS Lett 82: 303–306

Ivanov AG, Sane PV, Zeinalov Y, Malmberg G, Gardeström P, Huner NPA and Öquist G (2001) Photosynthetic electron transport adjustments in overwintering Scots pine (*Pinus sylvestris* L.). Planta 213: 575–585

Ivanov AG, Sane PV, Zeinalov Y, Huner NPA and Öquist G (2002) Seasonal responses of photosynthetic electron transport in Scots pine (*Pinus sylvestris* L.) studied by thermoluminescence. Planta 215: 457–465

Ivanov AG, Sane PV, Hurry V, Krol M, Sveshnikov D, Huner NPA and Öquist G (2003) Low-temperature modulation of the redox properties of the acceptor side of photosystem II: photoprotection through reaction centre quenching of excess energy. Physiol Plant 119: 376–383

Ivanov, AG, Krol M, Apostolova EL, Morgan-Kiss RM, Naydenova N, Huner NPA and Sane PV (2005) Oligomerization state of LHCII modulates the redox properties of the acceptor side of photosystem II in *Costata-2/133* mutant of pea. Physiol Mol Biol Plants 11: 199–207

Ivanov AG, Sane PV, Krol M, Gray GR, Balseris A, Savitch LV, Öquist G and Huner NPA (2006) Acclimation to temperature and irradiance modulates PS II charge recombination. FEBS Lett 580: 2797–2802

Ivanov AG, Sane PV, Hurry V, Öquist G and Huner NPA (2008) Photosystem II reaction centre quenching: mechanisms and physiological role. Photosynth Res 98: 565–574

Janda T, Weissner W, Paldi E, Mende D and Demeter S (1992) Thermoluminescence investigation of photoinhibition in the green alga, *Chlamydobotrys stellata* and *Pisum sativum* L. leaves. Z Naturforsch 47c: 585–590

Janda T, Szalai G, Giauffret C, Paldi E and Ducruet J-M (1999) The thermoluminescence 'afterglow' band as a sensitive indicator of abiotic stresses in plants. Z Naturforsch 54c: 629–633

Janda T, Szalai G and Paldi E (2000) Thermoluminescence investigation of low temperature stress in maize. Photosynthetica 38: 635–639

Johnson GN, Boussac A and Rutherford AW (1994) The origin of 40-50°C thermoluminescence bands in photosystem II. Biochim Biophys Acta 1184: 85–92

Johnson GN, Rutherford AW, Krieger A (1995) A change in the midpoint potential of the quinone Q_a in photosystem-II associated with photoactivation of oxygen evolution. Biochim Biophys Acta 1229: 202–207

Jursinic PA and Govindjee (1972) Thermoluminescence and temperature effects on delayed light emission (corrected for changes in quantum yield of fluorescence) in DCMU-treated algae. Photochem Photobiol 15**:** 331–348

Kafarov RS, Shenderova LV, Matorin DN and Venedictov PS (1988) Inhibition of photosynthesis accumulation of lipid peroxides and cell death in *Chlorella* under high light intensity. Fiziol Rastenii 35: 458–463 (In Russian)

Kimura Y, Mizusawa N, IIshii A, Nakazawa S and Ono T (2005) Changes in structural and functional properties of oxygen-evolving complex induced by replacement of D1-glutamate 189 with glutamine in photosystem II. Ligation of glutamate 189 carboxylate to the manganese cluster. J Biol Chem 280: 37895–37900

Klevanik AV (1995) Thermoluminescence during photosynthesis. 3. Isolated reaction centers of photosystem II. Molec Biol 29: 382–386

Koike H, Siderer Y, Ono TA and Inoue Y (1986) Assignment of thermoluminescence A band to $S_3Q_A^-$ charge recombination: sequential stabilization of S_3 and Q_A^- by a two-step illumination at different temperatures. Biochim Biophys Acta 850: 80–89

Kramer DM, Roffey RA, Govindjee and Sayre RT (1994) The A_I thermoluminescence band from *Chlamydomonas reinhardtii* and the effects of mutagenesis of histidine residues on the donor side of the photosystem II D1 polypeptide. Biochim Biophys Acta 1185: 228–237

Krieger A, Bolte S, Dietz KJ and Ducruet J-M (1998) Thermoluminescence studies on the facultative crassulacean acid-metabolism plant *Mesembryanthemum crystallinum L*. Planta 205: 587–594

Krieger A and Rutherford AW (1997) Comparison of chloride-depleted and calcium-depleted PSII: The midpoint potential of Q_A and susceptibility to photodamage. Biochim Biophys Acta 1319: 91–98

Krieger A, Weis E and Demeter S (1993) Low-pH-induced Ca^{2+} ion release in the water-splitting system is accompanied by a shift in the midpoint redox potential of the primary quinone acceptor $Q_{A.}$ Biochim Biophys Acta 1144: 411–418

Krieger-Liszkay A and Rutherford AW (1998) Influence of herbicide binding on the redox potential of the quinone acceptor in photosystem II: Relevance to photodamage and toxicity. Biochemistry 37: 17339–17344

Lurie S and Bertsch W (1974) Thermoluminescence studies on photosynthetic energy conversion. I. Evidence for three types of energy storage by photoreaction II of higher plants. Biochim Biophys Acta 357: 420–428

Mäenpää P, Miranda T, Tyystjarvi E, Tyystjarvi T, Govindjee, Ducruet J-M, Etienne AL and Kirilovsky D (1995) A mutation in the D-de loop of D1 modifies the stability of the $S_2Q_A^-$ and $S_2Q_B^-$ states in photosystem II. Plant Physiol 107: 187–197

Malkin S and Hardt H (1973) Kinetic characterization of T-jump thermoluminescence in isolated chloroplasts. Biochim Biophys Acta 305: 292–301

Mar T and Govindjee (1971) Thermoluminescence in spinach chloroplasts and in *Chlorella.* Biochim Biophys Acta 226: 200–203

Marco E, Ohad N, Schwarz R, Lieman-Hurwitz J, Gabay C and Kaplan A (1993) High CO_2 concentration alleviates the block in photosynthetic electron transport in an ndhB-inactivated mutant of *Synechococcus* sp. PCC 7942. Plant Physiol 101: 1047–1053

Marder JB, Droppa M, Caspi V, Raskin VI and Horvath G (1998) Light-independent thermoluminescence from thylakoids of greening barley leaves. Evidence for involvement of oxygen radicals and free chlorophyll. Physiol Plant 104: 713–719

Matorin, DN, Vasil'ev IR and Vedernikov VI (1992) Photoinhibition of primary reactions of photosynthesis in the natural communities of Black Sea phytoplankton. Soviet Plant Physiol 39: 285–290

Mayers SR, Dubbs MJ, Vass I, Hideg E, Nagy L and Barber J (1993) Further characterization of the *psbH* locus of *Synechocystis* sp. PCC 6803: Inactivation of *psbH* impairs

Q_A to Q_B electron transport in photosystem 2. Biochemistry 32: 1454–1465

Metwally A, Tsonev T and Zeinalov Y (1997) Effect of cytokinins on the photosynthetic apparatus in water-stressed and rehydrated bean plants. Photosynthetica 34: 563–567

Minagawa J, Narusaka Y, Inoue Y and Satoh K (1999) Electron transfer between Q_A and Q_B in photosystem II is thermodynamically perturbed in phototolerant mutants of *Synechocystis* sp. PCC 6803. Biochemistry 38: 770–775

Miranda T and Ducruet J-M (1995a) Effect of dark- and light-induced proton gradients in thylakoids on the Q and B thermoluminescence bands. Photosynth Res 43: 251–262

Miranda T and Ducruet J-M (1995b) Characterization of the chlorophyll thermoluminescence afterglow in dark-adapted or far-red-illuminated plant leaves. Plant Physiol Biochem 33: 689–699

Misra AN, Sahu SM, Misra M, Ramaswamy NK and Desai TS (1999) Sodium chloride salt stress induced changes in thylakoid pigment-protein complexes, photosystem II activity and thermoluminescence glow peaks. Z Naturforsch 54c: 640–644

Misra AN, Dilnawaz F, Misra M and Biswal AK (2001) Thermoluminescence in chloroplasts as an indicator of alterations in photosystem 2 reaction centre by biotic and abiotic stresses. Photosynthetica 39: 1–9

Mizusawa N, Kimura Y, Ishii A, Yamanari T, Nakazawa S, Teramoto H and Ono T (2004) Impact of replacement of D1 C-terminal alanine with glycine on structure and function of photosynthetic oxygen-evolving complex. J Biol Chem 279: 29622–29627

Noguchi T, Inoue Y and Sonoike K (1993) Thermoluminescence emission at liquid helium temperature from photosynthetic apparatus and purified pigments. Biochim Biophys Acta 1141: 18–24

Ohad I, Koike H, Shochat S and Inoue Y (1988) Changes in the properties of reaction center II during the initial stages of photoinhibition as revealed by thermoluminescence measurements. Biochim Biophys Acta 933: 288–298

Ohad I, Adir N, Koike H, Kyle DJ and Inoue Y (1990) Mechanism of photoinhibition *in vivo*. A reversible light-induced conformational change of reaction center II is related to an irreversible modification of the D1 protein. J Biol Chem 265: 1972–1979

Ono T and Inoue Y (1991) Biochemical evidence for histidine oxidation in photosystem II depleted of the Mn-cluster for O_2-evolution. FEBS Lett 278: 183–186

Ono T, Noguchi T and Yoshihiro N (1995) Characteristic changes of function and structure of photosystem II during strong-light photoinhibition under aerobic conditions. Biochim Biophys Acta 1229: 239–248

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, Springer, Dordrecht

Peeva V and Maslinkova L (2004) Thermoluminescence study of photosystem II activity in *Haberlea rhodopensis* and spinach leaves during desiccation. Plant Biol 6: 319–324

Pocock T, Sane PV, Falk S and Hüner NPA (2007) Excitation pressure regulates the activation energy for recombination events in PSII reaction centres of *Chlamydomonas reinhardtii*. Biochem Cell Biol 85: 721–729

Randall JT and Wilkins MHF (1945) Phosphorescence and electron traps. The study of trap distribution. Proc R Soc London A 184: 366–389

Rane SS and Sane PV (1985) Characterization of glow peaks of chloroplast membranes: Part IV: Identification of different components of delayed light and glow peaks of chloroplasts. Ind J Exp Biol 23**:** 370–374

Rantamäki S and Tyystjärvi E (2011) Analysis of $S_2Q_A^-$ charge recombination with the Arrhenius, Eyring and Marcus theories. J Photochem Photobiol B: Biol 104: 292–300

Rappaport F and Lavergne J (2009) Thermoluminescence: theory. Photosynth Res 101: 205–216

Rappaport F, Cuni A, Xiong L, Sayre RT and Lavergne J (2005) Charge recombination and thermoluminescence in photosystem II. Biophys J 88: 1948–1958

Regel RE, Ivleva NB, Zer H, Meurer J, Shestakov SV, Hermann RG, Pakrasi HB and Ohad I (2001) Deregulation of electron flow within photosystem II in the absence of the PsbJ protein. J Biol Chem 276: 41473–41478

Roman A and Ducruet J-M (2000) Evidence from leaf thermoluminescence for a decrease of the [NADPH plus ATP] energetic potential in cold sensitive *Pisum sativum* L. varieties upon hardening at 5°C. J Plant Physiol 157: 177–181

Rose S, Minagawa J, Seufferheld M, Padden S, Svensson B, Kolling DRJ, Crofts AR and Govindjee (2008) D1-arginine257 mutants (R257E, K, and Q) of *Chlamydomonas reinhardtii* have a lowered Q_B redox potential: analysis of thermoluminescence and fluorescence measurements. Photosynth Res 98: 449–468

Rosza Z and Demeter S (1982) Effect of inactivation of the oxygen evolving system on the thermoluminescence of isolated chloroplasts. Photochem Photobiol 36: 705–708

Rubin AB and Venediktov PS (1969) Storage of light energy by photosynthesizing organisms at low temperature. Biofizika 14: 105–109

Rutherford WA, Crofts AR and Inoue Y (1982) Thermoluminescence as a probe of photosystem II photochemistry; the origin of the flash-induced glow peaks. Biochim Biophys Acta 682: 457–465

Rutherford WA, Govindjee and Inoue Y (1984) Charge accumulation and photochemistry in leaves studied by thermoluminescence and delayed light emission. Proc Natl Acad Sci USA 81**:** 1107–1111

Rutherford WA, Renger G, Koike H and Inoue Y (1985) Thermoluminescence as a probe of PSII: The redox and protonation state of the secondary acceptor quinone and the O_2 evolving enzyme. Biochim Biophys Acta 767: 548–556

Sane PV (2004) Thermoluminescence: A technique for probing photosystem II. In: Carpentier R (ed) Methods in

Molecular Biology: Photosynthesis Research Protocols, pp 229–248. Humana Press Inc, Totowa

Sane PV and Rutherford AW (1986) Thermoluminescence from photosynthetic membranes. In: Govindjee, Amesz J and Fork DC (eds) Light Emission by Plants and Bacteria, pp 329–360. Academic Press, Orlando

Sane PV, Tatake VG and Desai TS (1974) Detection of the triplet states of chlorophyll *in vivo.* FEBS Lett 45: 290–294

Sane PV, Desai TS, Tatake VG and Govindjee (1977) On the origin of glow peaks in *Euglena* cells, spinach chloroplasts and subchloroplast fragments enriched in system I or II. Photochem Photobiol 26: 33–39

Sane PV, Desai TS and Tatake VG (1983a) Characterization of glow peaks of chloroplast membranes. Part I. – Relationship with "S" states. Ind J Exp Biol 21: 396–400

Sane PV, Desai TS, Rane SS and Tatake VG (1983b) Characterization of glow peaks of chloroplast membranes. Part II – Relationship with redox components. Ind J Exp Biol 21: 401–404

Sane PV, Ivanov AG, Sveshnikov D, Huner NPA and Öquist G (2002) A transient exchange of the photosystem II reaction center protein D1:1 with D1:2 during low temperature stress of *Synechococcus sp* PCC 7942 in the light lowers the redox potential of Q_B. J Biol Chem 277: 32739–32745

Sane PV, Ivanov, AG, Hurry, V, Huner NPA and Öquist G (2003) Changes in redox potential of primary and secondary electron-accepting quinones in photosystem II confer increased resistance to photoinhibition in low temperature acclimated Arabidopsis. Plant Physiol 132: 2144–2151

Shen JR, Qian M, Inoue Y and Burnap RL (1998) Functional characterization of *Synechosystis* sp. PCC 6803 delta *psbU* and delta *psbV* mutants reveals important roles of cytochrome *c*-550 in cyanobacterial oxygen evolution. Biochemistry 37: 1551–1558

Shuvalov VA and Litvin FF (1969) Mechanism of prolonged after-luminescence of plant leaves and energy storage in the photosynthetic reaction centers. Molec Biol 3: 59–73

Sicora C, Wiklund R, Jansson C and Vass I (2004) Charge stabilization and recombination in photosystem II containing the D1' protein product of the *psbA1* gene in *Synechocystis* 6803. Phys Chem Chem Phys 6: 4832–4837

Skotnica J, Fiala J, Ilik P and Dvorak L (1999) Thermally induced chemiluminescence of barley leaves. Photochem Photobiol 69: 211–217

Skotnica J, Matouskova M, Naus J, Lazar D and Dvorak L (2000) Thermoluminescence and fluorescence study of changes in photosystem II photochemistry in desiccating barley leaves. Photosynth Res 65: 29–40

Skotnica J, Gilbert M, Weingart I and Wilhelm C (2003) Thermoluminescence as a tool for monitoring ozone-stressed plants. Environ Poll 123: 15–20

Skotnica J, Gilbert M, Weingart I and Wilhelm C (2005) The mechanism of the ozone-induced changes in thermoluminescence glow curves of barley leaves. Photosynthetica 43: 425–434

Solntsev MK (1995) The influence of spectral composition of exiting light on bean leaf thermoluminescence at 40-70°C. Biofizika 40:417–421

Sonoike K, Koike H, Enami I and Inoue Y (1991) The emission spectra of thermoluminescence from photosynthetic apparatus. Biochim Biophys Acta 1058: 121–130

Stallaert VM, Ducruet J-M, Tavernier E and Blein JP (1995) Lipid peroxidation in tobacco leaves treated with the elicitor cryptogein: Evaluation by high-temperature thermoluminescence emission and chlorophyll fluorescence. Biochim Biophys Acta 1229: 290–295

Strehler B and Arnold W (1951) Light production by green plants. J Gen Physiol 34: 809–820

Sveshnikov D, Ensminger I, Ivanov AG, Campbell D, Lloyd J, Funk C, Huner NPA and Öquist G (2006) Excitation energy partitioning and quenching during cold acclimation in Scots pine. Tree Physiol 26: 325–336

Tal S, Keren N, Hirschberg J and Ohad I (1999) Photosystem II activity and turnover of the D1 protein are impaired in the *psbA* Y112L mutant of *Synechocystis* PCC6803 sp. J Photochem Photobiol B: Biol 48: 120–126

Tatake VG, Desai TS and Bhattacharjee SK (1971) Variable temperature cryostat for thermoluminescence studies. J Phys E Sci Instrum 4: 755–757

Tatake VG, Desai TS, Govindjee, and Sane PV (1981) Energy storage states of photosynthetic membranes: Activation energies and lifetimes of electrons in the trap states by thermoluminescence method. Photochem Photobiol 33: 243–250

Tollin G and Calvin M (1957) The luminescence of chlorophyll-containing plant material. Proc Natl Acad Sci USA 43: 895–908

Tyystjärvi E and Rantamäki S (2009) Connectivity of photosystem II is the physical basis of retrapping in photosynthetic thermoluminescence. Biophys J 96: 1–9

Vass I (2003) The history of photosynthetic thermoluminescence. Photosynth Res 76: 303–318

Vass I and Govindjee (1996) Thermoluminescence from the photosynthetic apparatus. Photosynth Res 48: 117–126

Vass I and Inoue Y (1986) pH dependent stabilization of $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs studied by thermoluminescence. Photosynth Res 10: 431–436

Vass I, Horwath G, Herczeg T and Demeter S (1981) Photosynthetic energy conservation investigated by thermoluminescence. Activation energies and half lives of thermoluminescence bands of chloroplasts determined by mathematical resolution of glow curves. Biochim Biophys Acta 634: 140–152

Vass I, Ono T and Inoue Y (1987) Removal of 33 kDa extrinsic protein specifically stabilizes the $S_2Q_A^-$ charge pair in photosystem II. FEBS Lett 211: 215–220

Vass I, Mohanty N and Demeter S (1988) Photoinhibition of electron transport activity of photosystem II in isolated

thylakoids studied by thermoluminescence and delayed luminescence. Z Naturforsch 43c: 871–876

Vass I, Chapman DJ and Barber J (1989) Thermoluminescence properties of the isolated photosystem two reaction center. Photosynth Res 22: 295–301

Vavilin DV and Ducruet J-M (1998) The origin of the 115–130°C thermoluminescence bands in chlorophyll-containing material. Photochem Photobiol 68: 191–198

Vavilin DV, Vermaas WFJ (2000) Mutations in the CD-loop region of the D2 protein in *Synechocystis* sp. PCC 6803 modify charge recombination pathways in photosystem II *in vivo*. Biochemistry 39: 14831–14838

Vavilin DV, Ducruet J-M, Matorin DN, Venediktov PS and Rubin AB (1998) Membrane lipid peroxidation, cell viability and photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. J Photochem Photobiol B: Biol 42: 233–239

Vidyasagar PB, Thomas S, Banerjee M, Hegde U and Shaligram AD (1993) Determination of peak parameters for thermoluminescence glow curves obtained from spinach thylakoid preparations, using mathematical models based on general order kinetics. J Photochem Photobiol B Biol 19: 125–128

Walters RG and Johnson GN (1997) The effect of elevated light on photosystem II function: A thermoluminescence study. Photosynth Res 54: 169–183

Wiessner W, Mende D, Demeter S (1992) Thermoluminescence study of the *in vivo* effects of bicarbonate depletion and acetate/formate presence in the algae *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*. Photosynth Res 34: 279–285

Zeinalov Y and Maslenkova L (1996) A computerized equipment for thermoluminescence investigations. Bulg J Plant Physiol 22: 88–94

Chapter 20

Regulation of Photosystem II Electron Transport by Bicarbonate

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Summary

In oxygenic photosynthesis, carbon dioxide is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and further reduced to carbohydrates. However, CO_2, in the form of carbonate or bicarbonate, is also directly involved in the "light reactions" through structural and regulatory roles within Photosystem II (PS II). A notable feature is antagonistic interactions between bicarbonate (carbonate) and monovalent anions such as formate within PS II. Incubation of PS II-containing samples with formate results in the inhibition of electron flow activity, which can be restored only by the addition of bicarbonate. This "bicarbonate effect" influences molecular processes associated with both the electron acceptor and electron donor sides of PS II. The bicarbonate interaction on the acceptor side is located in the region of the primary and secondary quinones and contributes to the protonation states associated with quinol formation. At physiological pH, bicarbonate (carbonate) is a ligand to the non-heme iron and forms hydrogen bonds to several amino acids of the D1 and D2 proteins. Bicarbonate may stabilize, through conformational means, the reaction center proteins by protonation of certain amino acids near the secondary quinone electron acceptor. A possible functional role in vivo is that it controls PS II electron flow in order to ameliorate the impact of stress conditions leading to, for instance, photoinhibition or thermoinactivation. The role of bicarbonate on the donor of PS II has been the subject of renewed interest and bicarbonate has been suggested to play a role in the assembly of the Mn_4Ca cluster during photoactivation. Additionally, a role as a catalytic base or proton transporter on the donor side of PS II has been proposed. However, while clear evidence for bicarbonate's role on the acceptor side has been established, experiments designed to elucidate the putative role of bicarbonate on the donor side of PS II have not yet provided convincing evidence.

I. Discovery of the Bicarbonate Effect and Early Work

The role of carbon dioxide (CO_2) or bicarbonate in photosynthesis was clear to researchers from the very beginning of the history of photosynthesis. Joseph Priestley observed in 1775 that "a green matter" deposited on the walls of water containers, formed bubbles of pure "dephlogisticated air" (oxygen) (Priestley, 1776). While Ingen-Housz (1779, 1796) discovered the importance of light in this process, it was Senebier (1782) who demonstrated that the production of oxygen by plants depends on the presence of "fixed air" (CO_2) (see also Chapter 30).

Today it is obvious that plants cannot perform the process of photosynthesis without CO_2. In the reactions of photosynthesis, CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and further reduced to carbohydrate (see Chapter 26). To capture light energy to drive CO_2 fixation, two light reactions in series are driven by two photosystems associated with the thylakoid membrane (for the evolution of the two-light reactions and two-photosystems scheme, see Govindjee and Björn, 2011). Photosystem II (PS II) is a water:plastoquinone oxidoreductase (Wydrzynski and Satoh, 2005; Govindjee et al., 2010). It carries out photochemical reactions including primary charge separation and the subsequent electron transfer from water to plastoquinone. Reduced plastoquinone is oxidized by the cytochrome b_6f complex; the latter reduces plastocyanin (see Chapter 21). The second photosystem in the pathway is Photosystem I (PS I) (Golbeck, 2006), which transfers the electrons from reduced plastocyanin ultimately to nicotinamide adenine dinucleotide phosphate ($NADP^+$), which after reduction is used in the

Abbreviations: CA – Carbonic anhydrase; DBMIB – Dibromothymoquinone; DCBQ – 2,5-dichloro-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenolindophenol; DPC – 1,5-diphenylcarbazide; EPR – Electron paramagnetic resonance; FTIR – Fourier transform infrared spectroscopy; K_d – Apparent dissociation constant; OEC – Oxygen-evolving complex; PQ – Plastoquinone; PS I, PS II – Photosystem I, Photosystem II; Q_A – Primary plastoquinone electron acceptor of PS II; Q_B – Secondary plastoquinone electron acceptor of PS II; Y_D – Redox active tyrosine-160 of the D2 protein of PS II; Y_Z – Redox active tyrosine-161 on the D1 protein of PS II

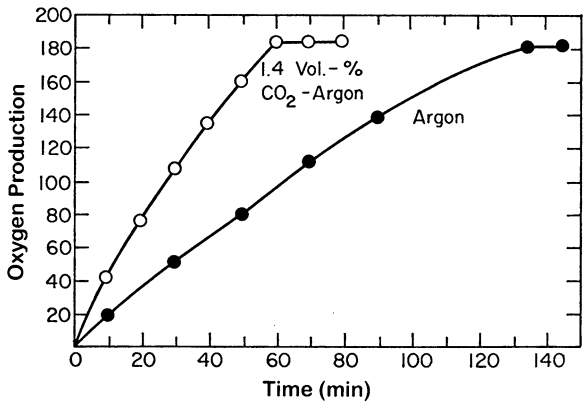

Fig. 20.1. Hill reaction rates of isolated kohlrabi grana suspended in 0.1% KCl. Electron acceptor: 2.1 mg quinone; gas phase: Argon or Argon plus 1.4% CO_2 by volume (Warburg and Krippahl, 1958).

reactions converting fixed CO_2 into C-3 sugars by the Calvin-Benson cycle (Bassham, 2005; Benson, 2005).

During the preparation of chloroplasts one of the electron carriers (ferredoxin) is lost and therefore these preparations do not show oxygen evolution in light. This is because the organelle is broken open during the procedure resulting in thylakoid membranes being obtained. Only after the addition of an oxidant, such as potassium ferricyanide or methyl viologen, do such isolated thylakoids show oxygen evolution upon illumination: this is known as the Hill reaction, named after Robin Hill. While it was assumed for a long time that CO_2 did not play a role in the Hill reaction, Warburg and Krippahl (1958, 1960) discovered that this reaction was dependent on the presence of CO_2. Using grana suspensions isolated from kohlrabi leaves, and adding quinone as an electron acceptor, they found oxygen evolution to occur at a higher rate when the argon gas in the vessel contained 1.2% CO_2 (Fig. 20.1). Abeles et al. (1961) confirmed this effect of CO_2 in kohlrabi thylakoids and showed by mass spectrometry that this effect was specific for oxygen evolution since no metabolism of CO_2 was observed. Because they did not find a substantial effect in sugar beet chloroplast preparations, they concluded that this CO_2 effect was not a general one. A CO_2 requirement for the Hill reaction was shown by Stern and Vennesland (1960, 1962), not only for kolhlrabi but also for isolated spinach thylakoids. They first removed CO_2 by a prolonged incubation of the sample in the dark with KOH in the center well of the Warburg vessel. The Hill reaction rates with ferricyanide and catalytic amounts of trichlorophenol were higher in the presence of CO_2. Because buffered media were used, this was not a pH effect. After the addition of bicarbonate to CO_2-depleted samples, a large stimulation of the Hill reaction rate occurred. This effect was present in thylakoids isolated from different plants and using different electron acceptors.

It was Izawa (1962) who compared the different techniques used, in various studies, in producing the CO_2 effect. While Abeles et al. (1961) eliminated buffer from the reaction media (and used no pre-treatment), others used very long pre-incubation of the chloroplast preparations with CO_2-removing methods. Izawa realized that these methods induced a substantial loss of Hill reaction activity of the thylakoid membranes or a change in pH. Therefore, he added carbonic anhydrase (CA) in combination with KOH in the center well of the Warburg vessel. In this way, the equilibration between free CO_2 and carbonic acid was speeded up, and he obtained a CO_2 effect within 15 min. Good (1963) was equally engaged in evaluating the very different methods that were being used to produce the CO_2 effect. He observed that the loss of Hill reaction activity by incubating isolated thylakoids under a CO_2-free atmosphere is greater in the presence of small anions, especially of formate, acetate, fluoride or chloride.

While the topic suffered a while because of lack of reliable methods to reproduce the CO_2 effect, Stemler and Govindjee (1973) made the key finding that the monovalent anions were much more active if the isolated thylakoids were pretreated at a pH below 6.0. By incubating thylakoids at this low pH and in the presence of a high concentration of monovalent anions, the bicarbonate effect becomes highly reproducible. A reliable effect could now be produced by depletion of CO_2 from thylakoid membranes by flushing the suspensions in the dark with CO_2-free air or nitrogen gas, while the chloroplasts are suspended in a medium of a pH between 5.5 and 6.0, and containing a high concentration of anion, usually formate. The resulting electron transport rate is then measured in a medium of usually pH 6.5; and it is indeed very low. Because the stimulation is evoked by the addition of a bicarbonate solution to the CO_2-depleted samples, the phenomenon is named the *bicarbonate effect*.

Stemler (1989) has challenged the hypothesis that formate inhibition of photosynthetic electron transport functions by displacing bicarbonate, because he found no CO_2 release after formate addition to maize thylakoids. However, Govindjee et al. (1991) clearly demonstrated that within minutes after the addition of 100 mM sodium formate, both pea and spinach thylakoids release CO_2, the amount being much greater (about 1 CO_2 per PS II reaction center) at pH 6.5 than at pH 6.0.

The stimulation of the Hill reaction by CO_2 was discussed by Otto Warburg (Warburg, 1964) as a proof of his theory that CO_2 was the source of photosynthetically-evolved oxygen, and not water. One of the very few who picked up Warburg's ideas was Alan Stemler in Govindjee's laboratory (see Stemler, 1998). In the mid 1970s, Thomas Wydrzynski and Govindjee found that bicarbonate has a clear and large effect on the acceptor side of PS II (Wydrzynski and Govindjee, 1975). This effect was very easy to reproduce and since then much attention has been given to this acceptor side effect. However, work from the group of Vyacheslav Klimov (Van Rensen and Klimov, 2005) supported the early work of Stemler and Govindjee (Stemler and Govindjee, 1973; Stemler et al., 1974), who had shown an effect of bicarbonate on the donor side of PS II.

The bicarbonate effect is thought to require carbon dioxide as the diffusing species and bicarbonate and carbonate as active species. For an early review on the bicarbonate effect, see Govindjee and Van Rensen (1978); for an elaborate review, see Blubaugh and Govindjee (1988a). Also see reviews by Diner et al. (1991), Govindjee and Van Rensen (1993), Van Rensen et al. (1999), Klimov and Baranov (2001), Stemler (2002), Van Rensen (2002) and that by Van Rensen and Klimov (2005).

II. Regulation of Electron Flow at the Acceptor Side of Photosystem II

Wydrzynski and Govindjee (1975) were the first to locate an effect of bicarbonate on the acceptor side of PS II. They measured chlorophyll *a* fluorescence induction kinetics in isolated thylakoid membranes in which artificial electron donors replaced water. The variable chlorophyll fluorescence monitors the redox state of Q_A: Q_A is a quencher of fluorescence, whereas Q_A^- is not (see chapters in Papageorgiou and Govindjee, 2004). The accumulation of Q_A^- can be monitored by the increase of the fluorescence yield. Inhibition of electron transport beyond Q_A results in the rapid increase of chlorophyll *a* fluorescence. CO_2 depletion causes a fast increase of the fluorescence yield, similar but not identical to that observed when the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added. It is known that DCMU inhibits the reoxidation of Q_A^- by the secondary electron acceptor Q_B since it replaces it (Velthuys and Amesz, 1974; Van Rensen, 1982).

The bicarbonate effect is not observed in PS I (Khanna et al., 1977) nor in photosynthetic bacterial reaction centers (Shopes et al., 1989; Wang et al., 1992). Thus, bicarbonate is required for the functioning of PS II, and a major effect is at the electron acceptor side.

A. The Nature of the Active Species

Good (1963) observed that the loss of Hill reaction activity in bicarbonate-depleted thylakoid membranes is related to the presence of other small anions. Rather high concentrations of formate, acetate, fluoride or chloride must be present before the removal of CO_2 results in a major reduction of the Hill reaction rate. The effect of CO_2 removal is fully reversible. After CO_2 depletion the thylakoid membranes become completely inactive, but exposure to CO_2 results in full recovery of their Hill reaction activity (Fig. 20.2). The correlation of the CO_2 dependence with the presence of such small anions suggested that bicarbonate, and not CO_2, is the important species.

In water, dissolved CO_2 has the following equilibria:

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow \quad (1) \qquad (2)$$

$$H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad (3)$$

The concentration of H_2CO_3 is essentially dependent upon dissolved CO_2; the pKa of reaction (2) is 6.37. The pKa of reaction (3) is about 10.2; steps (2) and (3) are fast compared to step (1). The hydration of CO_2 or the dehydration

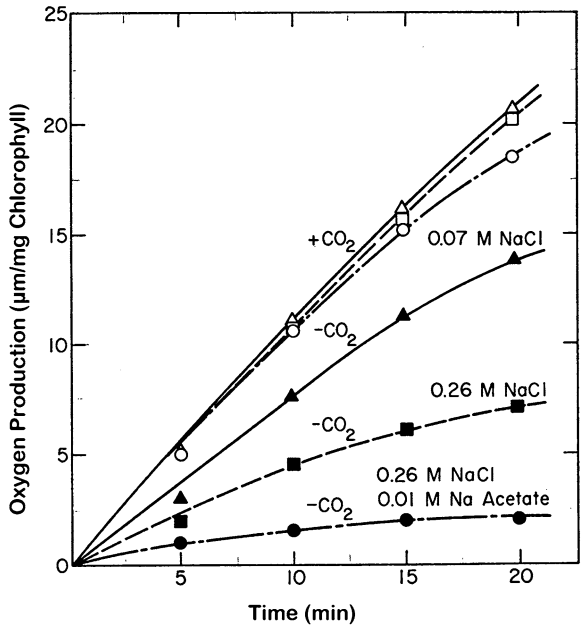

Fig. 20.2. The influence of chloride and acetate on the CO_2 dependence of the Hill reaction. The pea chloroplasts at a concentration of 126 μg chlorophyll were incubated for 2.5 h at 16°C in dim light and in 50 mM phosphate buffer containing the indicated concentrations of chloride and acetate plus dichlorophenol indophenol, DCPIP. (The unit of rate of oxygen production in the ordinate is micromol.) Final pH 6.6; final volume 1.9 ml. The center well of the Warburg manometer vessels contained either KOH or CO_2 buffer. The concentration of the CO_2 in the gas phase in equilibrium with this CO_2 buffer was about 1.1%. Twenty μmol of potassium ferricyanide in 0.1 ml water were tipped into the vessel from the side arm after the incubation period and before the start of illumination. The three lower curves illustrate the progress of ferricyanide reduction in the absence of CO_2; the three upper curves are their controls with CO_2 (Good, 1963).

of H_2CO_3 is the slowest step: at pH < 8 and at 18°C a dissolved CO_2 molecule lives on the average about 1 min before it is hydrated; this step can be speeded up by CA. Stemler and Govindjee (1973) measured the bicarbonate effect at pH 5.8 and 6.8. At pH 6.8 the concentration of bicarbonate is much higher than that of CO_2, while at pH 5.8 the concentration of CO_2 is about 4-times that of bicarbonate. Their finding that at subsaturating concentrations of bicarbonate the stimulation was much higher at pH 6.8 compared with that at pH 5.8, suggests that bicarbonate is the active species.

The pH profile of the bicarbonate effect has an optimum around pH 6.5 (Khanna et al., 1977; Vermaas and Van Rensen, 1981). While these reports confirmed the results of Stemler and Govindjee (1973), the decrease of the stimulation by bicarbonate at pH values above 6.5 does not correspond with bicarbonate being the active species, because the concentration of bicarbonate increases above that pH value. Vermaas and Van Rensen (1981) suggested that bicarbonate is not the only species required. They proposed that bicarbonate is the species that binds at the thylakoid membrane, but that the molecule reaches its binding site in CO_2 form (also see Sarojini and Govindjee, 1981). The thylakoid membrane is negatively charged at the stroma side and is therefore not readily accessible to the charged HCO_3^-; however, the uncharged CO_2 can easily pass.

That bicarbonate was indeed the active species was demonstrated by Blubaugh and Govindjee (1986). They took advantage of the pH dependence of the equilibrium ratio of $[CO_2]$ to $[HCO_3^-]$ to effectively hold one concentration constant while varying the other. The restoration of the Hill reaction after CO_2 depletion was shown to be dependent only on $[HCO_3^-]$ and was apparently independent of $[CO_2]$, $[H_2CO_3]$ or $[CO_3^{2-}]$ over the pH range of 6.3–6.9, which spanned both sides of the pH optimum. Although these results indicated that HCO_3^- is the binding species, they leave open a possible role for CO_2 as the diffusing species to the binding site, since they were performed under equilibrium conditions.

B. Bicarbonate Acts at the Electron Acceptor Side of Photosystem II

As mentioned above, the chlorophyll *a* fluorescence induction experiments of Wydrzynski and Govindjee (1975) were the first indications for an action site at the electron acceptor (also referred to as "reducing") side of PS II. Further work located the site of action at the reducing site more precisely.

While fluorescence increase monitors the reduction of Q_A to Q_A-, the decay of the chlorophyll fluorescence yield after a saturating flash of light monitors the reoxidation of Q_A^-. Important information can be obtained by measuring the decay of the fluorescence yield, after various numbers of short saturating flashes, which monitors the following events:

After an odd number of flashes:

$$Q_A Q_B \xrightarrow{h\upsilon} Q_A^- Q_B \rightarrow Q_A Q_B^-$$

And after an even number of flashes:

$$Q_A Q_B^- \xrightarrow{h\upsilon} Q_A^- Q_B^- \rightarrow Q_A Q_B^{2-}$$

Then, Q_B^{2-} becomes protonated and exchanges with the PQ pool:

$$Q_A Q_B^{2-} + 2H^+ + PQ \rightarrow Q_A Q_B + PQH_2$$

Govindjee et al. (1976) found no differences in the decay of chlorophyll *a* fluorescence yield after various numbers of flashes in control and in CO_2-depleted samples, to which bicarbonate was added. However, in CO_2-depleted thylakoid membranes, they found little effect on the fluorescence intensity measured 160 ms after one or two flashes, but a much higher fluorescence after three or more flashes. Robinson et al. (1984) measured the fluorescence decay in a time range up to 10 ms and showed that in CO_2-depleted thylakoids, the Q_A^- decay after one flash is about 5 times slower than in control samples, but after three or more flashes it is 36-fold slower. Although the absolute values of the rates of the Q_A^- decay in this type of experiment depend on the conditions of the experiment, it is clear that there is a small inhibition by CO_2 depletion on the reoxidation of Q_A^- by Q_B, a larger effect on of the reoxidation by Q_B^-, and a much larger inhibition of the protonation of Q_B^{2-} and exchange with the plastoquinone pool. In CO_2-depleted thylakoid membraness three electrons can be stored leading to $Q_A^- Q_B^{2-}$. For a detailed study, see Eaton-Rye and Govindjee (1988a, b).

The localization of the bicarbonate effect was also determined by studying its effect on various parts of the electron transport chain (Khanna et al., 1977; Eaton-Rye and Govindjee, 1984). Electron transport from reduced diaminodurene to methyl viologen was not affected by CO_2 depletion, indicating the absence of an effect on PS I-dependent electron flow. A large bicarbonate-effect was observed on the electron transport from water to oxidized diaminodurene in the presence of dibromothymoquinone (DBMIB), indicating an effect before the plastoquinone pool. Because the electron flow from water to silicomolybdate in the presence of DCMU was not affected by CO_2 depletion, Khanna et al. (1981) concluded that the bicarbonate-effect is located between Q_A and the PQ pool. The same conclusions were reached by Van Rensen and Vermaas (1981) because there was no effect of bicarbonate depletion on electron transport in trypsin-treated thylakoids in which ferricyanide accepts electrons directly at the Q_A site.

The site of inhibition by the absence of bicarbonate was further inferred from the interaction of bicarbonate (or formate) with PS II-inhibiting herbicides, as studied by Khanna et al. (1981), Van Rensen and Vermaas (1981), Vermaas et al. (1982) and Snel and Van Rensen (1983). By adding different concentrations of bicarbonate to CO_2-depleted thylakoids, various rates of restoration of the Hill reaction can be obtained. The activity of the system represented by the thylakoid membrane versus bicarbonate concentration has Michaelis-Menten kinetics and can be treated like an enzyme system versus substrate. From the double reciprocal plots of the rate of the Hill reaction as a function of the bicarbonate concentration (Fig. 20.3)

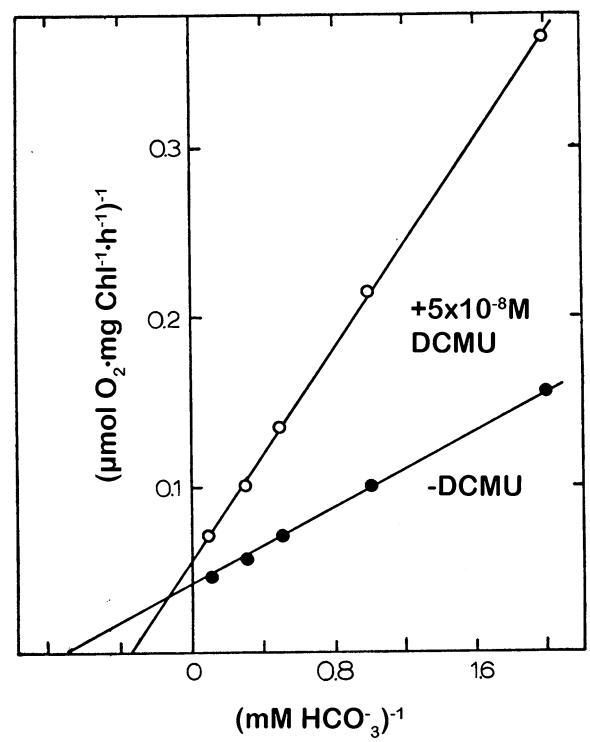

Fig. 20.3. Double reciprocal plot of the Hill reaction rate as a function of the bicarbonate concentration in CO_2-depleted pea chloroplasts in the absence and in the presence of 5×10^{-8} M DCMU. Reaction medium: 50 mM Na-phosphate, 100 mM Na-formate, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM potassium ferricyanide (pH 6.5) and 33 μg chlorophyll ml^{-1}.

the apparent dissociation constant (K_d) of the thylakoid-bicarbonate complex could be calculated. When 100 mM formate is present in the reaction medium, the apparent K_d appears to be about 1 mM bicarbonate. The K_d for bicarbonate depends on the presence of both formate and of herbicides. In the presence of lower concentrations of formate, the apparent K_d decreases, approaching 80 μM $NaHCO_3$ in the absence of formate (Stemler and Murphy, 1983; Snel and Van Rensen, 1984). In the presence of urea, triazine or phenol-type herbicides, the K_d for bicarbonate increases by at least two-fold (Fig. 20.3), indicating that these herbicides decrease the apparent affinity of the thylakoid membrane for bicarbonate.

Because these PS II herbicides bind at the D1 protein, the above mentioned interactions of bicarbonate with the herbicides suggested the involvement of the D1 protein in the bicarbonate effect. A further indication was obtained using herbicide-resistant mutants. Khanna et al. (1981) observed an increased binding constant for bicarbonate in thylakoids of triazine-resistant *Amaranthus hybridus*, in which Ser264 is replaced by glycine in the D1 protein. In the presence of 100 mM formate, the binding constant of bicarbonate increases from 1 mM in thylakoids of the sensitive (wild type) plants to about 2 mM in the resistant ones. That implies that the affinity for bicarbonate is lowered in the resistant membranes (see also Srivastava et al., 1995).

C. Structural Binding Environment of Bicarbonate in Photosystem II

The arguments presented in the previous section lead to the idea that the binding site of bicarbonate is located on the D1 protein of PS II. Studies by Blubaugh and Govindjee (1988b) indicated two binding sites or domains: one binding site at the non-heme iron and the other at an amino acid residue.

The first indication that the non-heme iron is involved in bicarbonate action was reported by Vermaas and Rutherford (1984). They showed that formate addition to thylakoids increases the amplitude of the g = 1.82 electron paramagnetic resonance (EPR) signal of $Q_A^-Fe^{2+}$ ten-fold. Michel and Deisenhofer (1988) compared the primary structure of the L and M polypeptides of the bacterial reaction centers with the D1 and D2 polypeptides of PS II and suggested that glutamate in bacteria is replaced by bicarbonate in PS II as a bidentate ligand to the non-heme iron. Van Rensen et al. (1988) showed that the kinetics of bicarbonate binding to thylakoids are influenced by the redox state of the non-heme iron. NO has been shown to be able to ligate to the non-heme iron (Diner and Petrouleas, 1990; Petrouleas and Diner, 1990). Kinetic measurements of electron transport between Q_A and Q_B indicated that NO treatment shows the same effect of slowing down electron transport as does formate. This slowing is completely reversed by the addition of bicarbonate, indicating that NO, like formate, displaces bicarbonate from the reaction center. These data also argue in favor of bicarbonate as a ligand to the non-heme iron.

A formate-bicarbonate effect was demonstrated by measurements of EPR spectra of the quinone-iron or $Q_A^-FeQ_B$ complex with and without bicarbonate (Bowden et al., 1991). The Mössbauer spectrum of Fe is affected by formate and is returned to its original form by re-addition of bicarbonate, indicating that the Fe is a key element in the binding of formate that is displaced by bicarbonate (Diner and Petrouleas, 1987; Semin et al., 1990; also see Petrouleas et al., 1994). Moreover, Diner et al. (1991) suggested that iron-bound bicarbonate may be one of the pathways for the protonation of Q_B. A Fourier transform infrared (FTIR) difference spectroscopy study using ^{13}C-labeled bicarbonate has established that bicarbonate is a bidentate ligand of the non-heme iron (Hienerwadel and Berthomieu, 1995); further, spectroscopy indicated that the bicarbonate ion switches from a chelating to a monodentate binding mode when the iron is oxidized (Hienerwadel and Berthomieu, 1995).

In addition to the bicarbonate, the non-heme iron appears to be liganded by four histidines, D1-His215, D1-His272, D2-His214, and D2-His268. The binding site for bicarbonate at the non-heme iron was modeled by Xiong et al. (1996). The residues that form the binding pocket are positively charged and hydrophobic. They were suggested to include D1-Leu233, D1-Val219, D2-Asn230, D2-Thr231, D2-Phe232, D2-Arg233, D2-Ala234, D2-Pro237, and D2-Lys264. The hydroxyl oxygen of the bicarbonate is separated from the main-chain hydrogen of D2-Arg233

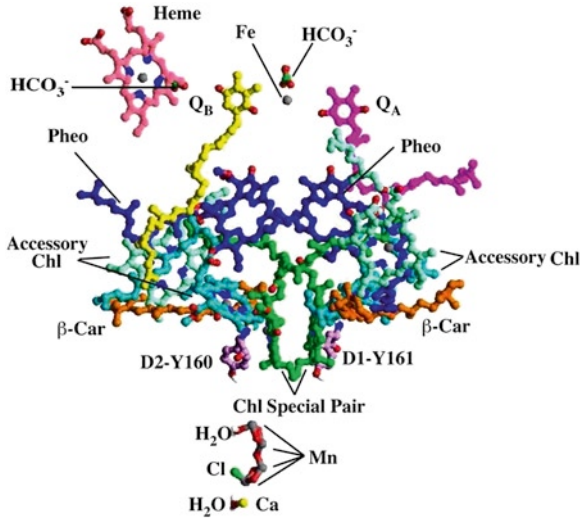

Fig. 20.4. The modeled cofactors in the PS II reaction center (Modified from Van Rensen, 2002; originally from Xiong et al., 1998a).

by 4.8 Å. However, D2-Lys264 appeared to be the most likely candidate to interact directly with the bicarbonate ion.

The structure of PS II at 3.5 Å from the cyanobacterium *Thermocynechococcus elongatus* reported by Ferreira et al. (2004) indicates that the non-heme iron is associated with an electron density sufficient to accommodate the bicarbonate ion. Close to this non-protein density are D1-Tyr246 and D2-Lys264, positioned and oriented such that they could stabilize the bicarbonate by hydrogen bonding. In Fig. 20.4, the 1998 model of the bicarbonate-binding niche is presented (Van Rensen and Klimov, 2005).

Working with spinach thylakoid membranes, the second binding site was suggested by Blubaugh and Govindjee (1988a) to be at the D1 protein at Arg257 and His252, the bicarbonate ion at both sites binding cooperatively. Characterization of Q_B cyanobacterial mutants that are also herbicide-resistant yielded more information about the bicarbonate-binding environment (Cao et al., 1992; Mäenpää et al., 1995; Vernotte et al., 1995). Because anionic bicarbonate/formate is very probably the active species involved, it may be expected that the binding would be electrostatic in nature and therefore positively charged amino acid residues are likely to participate in anion binding. While the Q_B binding niche is composed of the residues including D1-Met214, D1-Leu218, D1-Ala251, D1-Phe255, and D1-Leu271 (Ferreira et al., 2004), only three positively charged D1 residues are found near the Q_B-binding environment: D1-His252, D1-Arg257, and D1-Arg269. Sequence analysis of the D1 protein indicates that D1-Arg257 is closer to the Q_B binding niche than D1-Arg269, making it a more likely residue to be involved in the protonation of Q_B. This residue is thought to be located on the stromal/cytosolic side between the putative transmembrane helices D and E of D1 and may be located within or close to the D1-de helix. Xiong et al. (1998b) constructed and characterized two site-directed mutants in *Chlamydomonas reinhardtii*: D1-Arg257Glu and D1Arg257Met. They concluded that D1-Arg257 with its positively charged side chain is important for the binding of formate, and in all likelihood also for bicarbonate. Modifications at D1-Arg257 also appear to impact on the effective redox potential of the Q_B/Q_B^- pair (Rose et al., 2008). However, to date only one density in the cyanobacterial PS II structure from *Thermosynechococcus elongatus* has been assigned to bicarbonate and confirmation of the second site may require improvements in the resolution of the structural information and/or a structure from a eukaryotic system (Guskov et al., 2009). Takahashi et al. (2009), on the other hand, have proposed from FTIR measurements of PS II core complexes that D1-Tyr246 (or D2-Tyr244) provides a hydrogen bond to the oxygen of the bicarbonate ligand (Fig. 20.5). They further suggest "the Tyr residue coupled to the non-heme iron may play a key role in the regulatory function of the iron-bicarbonate center by stabilizing the bicarbonate ligand and forming a rigid hydrogen bond network around the non-heme ion." The most recent atomic level structure, at 1.9 Angstrom resolution of PS II, has now provided the final picture (Umena et al., 2011); the closest amino acids to bicarbonate are : D2-Tyr244 and D1-Tyr246. Further new experiments with mutants of D2-Lys264, D2-Tyr244 and D1-Tyr246 are needed to investigate the definitive role of bicarbonate on the electron acceptor side of PS II (also see McEvoy and Brudvig, 2008).

D. Functional Role(s) of Bicarbonate

In the preceding section two bicarbonate binding sites were introduced at the reducing side of PS II; one at the non-heme iron (close to D2-Lys264 and/or one of the tyrosines D1-Tyr246

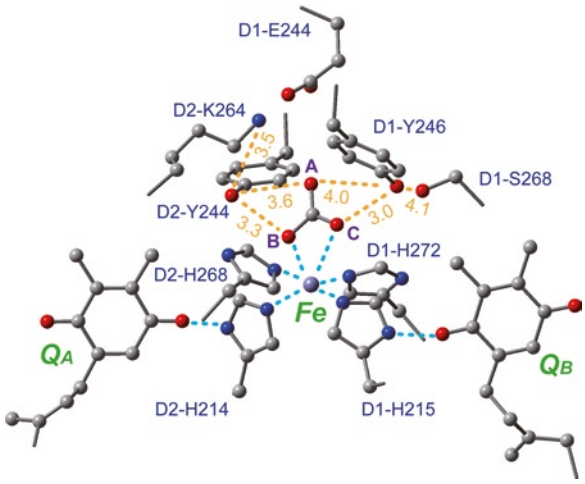

Fig. 20.5. The iron-quinone center of PS II from *Thermosynechococcus elongatus* (Guskov et al., 2009; Protein Data Base file 3BZ1). The ligand environments around Fe and bicarbonate are depicted. Nitrogens are shown in blue, oxygens in red. The oxygen atoms of the bicarbonate are designated *A*, *B* and *C*. The distances (in Å) from D1-Tyr246 and D2-Tyr244 to bicarbonate and other nearby residues are shown in orange (Modified from Takahashi et al. [2009]) . Also, see a figure on bicarbonate binding in the supplementary material associated with the paper of Umena et al. (2011) for more accurate distances.

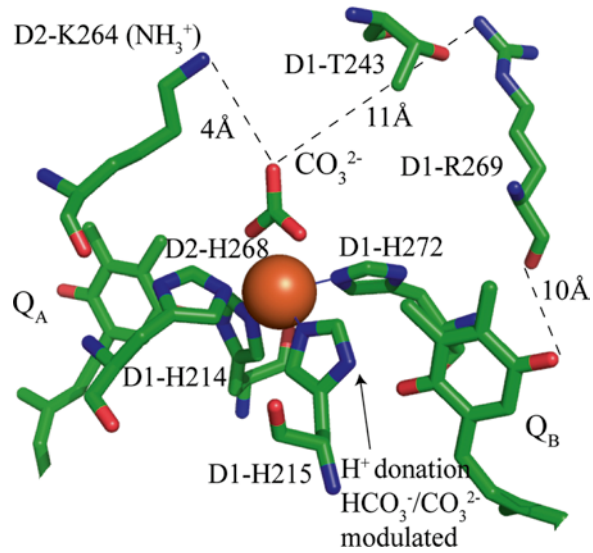

Fig. 20.6. The ligand environment of the non-heme iron of PS II showing the amino acids in the vicinity of bound carbonate taken from the Protein Data Base file 2AXT (Loll et al., 2005) (Adapted from Cox et al. [2009] [cf. Fig. 20.5; and the figure in the supplementary material of Umena et al. (2011), reproduced as Figure 10 in Govindjee and Shevela (2011)]).

(or D2-Tyr244)) and the other at D1-Arg257. What does it do there? One role may be to stabilize the Q_A-Fe-Q_B structure. Upon removal of bicarbonate, the distance between Q_A and Q_B may be altered, slowing down the rate of electron transport from Q_A^- to Q_B, although a larger effect is on the protonation of reduced Q_B^{2-}.

Because the absence of bicarbonate drastically decreases the rate of reduction of Q_B by Q_A^- after a second actinic flash, and the pKa of $H_2O + CO_2/HCO_3^- + H^+$ is about 6.3, it appears that bicarbonate is involved in the protonation of Q_B^{2-} (see Eaton-Rye et al., 1986; Govindjee and Eaton-Rye, 1986). Formate is not able to function in such a way because the pKa of formate is 3.8. Evidence for such a function was reported by Eaton-Rye and Govindjee (1987, 1988a, b), Van Rensen et al. (1988), and Xu et al. (1991). Xiong et al. (1996) modeled a bicarbonate and a water molecule in the Q_B binding pocket and proposed a hypothesis for the role of bicarbonate in the protonation of Q_B^{2-}. The bicarbonate, stabilized by D1-Arg257, could donate a proton to Q_B^{2-} through the intermediate D1-His252, and a water molecule would donate another proton to Q_B^{2-}. Based on the discovery of a water transport channel in the bacterial reaction center, an analogous channel for transporting water and bicarbonate can be proposed in the PS II reaction center. Such a putative channel would be positively charged near Q_B and the non-heme iron.

E. Future Directions for Research on the Acceptor Side

At least two pressing questions regarding bicarbonate on the acceptor of PS II remain. In the cyanobacterial reaction center, there is only evidence for a single bound bicarbonate at the non-heme iron. Is there only a single site or is there a single site in cyanobacterial PS II while two sites (as inferred by Blubaugh and Govindjee, 1988a and the model of Xiong et al., 1996) exist in eukaryotic PS II? The evidence for "bicarbonate" as ligand to the quinone-iron complex leaves little doubt for a role for this ligand in vivo (see Umena et al., 2011). What specific advantage does an exchangeable ligand at this site confer on the operation of PS II? Intriguingly, recent evidence using EPR spectroscopy and Density Functional Theory calculations suggests that it is carbonate, rather than bicarbonate, that is the ligand to iron in the quinone-iron complex of spinach PS II (Cox et al., 2009; and see Fig. 20.6).

Future studies will no doubt be directed at understanding the contribution of bicarbonate to electron transfer between Q_A and Q_B and the full elucidation of how bicarbonate/carbonate contributes to quinol formation and exchange at the Q_B binding site (Sedoud et al., 2011). Such studies will lead to a full understanding of the physiological gain conferred by the presence of bicarbonate on the acceptor side of PS II.

III. Bicarbonate Effects on the Donor Side of Photosystem II

A. Historical Perspective

As noted above, inorganic carbon was first recognized as being an important factor in photosynthetic electron transport by Warburg and Krippahl (1958, 1960) (see Fig. 20.1). This observation has been repeated multiple times in different laboratories (Stern and Vennesland, 1960; Izawa, 1962; Batra and Jagendorf, 1965; West and Hill, 1967). Warburg considered his observation of the oxygen-evolving reaction's requirement for CO_2 as support for his view that CO_2 was the source of oxygen produced in photosynthesis (Warburg, 1958). However, the contemporary view that bicarbonate also has an effect on donor side PS II electron transport arose initially from experiments by Stemler and Govindjee (1973). The hypothesis stated that if bicarbonate was to be involved at the oxygen-evolving complex (OEC) then there should be no effect on the rate of electron transport from the redox active tyrosine Y_Z through to the acceptor side. This was tested in an experiment with broken chloroplasts where the OEC had been destroyed by heat treatment at 49°C. In this experiment PS II accepted electrons at Y_Z from the artificial electron donor diphenylcarbazide (DPC) in the presence of an added electron acceptor 2,6 dichlorophenol indophenol (DCPIP). The rate of electron transfer from DPC to DCPIP in the absence of a functional OEC was not dependent on the bicarbonate concentration while electron transfer from water to DCPIP was dependent. Although this result was not confirmed by subsequent studies (Wydrzynski and Govindjee, 1975; Khanna et al., 1977 and see Section II above) the work of Stemler and Govindjee (1973) indicated that a site of bicarbonate action was possibly also at the OEC. Putative binding of $^{14}CO_2$ to the OEC also supported this view (Stemler, 1980a) and bicarbonate depletion increased damping of S-state cycling of the OEC (Stemler et al., 1974; for a discussion of S states see Chapter 17). Moreover, addition of 100 mM formate could slow the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ S-state transitions specifically by apparently displacing a CO_2 bound to the OEC (Stemler, 1980b).

In the PS II structure from *Thermosynechococcus elongatus* obtained at a resolution of 3.5 Å by Ferreira et al. (2004), a non-protein electron density, that could accommodate a carbonate (deprotonated bicarbonate) moiety, was modeled near the active site of the OEC. However, subsequent crystallographic studies at 3.2- to 2.9-Å resolution did not replicate this non-protein density (Biesiadka et al., 2004; Loll et al., 2005; Guskov et al., 2009, 2010) (We note that Umena et al. (2011), at 1.9 Å resolution, were unable to see any bicarbonate moiety on the electron donor side of PSII.) Moreover, structure-based computational studies have not supported the presence of a bicarbonate either (Siegbahn and Lundberg, 2005; Sproviero et al., 2006). Nevertheless, the structure-based suggestion that bicarbonate might interact directly with the OEC stimulated this area of research. The following sections explain the aspects of donor side PS II structure and function in which bicarbonate has been perceived to play a role.

B. Evidence and Absence of Evidence for Bicarbonate Binding on the Donor Side of Photosystem II

There is no large effect on the rate of oxygen evolution when bicarbonate is added to untreated PS II-enriched preparations. However, if such PS II preparations are first treated to remove bicarbonate, and then bicarbonate is added back, differences can be seen between the depleted and reconstituted samples. Bicarbonate depletion is achieved by adding formate or acetate to compete for binding sites, or by introducing a 100–1,000-fold dilution of the bicarbonate concentration by removing dissolved CO_2 from solution. Often, the pH of the solution is also shifted to below carbonic acid's pK_a (6.4) (see Section II.A above).

Apparent K_d values for bicarbonate in inorganic carbon-depleted PS II samples have been calculated (Allakhverdiev et al., 1997). There appeared to be two binding sites (cf. Blubaugh and Govindjee, 1988b). Lineweaver-Burk plots of oxygen evolution versus bicarbonate concentration in a Mn-containing medium with Mn-depleted PS II-enriched preparations indicated K_d values of approximately 2.5 μM and 34 μM (Allakhverdiev et al., 1997). If the exogenous electron acceptor used acted at or before Q_A only the lower-affinity site with a K_d of 34 μM remained. That result indicated that the high-affinity site was on the acceptor side while the low-affinity site was before Q_A. If the samples were not bicarbonate depleted then the high-affinity site (K_d=2.5 μM) site was not detectable. This result is expected since the bicarbonate concentration in solution at pH 5.5 from equilibration with CO_2 in the atmosphere would be above 2.5 μM. Under the same conditions, but where chlorophyll *a* variable fluorescence is examined instead of oxygen evolution, binding sites exhibiting a K_d of 20 μM, and another site with a K_d of less than 10 μM are detected (Allakhverdiev et al., 1997).

A distinct effect of bicarbonate depletion on chlorophyll *a* variable fluorescence yield attributed to changes on the donor side has been demonstrated by Klimov et al. (1995a, b; also see Stemler and Govindjee, 1974). Using PS II samples prepared according to Shutilova et al. (1975), and depleted of bicarbonate by flushing with CO_2 free air with or without 5–20 μM formate, electron flow on the donor side was inhibited and then restored by the reintroduction of bicarbonate. This donor side effect is distinguished from the well-characterized acceptor side effect discussed in Section II which is typically observed by the addition of millimolar levels of formate.

Absorption measurements of the OEC at 295 nm, measured as a function of the different S-states, have shown that the binding of formate can suppress the periodicity-of-four, typical of S-state cycling by the OEC. The addition of bicarbonate restores the typical absorption pattern (Wincencjusz et al., 1996). This work was carried out on a PS II sample prepared according to Shutilova et al. (1975); the same treatment did not affect the more commonly used PS II samples prepared according to Berthold et al. (1981). The only other difference noted between these two procedures for obtaining enriched PS II preparations is that those obtained by the procedure described by Shutilova et al. (1975) have lower oxygen-evolving activity and this may suggest that a notable fraction of PS II centers are damaged by this method (Wincencjusz et al., 1996; Hulsebosch et al., 1998).

The effects of formate and bicarbonate were further examined by Klimov et al. (1997a) in PS II membranes prepared according to Shutilova et al. (1975). EPR was used to probe the state of the Mn in the system. Specifically, Signal II, which is attributed to the oxidation state of Y_Z (and Y_D), and the six-line signal from soluble Mn^{2+} were examined. This work showed that bicarbonate was required for Mn binding to a Mn-depleted PS II preparation after formate treatment. Bicarbonate was also found to be necessary for the restoration of the fast phase of Y_Z reduction. Another EPR-based study examining the interaction of formate at the OEC noted that adding bicarbonate when treating PS II-enriched membranes with formate maintained the S_2-state multi-line signal at the expense of the g=4.1 signal (Feyziev et al., 2000).

Initial attempts to directly observe bicarbonate binding to the OEC via FTIR spectroscopy were performed by Yruela et al. (1998). Light minus dark, bicarbonate minus bicarbonate-depleted, double difference spectra were presented in addition to a light minus dark, ^{12}C bicarbonate minus ^{13}C labeled bicarbonate double difference spectrum. Yruela et al. (1998) concluded that bicarbonate might indeed bind to the donor side of PS II. However, the conditions under which those data were collected (at room temperature and under constant illumination) rendered the spectra difficult to interpret. The conditions required to make solid conclusions about effects on the OEC require flashing light and cooling (Hillier and Babcock, 2001b; Noguchi, 2007). Aoyama et al. (2008), using FTIR spectroscopy, have examined double difference spectra per S state (S_2-*minus*-S_1, S_3-*minus*-S_2, S_0-*minus*-S_3, S_1-*minus*-S_0) with ^{13}C labeled and non-labeled (^{12}C) bicarbonate at 10°C. These data provided no evidence for bicarbonate binding other than to the acceptor side non-heme iron.

Further investigations into bicarbonate binding have been made using mass spectrometric techniques to detect the presence of a putative

non-exchanging bicarbonate incorporated into the structure of PS II. Hydroxylamine was used to reduce and destroy the Mn_4Ca cluster of PS II-enriched preparations in a slightly acidic medium containing added CA (Ulas et al., 2008). PS II membranes prepared with the Mn_4Ca cluster already removed prior to the experiment were used as a control. There was no discernable difference in the amount of CO_2 produced on hydroxylamine treatment between these two conditions, finding approximately 0.26 bicarbonate ions per PS II center – clearly not from the OEC. A similar approach employed a formate pretreatment and found approximately 0.3 bicarbonate ions per PS II but did not detect any release of CO_2 after the subsequent addition of hydroxylamine (Shevela et al., 2008). Both of these studies concluded there was no non-exchangeable bicarbonate bound to PS II.

C. Does Bicarbonate Stabilize the Donor Side of Photosystem II?

Some studies have indicated that bicarbonate increases stability and protects against inactivation by exposure to heat and light (Klimov et al., 1997b, 2003). Bicarbonate protected against a drop in electron transport rates through PS II during a 20 min exposure to photoinhibitory light. A similar bicarbonate dependent protective effect was seen against thermal inactivation by incubation at 40°C over 30 min. Pobeguts et al. (2007) have suggested that bicarbonate plays a role in binding of the PsbO lumenal extrinsic subunit to the membrane intrinsic components of PS II. Attempts to remove PsbO with urea washes are less successful when 5 mM bicarbonate is present but this is expected to be independent of any interaction with the Mn of the OEC.

A further stabilizing effect was hinted at in experiments involving reduction of the OEC Mn cluster to non-native states. The usual S-state cycle includes S_0 to S_4 states; however, an S_{-1} or lower state can be produced by incubating samples with a reductant such as hydrazine (Messinger et al., 1997). If bicarbonate is present during incubation with hydrazine, the reduction to super-reduced S-states is impaired and higher rates of oxygen-evolving activity are observed following reduction than if hydrazine is applied alone (Shevela et al., 2006). Shevela et al. (2006) suggested the S-state dependence of the data indicated a direct Mn-bicarbonate interaction that protected against the effects of hydrazine. Further examination indicated it unlikely that bicarbonate had a role on the S-state cycle as bicarbonate depletion did not affect the rate of OEC reduction by hydrazine, neither did it appear to alter S-state oxidation potentials (Shevela et al., 2007). Also it was noted that CO_2 diffuses back into purged samples very quickly when small sample volumes are handled – potentially explaining any varying accounts of the effect of bicarbonate depletion in the literature. This seems to agree with previous work indicating bicarbonate does not prevent formate from destabilizing the S_1 state (Stemler and Lavergne, 1997).

D. Bicarbonate has a Role in Photoactivation

Photoactivation is a light-driven process where free inorganic cofactors are bound and oxidized by apo-PS II to assemble the OEC inorganic core (Mn_4Ca) into a functional PS II unit that is competent for oxygen evolution (DasGupta et al., 2008). This process has been studied for some time (Cheniae and Martin, 1967; Frasch and Sayre, 2001; Dismukes et al., 2005). However, that bicarbonate can have a role in this process has only recently been discovered. Photoactivation is frequently studied by biochemically removing the OEC from PS II and observing reassembly in vitro. This approach may resemble two in vivo processes: PS II biogenesis and repair after photodamage.

The photoactivation process absolutely requires several different cofactors (Cheniae and Martin, 1967). These are Cl^-, Mn^{2+} and Ca^{2+} in addition to an external electron acceptor. Light is also essential to the process. (The water-soluble lumenal protein subunits that sit between the Mn_4Ca cluster and the lumen: PsbO, and typically PsbP and PsbQ in the majority of photosynthetic eukaryotes, are not essential.) It appears to be a multi-step reaction and Ono and Inoue (1983) have suggested that it begins with inorganic ion binding followed by further light-dependent steps.

The first evidence that bicarbonate could play a role in the photoactivation process came with measurements of the recovery of the variable chlorophyll *a* fluorescence yield in PS II-enriched membranes in which the Mn_4Ca cluster had been

removed (Klimov et al., 1995a, b). It was clearly shown that when micromolar concentrations of Mn^{2+} and millimolar concentrations of bicarbonate were present, the variable fluorescence recovered faster and to a greater extent than in the cases where just Mn^{2+} or bicarbonate had been added. This showed that more PS II centers were competent for electron transfer with the addition of bicarbonate. However, the addition of just Mn^{2+} did produce some restoration, perhaps via direct oxidation to Mn^{3+}. This work concluded that the combined effect was specific to the co-addition of bicarbonate, and that other anions did not act in the same way (Klimov et al., 1995a, b).

Further experiments using similar treatments indicated that the recovery of chlorophyll fluorescence was stimulated to the same level, irrespective of whether the preparations had been previously depleted of bicarbonate, by the addition of 0.4 mM bicarbonate and 2–4 Mn per reaction center (Allakhverdiev et al., 1997). This same work indicated two separate K_d values for bicarbonate binding to PS II as described above. However, this did not mean that bicarbonate is essential, as the process was still able to proceed to some degree without bicarbonate addition in depleted conditions.

Bicarbonate's role in photoactivation has also been examined by using EPR to follow changes in the S_2-state multiline signal from the OEC (Hulsebosch et al., 1998). This approach showed that the S_2-state multiline signal of PS II is restored to around 30% of the original signal strength after photoactivation in the presence of bicarbonate and Mn^{2+} in PS II-enriched membranes. Under conditions where only bicarbonate or only Mn^{2+} was added no signal was detectable. Furthermore, the restored signal appeared to have the same structure as the untreated multiline signal indicating that not only the OEC was being reassembled under these conditions, but the centers structurally resembled their native counterparts (Fig. 20.7). This is an important result as it is a direct observation of an effect of bicarbonate on photoassembly.

Further work on bicarbonate's role in photoactivation demonstrated a bicarbonate-dependent increase in the yield of photoactivation and also an increase in the rate of the process (Baranov et al., 2000). This study used an oxygen electrode that gave results with time resolution of a millisecond; further it used a new light regime for photoactivation that reduced photodamage.

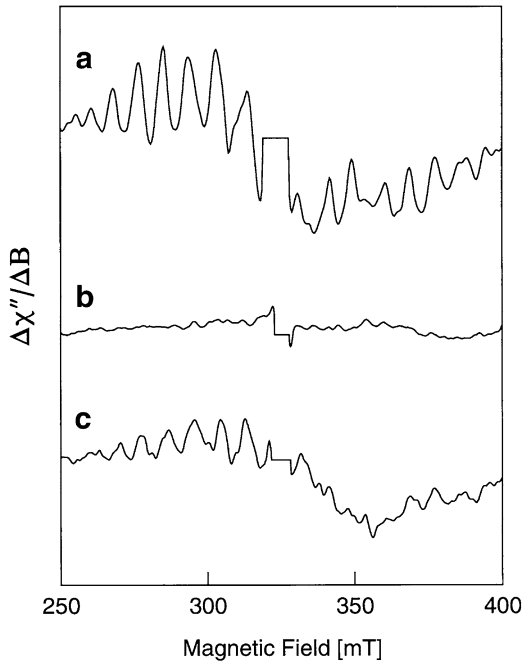

Fig. 20.7. Light minus dark EPR spectra indicating the bicarbonate's effect on photoactivation to the S_2 state compared with the native S_2 state (**a**) untreated PS II enriched membranes in the native S_2 state (**b**) Photoactivated PS II membranes washed with CO_2 free medium (**c**) As in panel b but with 1 mM bicarbonate added. Spectra are light minus dark where the light spectrum was produced by illumination at 190 K for 10 min. All spectra collected at 10 K with 10 G modulation amplitude, 1 mW microwave power, and a microwave frequency of 9.15 Ghz with PS II samples of 4 mg ml^{-1} chlorophyll (Adapted from Hulsebosch et al. [1998]).

An important distinction from other work was that, here, no attempt was made to deplete bicarbonate from the PS II samples used in this study. The addition of 25 μM bicarbonate increased the rate of photoactivation. However, adding 4 mM bicarbonate reduced the final yield of activated centers. A relationship between Mn^{2+} availability per reaction center and bicarbonate concentration was shown. At concentrations of Mn^{2+} up to 4 Mn per reaction center, bicarbonate reduces the time to begin photoactivation after illumination begins. This is the t_{lag} phase. Above 4 Mn per reaction center the bicarbonate concentration is not a limiting factor. Also at any Mn^{2+} concentration, a bicarbonate concentration above 150 μM does not decrease the t_{lag} or the final yield of activated centers. The addition of millimolar amounts of Ca^{2+}, in addition to micromolar to millimolar concentrations of bicarbonate, reduced t_{lag} but did

not increase the overall yield. A comparison of these results was made with the experiments also performed in the same study where the reaction medium was made alkaline. Alkalization produced an increase in the rate of the photoactivation process, but not a concomitant increase in the yield: that is, alkalization is not equivalent to adding bicarbonate.

This line of investigation led to the proposal of two possible models of photoactivation (Zaltsman et al., 1997; Baranov et al., 2000). A major step in these models is the formation of the state IM_1 where a Mn^{2+} is bound and then photo-oxidized with a bicarbonate ion bound which then becomes an integral component of the OEC. Alternatively, a hydroxide is bound with the Mn^{2+} and bicarbonate acts to either accelerate the formation of IM_1 or reduce the rate of its decay. Bicarbonate may achieve this by acting as a Bronsted base by accepting a proton resulting from the light-dependent reaction. Yet another possibility is that bicarbonate could donate a hydroxide to assist in forming IM_1, releasing CO_2 as a by-product. After this stage a further hydroxide ligand would bind, finally forming IM_1. Following the IM_1 stage, Ca^{2+} binds in a light-independent manner forming IM_2 then further light exposure is required to fully form the functional water-oxidizing inorganic core of the OEC. Another suggestion for the role of bicarbonate was that it might act in attracting Mn^{2+} to a binding site via electrostatic attraction as a membrane-soluble anion. A further binding affinity is determined here (Baranov et al., 2000) as approximately 6.5 μM. This site is only detectable when the Mn to PS II stoichiometry is equal or below 4 Mn^{2+} per reaction center.

Bicarbonate's role in photoactivation was probed again (Baranov et al., 2004) using higher Mn^{2+} and Ca^{2+} concentrations than previously used. This resulted in the discovery that a ratio of 1:500 Mn^{2+}:Ca^{2+} added to the reaction produces the fastest rate of activation. Addition of bicarbonate stimulated the rate of photoactivation 300 times and the final yield 50 times compared to the absence of added bicarbonate under these conditions.

Any claim that bicarbonate is essential for the process does not seem completely correct. In work of Hulsebosch et al. (1998), no effect is seen without a combination of bicarbonate and Mn but in other studies of Baranov et al. (2000), some degree of photoactivation is visible without the addition of bicarbonate (although 4 μM would be present under the conditions used). However, it does seem likely that bicarbonate would be present in the native system and since its presence increases both the rate and the yield of the process it is likely to be used in vivo, absolutely required or not (Baranov et al., 2000). There is no in vivo demonstration of bicarbonate-dependent photoactivation at the time of writing this chapter.

The possibility of a role for bicarbonate in photoactivation (Baranov et al., 2004) also spurred the idea that bicarbonate could have been the substrate of an ancestral (pre)-water-oxidizing "PS II" (Dismukes et al., 2001). That ancestral "PS II" would have existed in the oceans of the Archean era 2.5 billion years ago. At that time the atmospheric CO_2 levels were high enough to have produced an appreciable bicarbonate concentration in any body of water. Bicarbonate's intermediate oxidation potential, sitting above single-electron donors and under water, may have acted as a transitional substrate from bacterial reaction-center-like single-electron photochemistry to current PS II photochemistry coupled to four-electron water oxidation. This in turn led to speculation that PS II might retain some of that hypothetical ancestral capacity to oxidize bicarbonate (Dismukes et al., 2001).

E. Bicarbonate as an Alternative Substrate of Photosystem II

The idea that inorganic carbon could be involved in the oxygen-evolving chemistry of PS II was first advocated by Warburg (1958) with his 'photolyte' theory whereby the primary electron donor reacted directly with chlorophyll. The primary electron donor in this case was hypothesized to be CO_2 (Warburg and Krippahl, 1958; cf. Kamen, 1989), even though the evidence available at the time included isotopic measurements with ^{18}O labeled water which indicated that the O_2 produced indeed came from water (Ruben et al., 1941). Later it was proposed that bicarbonate might be necessary for the advancement of the OEC through each S-state (Stemler and Govindjee, 1973; Stemler et al., 1974). Additional work also indicated that the active species was bicarbonate and not CO_2 (Blubaugh and Govindjee, 1986) and since bicarbonate seemed to have an effect on the system, this lead to further investigation.

The oxidation of water is a thermodynamically demanding process where the oxidation potential required is the highest known in biology (Hillier and Babcock, 2001a). This led to alternative hypotheses regarding the perceived role of PS II in directly oxidizing water, especially all four electrons in just one step of the five steps of the S-state cycle, as is the current understanding (Hillier and Messinger, 2005). One such hypothesis was put forward by Metzner (1978) where he stated that it might in fact be bicarbonate that is oxidized by PS II and not water (see Eq. 20.1 below). Helmut Metzner argued that water was indeed the ultimate source of the oxygen meaning his hypothesis could not be disproved by the results of Ruben et al. (1941), but that it was first combined with a CO_2 molecule converting it to bicarbonate.

$$2\,HCO_3^- + 2H^+ \rightarrow\ 2\,CO_2 + O_2 + 4e^- + 4H^+ \quad (20.1)$$

The concept of bicarbonate's involvement at the OEC as an electron donor was advanced in work suggesting that formate binding could delay the progress of the OEC through the S states (Stemler, 1980b). In this paper, Alan Stemler stated that bicarbonate could be an electron donor to PS II and included a reaction scheme for this process that mentions the production of CO_2 as a by product of the reaction.

To investigate the bicarbonate oxidation hypothesis two experiments to detect labeled photosynthetic oxygen produced from ^{18}O labeled bicarbonate and ^{18}O labeled water, respectively, were performed (Radmer and Ollinger, 1980). All the photo-generated oxygen had the isotopic ratio of the water in the reaction, not the bicarbonate. In other words all the oxygen was produced from the water. This seemed to be a conclusive result. However, the discovery that thylakoid preparations contain a CA activity (Komarova et al., 1982) provided a putative mechanism that would satisfy Helmut Metzner's hypothesis – thus it was still possible for bicarbonate to be a substrate. The labeled bicarbonate results of Radmer and Ollinger (1980) might have been confounded by the labeled bicarbonate being exchanged into water (Eq. 20.2, see below) or vice versa on a time scale shorter than the measurements.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad (20.2)$$

Metzner produced mass spectrometric data to support his hypothesis of bicarbonate oxidation (Metzner et al., 1979, 1981) but this work has not been replicated. Shortly after Radmer and Ollinger (1986) refined earlier isotopic measurements: water exchange in the S_3 state was examined at exchange times of 1960s. These flash-based measurements (as opposed to continuous illumination) with $H_2{}^{18}O$ supported the concept of water being the substrate. Among their conclusions they found no evidence for exchangeable bound water oxidation intermediates. This could have been extended to include a slowly exchanging bicarbonate intermediate. While adding yet more information to the picture of the water oxidation mechanism this did not address the CA activity.

The possibility that PS II might actually oxidize bicarbonate as the ultimate substrate or as an alternative substrate to water was readdressed by the following two mass-spectrometry-based approaches. In Metzner's equations (Metzner, 1978; also see Kreutz, 1974), CO_2 must be produced as bicarbonate is oxidized (see Eq. 20.1 above). Clausen and Junge (2004) have shown that if the partial pressure of O_2 is increased then PS II can not release O_2, and the reactions appear to be paused. This approach was repeated using CO_2 instead of O_2. The oxygen producing reaction was not stopped (Clausen et al., 2005). These results are less clear given that the effect of oxygen back-pressure has since been disputed (Haumann et al., 2008). The hypothetical CO_2 in Metzner's equation could also not be detected by a mass spectrometer sampling the gas phase of the reaction (Clausen et al., 2005). The conclusion was that bicarbonate's involvement in water oxidation seemed unlikely but they could not rule out the production of CO_2 that was not freely exchangeable with the reaction medium.

To directly address the confounding effect of the PS II-associated CA activity from PS II-catalyzed water oxidation, another mass spectrometric approach was used. The associated CA activity exhibited by PS II preparations from three different organisms (spinach PS II-enriched membrane preparations; *Thermosynechococcus elongatus*, the species from which high resolution crystal structures were obtained; and *Arthrospira maxima* which requires high concentrations of bicarbonate for growth) was quantified in terms of how much ^{18}O labeled bicarbonate the system

would convert into ^{18}O labeled water as a function of time. That rate of $H_2{}^{18}O$ production was taken into account when measuring the flux of ^{18}O labeled oxygen from $HC^{18}O_3^-$ to photo-generated labeled O_2 via PS II. The results indicated that perhaps one in five thousand PS II reaction centers might actually be capable of oxidizing bicarbonate. This level of activity was dismissed as physiologically irrelevant (Hillier et al., 2006). Furthermore, if PS II has all inorganic carbon removed by complete degassing in a membrane inlet cuvette attached to a mass spectrometer, PS II still exhibits more than three quarters its original oxygen-evolving capacity indicating that bicarbonate is in no way essential for water oxidation (Shevela et al., 2008).

Despite this wealth of evidence against it, hypotheses are still proposed suggesting intermediate substrates between water and molecular oxygen (Castelfranco et al., 2007). It seems almost certain that bicarbonate is not a substrate of PS II either directly or via exchange with water through the associated CA activity. The PS II-associated CA activity might have some other function specific to PS II, but this does not appear to be water oxidation.

F. Photosystem II-Associated Carbonic Anhydrase Activity?

By the early 1980s it was established that PS II preparations from C3 plants had an associated CA activity. This was first noticed in thylakoid preparations (Komarova et al., 1982) and subsequently in different PSII-enriched preparations (Stemler, 1986; Moskvin et al., 2004). This CA activity has been suggested to be involved with PS II oxygen-evolving activity. In an interesting parallel to the donor side/acceptor side bicarbonate effect debate of the 1980s, the PS II-associated CA activity has been suggested to be a property of the water-soluble extrinsic proteins in particular the PsbO subunit (also known as the 33-kDa manganese-stabilizing protein) (Lu and Stemler, 2002; Lu et al., 2005) and also interpreted as two or more different activities present in the extrinsic and intrinsic components of PS II (Moubarak-Milad and Stemler, 1994; Stemler, 1997; Moskvin et al., 2004).

The interest in a potential role of CA activity in oxygen evolution was driven by the finding that the green algae *Chlamydomonas reinhardtii* has a well-defined α-CA (the definition of various classes of CA is based on conserved amino acid sequences) known as Cah3, that was bound to PS II on the donor side (Karlsson et al., 1998). Additional results indicated this CA functioned to supply adequate CO_2 to Rubisco (Park et al., 1999). Further work was published claiming the activity of Cah3 might regulate the oxygen-evolving activity of PS II in this organism (Villarejo et al., 2002). This was later refuted in a study that re-examined those claims (Hanson et al., 2003) indicating the function of Cah3 activity was to assist in inorganic carbon uptake when it is found at low concentrations in the environment. This result was in fact what had originally identified the *cia3* mutant when it was isolated from a screen for the phenotype of being unable to grow at low inorganic carbon concentrations (Spreitzer and Mets, 1981; Spalding et al., 1983).

Evidence for a role of bicarbonate as a proton transporter in conjunction with Cah3 in *Chlamydomonas reinhardtii* has also been suggested by Shutova et al. (2008). The *cia3* mutant of PS II has oxygen-evolving activity of about 50% of that observed in wild type that is restored to the wild-type level by the addition of Cah3 and bicarbonate. However, bovine CA does not substitute for Cah3 and bicarbonate is not sufficient on its own. Oxygen release is delayed in the mutant by 0.6 ms compared to the wild type, and this is restored upon the addition of bicarbonate and Cah3 as well. Furthermore, the fact that these data are fitted by a biphasic rise component in PS II from the *cia3* mutant, due to a slower initial phase, indicated some slowing of a process immediately before oxygen release. Perhaps most tellingly, when considering a role for Cah3 in proton transport, the addition of the proton-accepting dye neutral red produces the same effect on PS II activity in the *cia3* mutant as if both bicarbonate and Cah3 were added. An H/D isotope effect was also present where the oxygen evolution rate is 1.78-times slower in the presence of deuterium in PS II from the *cia3* mutant compared to only 1.15-times slower in PS II from wild type. However, this putative role as a proton transporter seems to be currently limited to *Chlamydomonas reinhardtii* as no specific PS II-associated CA has been identified and isolated from higher plants.

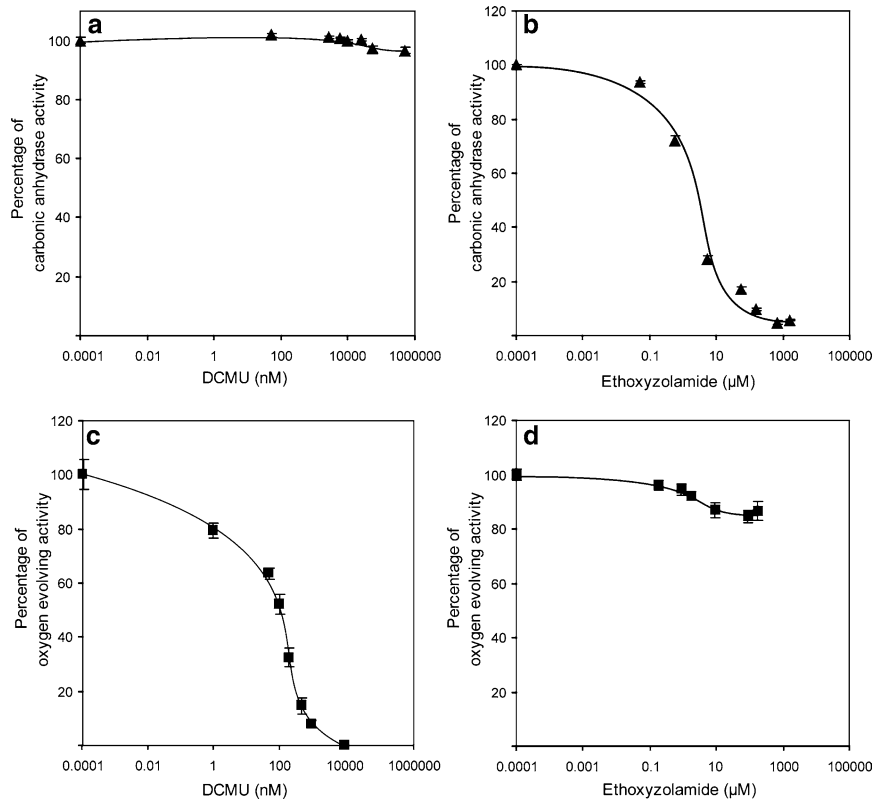

Fig. 20.8. Oxygen-evolving and carbonic anhydrase activities of PS II-enriched membranes are independent. DCMU (**a** and **c**) and ethoxyzolamide (**b** and **d**) titrations against the carbonic anhydrase (**a** and **b**) and oxygen-evolving activities (**c** and **d**) of PS II-enriched membranes (Adapted from McConnell et al. [2007]).

The potential diversity of CA enzymes (Tripp et al., 2001) was a factor in arguing that a CA activity could be associated with or make up a part of PS II. The possibility that the PS II-associated CA enzyme may not require a catalytic metal center (as might be the case with the PsbO protein), or might use Mn for that function, could not be ruled out on the basis that it would not resemble one of the currently well-characterized classes of CA. Much of the work on PS II-associated CA has been focused on trying to locate where the enzyme is in relation to PS II and hypothesizing a function. However, there has not been much in the way of attempts to confirm that the CA is associated with PS II.

McConnell et al. (2007) have examined the relationship between the oxygen-evolving activity of PS II and the associated CA activity. A comparison of associated CA activity against oxygen evolution found no relationship between the two activities. The CA activity did not increase with increasing purity of the PS II preparation nor did the CA respond to illumination. When PS II and CA inhibitor titrations were performed, the CA activity was found to be completely independent of oxygen-evolving activity and vice versa (Fig. 20.8). This is evidence that the CA activity is unrelated to the electron transport activity of PS II in higher plants and is most likely a contaminant in the preparation. Furthermore, it showed that the CA activity responded to inhibitors that are known to target the well known first three classes of CA: α, β, and γ. All of those require Zn as a catalytic metal. There is no evidence for the involvement of Zn in any PS II crystal structural determination to date except the structure of PsbQ where it is an artefact of preparation (Calderone et al., 2003; but see Jackson et al., 2010).

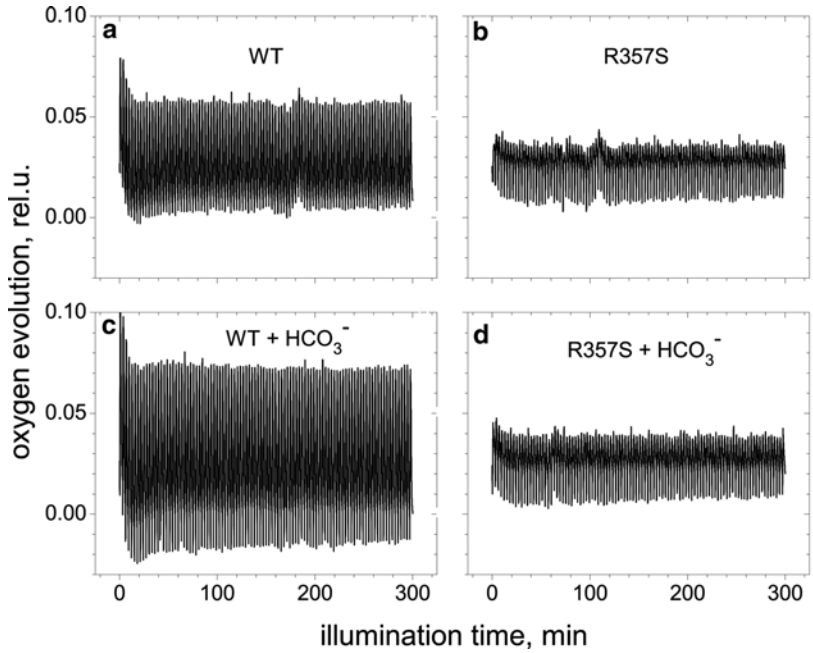

Fig. 20.9. Lack of bicarbonate response as shown by fast repetition rate fluorescence in whole cells from the CP43:R357S *Synechocystis* sp. PCC 6803 mutant compared to wild type. (**a**) wild type (**b**) mutant (**c**) as in panel a with 10 mM bicarbonate added (**d**) as in panel b with 10 mM bicarbonate added. Measurements were performed in a 'low bicarbonate' buffer (50 mM MES-NaOH pH 6.0, 35 mM NaCl and 300 mM sucrose) with less than 5 μM bicarbonate present from equilibration with the atmosphere (Adapted from Ananyev et al. [2005]).

G. Bicarbonate as a Catalytic Base

Experiments with the CP43-R357S mutant of *Synechocystis* sp. PCC 6803 (carrying an Arg to Ser substitution at amino acid 357 in the lumenal loop of the chlorophyll-binding protein CP43) may indicate a potential binding site for bicarbonate on the donor side of PS II (Ananyev et al., 2005). The wild type showed a two-fold increase in oxygen evolution when 10 mM bicarbonate was added compared to conditions without the additional inorganic carbon. However, in the mutant, oxygen evolution was independent of added inorganic carbon (Fig. 20.9). This result was obtained in whole cells and in isolated thylakoid membranes. The CP43 Arg357 to Ser mutation is relevant to the water oxidation chemistry as this change is lethal under photoautotrophic growth conditions, but when grown heterotrophically water oxidation proceeds at 20% of the level found in wild type with 60–70% of PS II assembled in the mutant compared to wild-type cells (Debus, 2005). The 3.5 Å crystal structure indicated that CP43 Arg357 could potentially ligate the non-protein electron density that was suggested to be a (bi)carbonate (Ferreira et al., 2004).

Further investigations of this residue with a CP43:R357K mutant indicate impaired S-state cycling (Hwang et al., 2007). That such a conservative mutation has an effect suggests that this residue is "highly tuned" to its function. The proximity of a basic residue to the OEC makes it a potential exit pathway for protons from the water-oxidation chemistry (Ferreira et al., 2004).

The OEC of PS II from *Arthrospira maxima* is also purported to have bicarbonate-dependent function (Carrieri et al., 2007). In the study by Carrieri et al. (2007), an in vivo effect is described by variable chlorophyll *a* fluorescence and oxygen evolution measurements using washed cells to lower bicarbonate levels and comparing these to bicarbonate complemented cells (cf. El-Shintinawy and Govindjee, 1990 for chlorophyll *a* fluorescence work on leaf discs showing a donor side effect as well as an acceptor side effect of bicarbonate).

H. Future Directions for Research on the Donor Side

It appears bicarbonate does not bind to the donor side of functional PS II in higher plants and therefore is unlikely to have an essential role in water oxidation. Further, bicarbonate may play a role as a proton transporter in the green alga *Chlamydomonas reinhardtii*. Another species-specific donor side role in *Arthrospira maxima* might also exist. A role seems well supported in photoactivation; however, precise information on just how it is acting is still required. A spectroscopic study that kinetically resolved each step of photoactivation would be valuable. Moreover, Pobeguts et al. (2010), using alterations in the fluorescence of the hydrophobic probe 8-anilino-1-naphthalene-sulfonic acid, have inferred bicarbonate-dependent structural re-arrangements in the PsbO protein of PS II, isolated from pea leaves. Further research is needed to relate these observations to the current understanding of the role of bicarbonate on the donor side of PS II.

IV. Conclusions

Bicarbonate appears to form a ligand to the non-heme iron between Q_A and Q_B. It also binds to amino acids of the D1 and D2 proteins. It is suggested to play a role in stabilization, by conformational means, of the reaction center protein that allows efficient electron flow and protonation of certain amino acids near Q_B^-.

Most of the reported effects of bicarbonate were obtained using isolated thylakoids or PS II-enriched preparations. One could ask: what is the importance of bicarbonate in PS II in vivo? The observation of a bicarbonate effect in vivo is difficult to distinguish, due to the obvious requirement for CO_2 in the Calvin-Benson cycle.

The binding constant for bicarbonate was estimated to be close to 80 μM (Stemler and Murphy, 1983; Snel and Van Rensen, 1984), whereas the CO_2 concentration in photosynthesizing chloroplasts is estimated to be only 5 μM (Hesketh et al., 1982). Thus, one may argue that under normal conditions, all binding sites are empty, and there may not be any real role for bicarbonate in vivo. However, since it is the bicarbonate concentration and not the CO_2 concentration, which determines the degree of functioning, there is no good reason to assume that the sites are empty. For example, if, in the vicinity of the binding site, the pH is 8, then the bicarbonate concentration in equilibrium with 5 μM CO_2 is 220 μM, well above the binding constant. The location of the bicarbonate binding site(s) is therefore important, and likely to be at the stroma side of the thylakoid membrane, which is more alkaline than the lumen side of the membrane.

There are several reports about an in vivo effect of bicarbonate on PS II; and a number are summarized below. Garab et al. (1983) demonstrated effects of bicarbonate on the energization of the thylakoid membrane in leaves. Mende and Wiessner (1985) studied the influence of bicarbonate in intact cells of the green alga *Chlamydobotrys stellata*, and found that both sides of PS II are affected by the absence of bicarbonate. Measurements of chlorophyll fluorescence and of electron flow in leaves under conditions of very low photosynthesis, independent of carbon assimilation (leaf discs infiltrated with methyl viologen acting as a terminal electron acceptor in stead of CO_2) have suggested that bicarbonate (CO_2) modifies the redox state of the quinone electron acceptors of PS II in vivo independently of carbon assimilation and thereby acts as a cofactor for efficient electron flow near PS II in the leaf (Ireland et al., 1987). Thermoluminescence of leaves provided evidence that CO_2 facilitates electron flow from Q_A^- towards PS I (Garab et al., 1988). Studies in formate-infiltrated spinach leaf discs indicated a site after the OEC but before Q_A that binds bicarbonate based upon the recovery of chlorophyll *a* fluorescence on addition of bicarbonate after hydroxylamine treatment (El-Shintinawy and Govindjee, 1990). This is curiously incongruous with similar treatments in broken chloroplasts that suggested a binding site at the OEC (Stemler and Govindjee, 1973). Govindjee et al. (1993) monitored CO_2-depletion effects in intact cells of the green alga *Scenedesmus obtusiusculus* and the cyanobacterium *Synechocystis* sp. PCC 6803 on State I-State II transitions of PS II. Xyländer and Hagen (2002) ascribed low-waves in chlorophyll fluorescence kinetics in filaments of the green alga *Haematococcus pluvialis* to deprivation of bicarbonate in the reaction

center of PS II. Using filaments of the alkalinophyllic cyanobacterium *Spirulina maxima* as a model organism, Fernandez-Velasco et al. (2005) reported that the stability of oxygen evolution is strongly dependent on bicarbonate; they suggested that bicarbonate has a direct role in the stability of the S-states.

What advantage is there for PS II reaction centers to have, unlike their bacterial counterparts (Shopes et al., 1989; Wang et al., 1992), a reversible ligand to the non-heme iron? One likely possibility is a regulatory one, in which binding of bicarbonate to PS II regulates the flow of electrons through PS II. Under conditions that photosynthesis can proceed well, enough bicarbonate is probably bound to PS II to function normally. However, under stress conditions like drought, high light intensity and high temperature, the stomata may close leading to a decrease of the internal CO_2 concentration. The consequent decrease of the bicarbonate concentration may limit the activity of PS II. It has been suggested several times that bicarbonate may be involved in the process of photoinhibition (Sundby, 1990; Sundby et al., 1992; Demeter et al., 1995). As photoinhibition occurs at high light intensity, the acceptor side of PS II becomes highly reduced. A reduced state is related to a decreased affinity of PS II for bicarbonate (Van Rensen et al., 1988). This may lead to the diffusion of bicarbonate from its binding site at the non-heme iron, resulting in an impaired rate of electron flow between Q_A and Q_B. Finally the reaction center may become overreduced. Because the area of PS II is rich in oxygen, photooxidation involving reactive oxygen species may occur, leading to the degradation of the D1 protein.

All the available research to date suggests that a renewed effort is necessary to establish the mechanistic role of bicarbonate/carbonate on both the donor and the acceptor sides of PS II.

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References

Abeles FB, Brown AH and Mayne BC (1961) Stimulation of the Hill reaction by carbon dioxide. Plant Physiol 36: 202–207

Allakhverdiev S, Yruela I, Picorel R and Klimov V (1997) Bicarbonate is an essential constituent of the water-oxidizing complex of photosystem II. Proc Natl Acad Sci USA 94: 5050–5054

Ananyev G, Nguyen T, Putnam-Evans C and Dismukes GC (2005) Mutagenesis of CP43-arginine-357 to serine reveals new evidence for (bi)carbonate functioning in the water oxidizing complex of photosystem II. Photochem Photobiol Sci 4: 991–998

Aoyama C, Suzuki H, Sugiura M and Noguchi T (2008) Flash-induced FTIR difference spectroscopy shows no evidence for the structural coupling of bicarbonate to the oxygen-evolving Mn cluster in photosystem II. Biochemistry 47: 2760–2765

Baranov SV, Ananyev GM, Klimov VV and Dismukes GC (2000) Bicarbonate accelerates assembly of the inorganic core of the water-oxidizing complex in manganese-depleted photosystem II: A proposed biogeochemical role for atmospheric carbon dioxide in oxygenic photosynthesis. Biochemistry 39: 6060–6065

Baranov SV, Tyryshkin AM, Katz D, Dismukes GC, Ananyev GM, Klimov VV (2004) Bicarbonate is a native cofactor for assembly of the manganese cluster of the photosynthetic water oxidizing complex. Kinetics of reconstitution of O_2 evolution by photoactivation. Biochemistry 43: 2070–2079

Bassham JA (2005) Mapping the carbon reduction cycle: a personal retrospective. In: Govindjee, Beatty JT, Gest H and Allen JF (eds) Discoveries in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 20, pp 815–832. Springer, Dordrecht

Batra P and Jagendorf AT (1965) Bicarbonate effects on the Hill reaction and photophosphorylation. Plant Physiol 40: 1074–1079

Benson AA (2005) Following the path of carbon in photosynthesis: a personal story. In: Govindjee, Beatty JT, Gest H and Allen JF (eds) Discoveries in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 20, pp 793–813. Springer, Dordrecht

Berthold DA, Babcock GT and Yocum CF (1981) A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes: EPR and electron-transport properties. FEBS Lett 134: 231–234

Biesiadka J, Loll B, Kern J, Irrgang K-D and Zouni A (2004) Crystal structure of cyanobacterial photosystem II at 3.2 Å resolution: a closer look at the Mn-cluster. Phys Chem Chem Phys 6: 4733–4736

Blubaugh DJ and Govindjee (1986) Bicarbonate, not CO_2, is the species required for the stimulation of photosystem II electron transport. Biochim Biophys Acta 848: 147–152

Blubaugh DJ and Govindjee (1988a) The molecular mechanism of the bicarbonate effect at the plastoquinone reductase site of photosynthesis. Photosynth Res 19: 85–128

Blubaugh DJ and Govindjee (1988b) Kinetics of the bicarbonate effect and the number of bicarbonate binding sites in thylakoid membranes. Biochim Biophys Acta 936: 208–214

Bowden SJ, Hallahan BJ, Ruffle SV, Evans MCW and Nugent JHA (1991) Preparation and characterization of photosystem II core particles with and without bound bicarbonate. Biochim Biophys Acta 1060: 89–96

Calderone V, Trabucco M, Vujicic A, Battistutta R, Giacometti GM, Andreucci F, Barbato R and Zanotti G (2003) Crystal structure of the PsbQ protein of photosystem II from higher plants. EMBO Rep 4: 900–905

Cao J, Ohad N, Hirschberg J, Xiong J and Govindjee (1992) Binding affinity of bicarbonate and formate in herbicide-resistant D1 mutants of *Synechococcus* sp. PCC-7942. Photosynth Res 34: 397–408

Carrieri D, Ananyev GM, Brown T and Dismukes GC (2007) *In vivo* bicarbonate requirement for water oxidation by photosystem II in the hypercarbonate-requiring cyanobacterium *Arthrospira maxima*. J Inorg Biochem 101: 1865–1874

Castelfranco PA, Lu YK and Stemler AJ (2007) Hypothesis: the peroxydicarbonic acid cycle in photosynthetic oxygen evolution. Photosynth Res 94: 235–246

Cheniae GM and Martin IF (1967) Photoreactivation of manganese catalyst in photosynthetic oxygen evolution. Biochem Biophys Res Commun 28: 89–95

Clausen J and Junge W (2004) Detection of an intermediate of photosynthetic water oxidation. Nature 430: 480–483

Clausen J, Beckmann K, Junge W and Messinger J (2005) Evidence that bicarbonate is not the substrate in photosynthetic oxygen evolution. Plant Physiol 139: 1444–1450

Cox N, Jin L, Jaszewski A, Smith PJ, Krausz E, Rutherford AW and Pace R (2009) The semiquinone-iron complex of photosystem II: structural insights from ESR and theoretical simulation. Evidence that the native ligand to the non-heme iron is carbonate. Biophys J 97: 2024–2033

DasGupta J, Ananyev GM and Dismukes GC (2008) Photoassembly of the water-oxidizing complex in photosystem II. Coord Chem Rev 252: 347–360

Debus R (2005) The catalytic manganese cluster: Protein ligation. In: Wydrzynski T and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 261–284. Springer, Dordrecht

Demeter S, Janda T, Kovacs L, Mende D and Wiessner W (1995) Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1229: 166–174

Diner BA and Petrouleas V (1987) Q_{400}, the non-heme iron of the photosystem II iron-quinone complex. A spectroscopic probe of quinone and inhibitor binding to the reaction center. Biochim Biophys Acta 895: 107–125

Diner BA and Petrouleas V (1990) Formation by NO of nitrosyl adducts of redox components of the photosystem II reaction center. II. Evidence that HCO_3^-/CO_2 binds to the acceptor side non-heme iron. Biochim Biophys Acta 1015: 141–149

Diner BA, Petrouleas V and Wendoloski JJ (1991) The iron-quinone electron-acceptor complex of photosystem II. Physiol Plant 81: 423–436

Dismukes GC, Klimov VV, Baranov SV, Kozlov YuN, DasGupta J and Tyryshkin A (2001) The origin of atmospheric oxygen on earth: The innovation of oxygenic photosynthesis. Proc Natl Acad Sci USA 98: 2170–2175

Dismukes GC, Gennady MA and Watt R (2005) Photoassembly of the catalytic manganese cluster. In: Wydrzynksi T and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 609–626. Springer, Dordrecht

Eaton-Rye JJ and Govindjee (1984) A study of the specific effect of bicarbonate on photosynthetic electron transport in the presence of methyl viologen. Photochem Photobiol 8: 279–288

Eaton-Rye JJ and Govindjee (1987) The effect of pH and flash frequency on electron transfer through the quinone acceptor complex of PS II in bicarbonate depleted or anion inhibited thylakoid membranes. In: Biggins J (ed) Progress in Photosynthesis Research, Vol II, pp 433–436. Martinus Nijhoff Publishers, Dordrecht

Eaton-Rye JJ and Govindjee (1988a) Electron transfer through the quinone acceptor complex of photosystem II in bicarbonate-depleted spinach thylakoid membranes as a function of actinic flash number and frequency. Biochim Biophys Acta 935: 237–247

Eaton-Rye JJ and Govindjee (1988b) Electron transfer through the quinone acceptor complex of photosystem II after one or two actinic flashes in bicarbonate-depleted spinach thylakoid membranes. Biochim Biophys Acta 935: 248–257

Eaton-Rye JJ, Blubaugh DJ and Govindjee (1986) Action of bicarbonate on photosynthetic electron transport in the presence or absence of inhibitory anions. In: Papageorgiou G, Barber J and Papa S (eds) Ion Interactions in Energy Transfer Biomembranes, pp 263–278. Plenum Press, New York

El-Shintinawy F and Govindjee (1990) Bicarbonate effect in leaf discs from spinach. Photosynth Res 24: 189–200

Fernandez-Velasco JG, Freeman J, Harms A, Dismukes GC and Wydrzynski T (2005) Bicarbonate stabilizes photosystem II electron transport in intact filaments of *Sprirulina maxima*. In: Van der Est A and Bruce D (eds) Photosynthesis: Fundamental Aspects to Global

Perspectives, Vol 1, pp 298–301. Alliance Communications Group, Lawrence

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Feyziev YM, Yoneda D, Yoshii T, Katsuta N, Kawamori A and Watanabe Y (2000) Formate-induced inhibition of the water-oxidizing complex of photosystem II studied by EPR. Biochemistry 39: 3848–3855

Frasch W and Sayre RT (2001) Obituary: Remembering George Cheniae, who never compromised his high standards of science. Photosynth Res 70: 245–247

Garab G, Sanchez Burgos AA, Zimányi L and Faludi-Dániel Á (1983) Effect of CO_2 on the energization of thylakoids in leaves of higher plants. FEBS Lett 154: 323–327

Garab G, Rozsa Z and Govindjee (1988) Carbon dioxide affects charge accumulation in leaves – measurements by thermo-luminescence. Naturwissenschaften 75: 517–519

Golbeck JH (ed) (2006) Photosystem I: The Light-Driven Plastocyanin:Ferrerdoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24. Springer, Dordrecht

Good NE (1963) Carbon dioxide & the Hill reaction. Plant Physiol 38: 298–304

Govindjee and Björn LO (2011) Dissecting oxygenic photosynthesis: The evolution of the "Z"-scheme for thylakoid reactions. In: Itoh S, Mohanty P and Guruprasad KN (eds) Photosynthesis: Overviews on Recent Progress and Future Perspectives, pp 1–27. IK Publishers, New Delhi

Govindjee and Eaton-Rye JJ (1986) Electron transfer through photosystem II acceptors: interaction with anions. Photosynth Res 10: 365–379

Govindjee and Shevela D (2011) Adventures with cyanobacteria: a personal perspective. Front Plant Sci 2: 28. doi:10.3389/fpls.2011.00028

Govindjee and Van Rensen JJS (1978) Bicarbonate effects on the electron flow in isolated broken chloroplasts. Biochim Biophys Acta 505: 183–213

Govindjee and Van Rensen JJS (1993) Photosystem II reaction center and bicarbonate. In: Deisenhofer J and Norris JR (eds) The Photosynthetic Reaction Center, Vol I, pp 357–388. Academic Press, New York

Govindjee, Pulles MJP, Govindjee R, Van Gorkom, HJ and Duysens LNM (1976) Inhibition of the reoxidation of the secondary electron acceptor of photosystem II by bicarbonate depletion. Biochim Biophys Acta 449: 602–605

Govindjee, Weger HG, Turpin DH, Van Rensen JJS, de Vos OJ and Snel JFH (1991) Formate releases carbon dioxide/bicarbonate from thylakoid membranes. Naturwissenschaften 78: 168–170

Govindjee, Snel JFH, De Vos OJ and Van Rensen JJS (1993) Antagonistic effects of light I and light II on chlorophyll *a* fluorescence yield and P700 turnover as monitors of carbon dioxide depletion in intact algal and cyanobacterial cells. Physiol Plant 89: 143–148

Govindjee, Kern JF, Messinger J and Whitmarsh J (2010) Photosystem II. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd, Chichester. DOI: 10.1002/9780470015902.a0000669.pub2

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A and Saenger W (2009) Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nat Struct Mol Biol 16: 334–341

Guskov A, Gabdulkhakov A, Broser M, Glöckner C, Hellmich J, Kern J, Frank J, Müh F, Saenger W and Zouni A (2010) Recent progress in the crystallographic studies of photosystem II. ChenPhysChem 11: 1160–1171

Hanson DT, Franklin LA, Samuelsson G and Badger MR (2003) The *Chlamydomonas reinhardtii cia3* mutant lacking a thylakoid lumen-localized carbonic anhydrase is limited by CO_2 supply to rubisco and not photosystem II function in vivo. Plant Physiol 132: 2267–2275

Haumann M, Grundmeier A, Zaharieva I and Dau H (2008) Photosynthetic water oxidation at elevated dioxygen partial pressure monitored by time-resolved X-ray absorption measurements. Proc Natl Acad Sci USA 105: 17384–17389

Hesketh JD, Wooley JT and Peters DB (1982) Predicting photosynthesis. In: Govindjee (ed) Photosynthesis vol 2: Development, Carbon Metabolism and Plant Productivity, pp 387–418. Academic Press, New York

Hienerwadel R and Berthomieu C (1995) Bicarbonate binding to the non-heme iron of photosystem II investigated by Fourier transform infrared difference spectroscopy and ^{13}C-labeled bicarbonate. Biochemistry 34: 16288–16297

Hillier W and Babcock GT (2001a) Photosynthetic reaction centers. Plant Physiol 125: 33–37

Hillier W and Babcock GT (2001b) S-state dependent Fourier transform infrared difference spectra for the photosystem II oxygen evolving complex. Biochemistry 40: 1503–1509

Hillier W and Messinger J (2005) Mechanism of photosynthetic oxygen production. In: Wydrzynski T and Satoh K (eds) Photosystem II: The Light-Driven Water: Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 567–608. Springer, Dordrecht

Hillier W, McConnell I, Badger MR, Boussac A, Klimov VV, Dismukes GC and Wydrzynski T (2006) Quantitative assessment of intrinsic carbonic anhydrase activity and the capacity for bicarbonate oxidation in photosystem II. Biochemistry 21: 2094–2102

Hulsebosch RJ, Allakhverdiev SI, Klimov VV, Picorel R and Hoff AJ (1998) Effect of bicarbonate on the S_2 multiline EPR signal of the oxygen-evolving complex in photosystem II membrane fragments. FEBS Lett 424: 146–148

Hwang HJ, Dilbeck P, Debus RJ and Burnap RL (2007) Mutation of arginine 357 of the CP43 protein of photosystem II severely impairs the catalytic S-state cycle of the H_2O oxidation complex. Biochemistry 46:11987–11997

Ingen-Housz J (1779) Experiments Upon Vegetables, Discovering Their Great Power of Purifying the Common

Air in Sunshine and Injuring It in the Shade and at Night. Elmsley and Payne, London

Ingen-Housz J (1796) Food of Plants and the Renovation of the Soil. Appendix to the Outlines of the Fifteenth Chapter of the Proposed General Report from the Board of Agriculture. Elmsley and Payne, London

Ireland CR, Baker NR and Long SP (1987) Evidence for a physiological role of CO_2 in the regulation of photosynthetic electron transport in intact leaves. Biochim Biophys Acta 893: 434–443

Izawa S (1962) Stimulatory effects of carbon dioxide upon the Hill reaction as observed with the addition of carbonic anhydrase to reaction mixture. Plant Cell Physiol 3: 221–227

Jackson SA, Fagerlund RD, Wilbanks SM and Eaton-Rye JJ (2010) Crystal structure of PsbQ from *Synechocystis* sp. PCC 6803 at 1.8 Å: Implications for binding and function in cyanobacterial photosystem II. Biochemistry 49: 2765–2767

Kamen M (1989) Onward into a fabulous half century. Photosynth Res 21: 137–144

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Park YI, Husic HD, Moroney JV and Samuelsson G (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient O_2. EMBO J 17: 1208–1216

Khanna R, Govindjee and Wydrzynski T (1977) Site of bicarbonate effect in Hill reaction. Evidence from the use of artificial electron acceptors and donors. Biochim Biophys Acta 462: 208–214

Khanna R, Pfister K, Keresztes A, Van Rensen JJS and Govindjee (1981) Evidence for a close spatial location of the binding sites for CO_2 and for photosystem II inhibitors. Biochim Biophys Acta 634: 105–116

Klimov VV and Baranov SV (2001) Bicarbonate requirement for the water-oxidizing complex of photosystem II. Biochim Biophys Acta 1503: 187–196

Klimov VV, Allakhverdiev SI, Baranov SV and Feyziev YaM (1995a) Effects of bicarbonate and formate on the donor side of photosystem II. Photosynth Res 46: 219–225

Klimov VV, Allakhverdiev SI, Feyziev YaM and Baranov SV (1995b) Bicarbonate requirement for the donor side of photosystem II. FEBS Lett 363: 251–255

Klimov VV, Hulsebosch R, Allakhverdiev S, Wincencjusz H, Van Gorkom H and Hoff A (1997a) Bicarbonate may be required for ligation of manganese in the oxygen evolving complex of photosystem II. Biochemistry 36: 16277–16281

Klimov VV, Baranov S and Allakhverdiev S (1997b) Bicarbonate protects the donor side of photosystem II against photoinhibition and thermoinactivation. FEBS Lett 418: 243–246

Klimov VV, Allakhverdiev SI, Nishiyama Y, Khorobrykh AA and Murata N (2003) Stabilization of the oxygen-evolving complex of photosystem II by bicarbonate and glycinebetaine in thylakoid and subthylakoid preparations. Funct Plant Biol 30: 797–803

Komarova Y, Doman N and Shaposhnikov G (1982) Two forms of carbonic anhydrase from bean chloroplasts. Biokhimiya 47: 1027–1034

Kreutz W (1974) Considerations on water-splitting in photosynthesis. In: Colhov K (ed) On the Physics of Biological Membranes, pp 419–429. Department of Physics, Simon Fraser University, Vancouver

Loll B, Kern J, Saenger W, Zouni A and Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. Nature 438: 1040–1044

Lu YK and Stemler AJ (2002) Extrinsic photosystem II carbonic anhydrase in maize mesophyll chloroplasts. Plant Physiol 128: 643–649

Lu YK, Theg SM and Stemler AJ (2005) Carbonic anhydrase activity of the photosystem II OEC33 protein from pea. Plant Cell Physiol 46: 1944–1953

McConnell IL, Badger M, Wydrzynksi T, Hillier W (2007) A quantitative assessment of the carbonic anhydrase activity in photosystem II. Biochim Biophys Acta 1767: 639–647

Mäenpää P, Miranda T, Tyystjärvi E, Tyystjärvi T, Govindjee, Ducruet JM, Etiene AL and Kirilovski D (1995) A mutation in the D-de loop of D1 modifies the stability of the $S_2Q_A^-$ and $S_2Q_B^-$ states in photosystem II. Plant Physiol 107: 187–197

McEvoy JP and Brudvig GW (2008) Redox reactions of the non-heme iron in photosystem II: An EPR spectroscopic study. Biochemistry 47: 13394–13403

Mende D and Wiessner W (1985) Bicarbonate in vivo requirement of photosystem II in the green alga *Chlamydobotrys stellata*. J Plant Physiol 118: 259–266

Messinger J, Seaton G, Wydrzynski T, Wacker U, Renger G (1997) S_{-3} state of the water oxidase in photosystem II. Biochemistry 36: 6862–6873

Metzner H (1978) Oxygen evolution as energetic problem. In: Metzner H (ed) Photosynthetic Oxygen Evolution, pp 59–76. Academic Press, London

Metzner H, Fischer K and Bazlen O (1979) Isotope ratios in photosynthetic oxygen. Biochim Biophys Acta 548: 287–295

Metzner H, Fischer K and Bazlen O (1981) The precursor of the photosynthetic oxygen. In: Akoyunoglou G (eds) photosynthesis II: Electron Transport and Photophosphorylation, pp 375–387. Balaban International Science Services, Philadelphia

Michel H and Deisenhofer J (1988) Relevance of the photosynthetic reaction center from purple bacteria to the structure of photosystem II. Biochemistry 27: 1–7

Moskvin OV, Shutova TV, Khristin MS, Ignatova LK, Villarejo A, Samuelsson G, Klimov VV and Ivanov BN (2004) Carbonic anhydrase activities in pea thylakoids. A photosystem II core complex associated carbonic anhydrase. Photosynth Res 79: 93–100

Moubarak-Milad M and Stemler A (1994) Oxidation-reduction potential dependence of photosystem II carbonic anhydrase in maize thylakoids. Biochemistry 33: 4432–4438

Noguchi T (2007) Light-induced FTIR difference spectroscopy as a powerful tool toward understanding the molecular mechanism of photosynthetic oxygen evolution. Photosynth Res 91: 59–69

Ono T-A and Inoue Y (1983) Requirement of divalent cations for photoactivation of the latent water-oxidation system in intact chloroplasts from flashed leaves. Biochim Biophys Acta 723: 191–201

Papageorgiou GC and Govindjee (eds) (2004) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19. Springer, Dordrecht

Park Y-I, Karlsson J, Rojdestvenski I, Pronina N, Klimov V, Oquist G and Samuelsson G (1999) Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*. FEBS Lett 444: 102–105

Petrouleas V and Diner BA (1990) Formation by NO of nitrosyl adducts of redox components of the photosystem II reaction center. I. NO binds to the acceptor-side non-heme iron. Biochim Biophys Acta 1015: 131–140

Petrouleas V, Deligiannakis Y and Diner BA (1994) Binding of carboxylate anions at the non-heme Fe(II) of PSII. 2. Competition with bicarbonate and effects on the Q_A/Q_B electron transfer rate. Biochim Biophys Acta 1188: 271–277

Pobeguts OV, Smolova TN, Zastrizhnaya OM and Klimov VV (2007) Protective effect of bicarbonate against the extraction of the extrinsic proteins of the water oxidising complex in the PSII membrane fragments. Biochim Biophys Acta 1767:624–632

Pobeguts OV, Smolova TN, Timoshevsky DS and Klimov VV (2010) Interaction of bicarbonate with the manganese-stabilizing protein of photosystem II. J Photochem Photobiol B: Biology 100: 30–37

Priestley J (1776) Experiments and Observations on Different Kinds of Air, Vol II, J. Johnson, London

Radmer R and Ollinger O (1980) Isotopic composition of photosynthetic O_2 flash yield in the presence of $H_2{}^{18}O$ and $HC^{18}O_3^-$. FEBS Lett 110: 57–61

Radmer R and Ollinger O (1986) Do the higher oxidation states of the photosynthetic O_2-evolving system contain bound H_2O? FEBS Lett 195: 285–289

Robinson HH, Eaton-Rye JJ, Van Rensen JJS and Govindjee (1984) The effects of bicarbonate depletion and formate incubation on the kinetics of oxidation-reduction reactions of the photosystem II quinone acceptor complex. Z Naturforsch 39c: 382–385

Rose S, Minagawa J, Seufferheld M, Padden S, Svensson B, Kolling DRJ, Crofts AR and Govindjee (2008) D1-arginine257 mutants (R257E, K, and Q) of *Chlamydomonas reinhardtii* have a lowered Q_B redox potential: analysis of thermoluminescence and fluorescence measurements. Photosynth Res 98: 449–468

Ruben S, Randall M, Kamen M and Hyde JL (1941) Heavy Oxygen ^{18}O as a tracer in study of photosynthesis. J Am Chem Soc 63: 877–879

Sarojini G and Govindjee (1981) On the active species in bicarbonate stimulation of Hill reaction in thylakoid membranes. Biochim Biophys Acta 634: 340–343

Sedoud A, Kastner L, Cox N, El-Alaoui S, Kirilovsky D and Rutherford AW (2011) Effects of formate binding on the quinone-iron electron acceptor complex of photosystem II. Biochim Biophys Acta 1807: 216–226

Semin BK, Loviagina ER, Aleksandrov AY, Kaurov YN and Novakova AA (1990) Effect of formate on Mössbauer parameters of the non-heme iron of PSII particles of cyanobacteria. FEBS Lett 270: 184–186

Senebier J (1782) Mémoires Physico-Chimiques sur L'Influence de la Lumière Solaire pour Modifier les Êtres de Trois Règnes, Surtout Ceux du Règne Végétal. 3 vols, Chirol, Genève

Shevela DN, Khorobrykh AN and Klimov VV (2006) Effect of bicarbonate on the water oxidising complex of photosystem II in the super reduced S-states. Biochim Biophys Acta 757: 253–261

Shevela DN, Klimov V and Messinger J (2007) Interactions of photosystem II with bicarbonate, formate and acetate. Photosynth Res 94: 247–264

Shevela DN, Su J-H, Klimov V and Messinger J (2008) Hydrogencarbonate is not a tightly bound constituent of the water-oxidizing complex in photosystem II. Biochim Biophys Acta 1777: 532–539

Shopes RJ, Blubaugh D, Wraight C and Govindjee (1989) Absence of a bicarbonate-depletion effect in electron transfer between quinones and reaction centers of *Rhodobacter sphaeroides*. Biochim Biophys Acta 974: 114–118

Shutilova NI, Klimov VV, Shuvalov VA and Kutyurin VM (1975) Isolation and investigation of photochemical and spectral properties of FS-2 subchloroplast fragments which are highly purified from FS-1 admixtures. Biofizika 20: 844–847

Shutova T, Kenneweg H, Buchta J, Nikitina J, Terentyev V, Chernyshov S, Andersson B, Allakhverdiev SI, Klimov VV, Dau H, Junge W and Samuelsson G (2008) The photosystem II-associated Cah3 in *Chlamydomonas* enhances the O_2 evolution rate by proton removal. EMBO J 27:782–791

Siegbahn PEM and Lundberg M (2005) The mechanism for dioxygen formation in PSII studied by quantum chemical methods. Photochem Photobiol Sci 4:1035–1043

Snel JFH and Van Rensen JJS (1983) Kinetics of the reactivation of the Hill reaction in CO_2-depleted chloroplasts by addition of bicarbonate in the absence or presence of herbicides. Physiol Plant 57: 422–427

Snel JFH and Van Rensen JJS (1984) Reevaluation of the role of bicarbonate and formate in the regulation of

photosynthetic electron flow in broken chloroplasts. Plant Physiol 75: 146–150

Spalding MH, Spreitzer RJ and Ogren WL (1983) Carbonic anhydrase-deficient mutant of *Chlamydomonas reinhardii* requires elevated carbon dioxide concentration for photoautotrophic growth. Plant Physiol 73: 268–272

Spreitzer RJ and Mets L (1981) Photosynthesis-deficient mutants of *Chlamydomonas reinhardii* with associated light-sensitive phenotypes. Plant Physiol 67: 565–569

Sproviero EM, Gascon JA, McEvoy JP, Brudvig GW and Batista VS (2006) QM/MM models of the O_2-evolving complex of photosystem II. J Chem Theor Comput 2: 1119–1134

Srivastava A, Strasser RJ and Govindjee (1995) Polyphasic rise of chlorophyll *a* fluorescence in herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii* 43: 131–141

Stemler A (1980a) Forms of dissolved carbon dioxide required for photosystem II activity in chloroplast membranes. Plant Physiol 65:1160–1165

Stemler AJ (1980b) Inhibition of photosystem II by formate: possible evidence for direct role of bicarbonate in photosynthetic oxygen evolution. Biochim Biophys Acta 593: 103–112

Stemler A (1986) Carbonic anhydrase associated with thylakoids and photosystem II particles from maize. Biochim Biophys Acta 850: 97–107

Stemler A (1989) Absence of a formate-induced release of bicarbonate from photosystem I. Plant Physiol 91: 287–290

Stemler A (1997) The case for chloroplast thylakoid carbonic anhydrase. Physiol Plant 99: 348–353

Stemler AJ (1998) Bicarbonate and photosynthetic oxygen evolution: an unwelcome legacy of Otto Warburg. Indian J Exp Biol 36: 841–848

Stemler AJ (2002) The bicarbonate effect, oxygen evolution, and the shadow of Otto Warburg. Photosynth Res 73: 177–183

Stemler A and Govindjee (1973) Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol 52: 119–123

Stemler A and Govindjee (1974) Effects of bicarbonate ion on chlorophyll *a* fluorescence transients and delayed light emission from maize chloroplasts. Photochem Photobiol 19: 227–232

Stemler A and Lavergne J (1997) Evidence that formate destabilizes the S_1 state of the oxygen-evolving mechanism in photosystem II. Photosynth Res 51: 83–92

Stemler AJ and Murphy JB (1983) Determination of the binding constant of $H^{14}CO_3^-$ to the photosystem II complex in maize chloroplasts: effects of inhibitors and light. Photochem Photobiol 38: 701–707

Stemler AJ, Babcock GT and Govindjee (1974) The effect of bicarbonate on photosynthetic oxygen evolution in flashing light in chloroplast fragments. Proc Natl Acad Sci USA 71: 4679–4683

Stern BK and Vennesland B (1960) The carbon dioxide requirement for the photoevolution of oxygen by chloroplast preparations J Biol Chem 235: PC 51–53

Stern BK and Vennesland B (1962) The effect of carbon dioxide on the Hill reaction. J Biol Chem 237: 596–602

Sundby C (1990) Bicarbonate effects on photo-inhibition. Including an explanation for the sensitivity to photoinhibition under anaerobic conditions. FEBS Lett 274: 77–81

Sundby C, Mattsson M and Schiött T (1992) Effects of bicarbonate and oxygen concentration on photoinhibition of thylakoid membanes. Photosynth Res 34: 263–270

Takahashi R, Boussac A, Sugiura M and Noguchi T (2009) Structural coupling of a tyrosine side chain with the non-heme iron center in photosystem II as revealed by light-induced Fourier transform infrared difference spectroscopy. Biochemistry 48: 8994–9001

Tripp BC, Smith K and Ferry JG (2001) Carbonic anhydrase: New insights for an ancient enzyme. J Biol Chem 276: 48615–48618

Ulas G, Olack G and Brudvig GW (2008) Evidence against bicarbonate bound in the O_2-evolving complex of photosystem II. Biochemistry 47: 3073–3075

Umena Y, Kawakami K, Shen JR and Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473: 55–60

Van Rensen JJS (1982) Molecular mechanisms of herbicide action near photosystem II. Physiol Plant 54: 515–521

Van Rensen JJS (2002) Role of bicarbonate at the acceptor side of photosystem II. Photosynth Res 73: 185–192

Van Rensen JJS and Klimov VV (2005) Bicarbonate interactions. In: Wydrzynski T and Satoh K (eds) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22, pp 329–345. Springer, Dordrecht

Van Rensen JJS and Vermaas WFJ (1981) Action of bicarbonate and photosystem II inhibiting herbicides on electron transport in pea grana and in thylakoids of a blue-green alga. Physiol Plant 51: 106–110

Van Rensen JJS, Tonk WJM and De Bruijn SM (1988) Involvement of bicarbonate in the protonation of the secondary quinone electron acceptor of photosystem II via the non-heme iron of the quinone-iron acceptor complex. FEBS Lett 226: 347–351

Van Rensen JJS, Xu C and Govindjee (1999) Role of bicarbonate in photosystem II, the water-plastoquinone oxidoreductase of plant photosynthesis. Physiol Plant 105: 585–592

Velthuys BR and Amesz J (1974) Charge accumulation at the reducing side of photosystem 2 of photosynthesis. Biochim Biophys Acta 333: 85–94

Vermaas WFJ and Rutherford AW (1984) EPR measurements on the effects of bicarbonate and triazine resistance

on the acceptor side of photosystem II. FEBS Lett 175: 243–248

Vermaas WFJ and Van Rensen JJS (1981) Mechanism of bicarbonate action on photosynthetic electron transport in broken chloroplasts. Biochim Biophys Acta 636: 168–174

Vermaas WFJ, Van Rensen JJS and Govindjee (1982) The interaction between bicarbonate and the herbicide ioxynil in the thylakoid membrane and the effects of amino acid modification on bicarbonate action. Biochim Biophys Acta 681: 242–247

Vernotte C, Briantais JM, Astier C and Govindjee (1995) Differential effects of formate in single and double mutants of D1 in *Synechocystis* sp. PCC 6714. Biochim Biophys Acta 1229: 296–301

Villarejo A, Shutova T, Moskvin O, Forssen M, Klimov V and Samuelsson G (2002) A photosystem II-associated carbonic anhydrase regulates the efficiency of photosynthetic oxygen evolution. EMBO J 21: 1930–1938

Wang X, Cao J, Maroti P, Stilz HU, Finkele U, Lauterwasse C, Zinth W, Oesterhelt D, Govindjee and Wraight C (1992) Is bicarbonate in photosystem II the equivalent of the glutamate ligand to the iron atom in bacterial reaction centers? Biochim Biophys Acta 1100: 1–8

Warburg O (1958) Photosynthesis. Science 128: 68–73

Warburg O (1964) Prefatory chapter. Ann Rev Biochem 33: 1–14

Warburg O and Krippahl G (1958) Hill-Reaktionen. Z Naturforsch 13b: 509–514

Warburg O and Krippahl G (1960) Notwendigkeit der Kohlensaure für die Chinon- und Ferricyanid-Reaktionen in Grünen Grana. Z Naturforsch 15b: 367–369

West J and Hill R (1967) Carbon dioxide and the reduction of indophenol and ferricyanide by chloroplasts. Plant Physiol 42: 819–826

Wincencjusz H, Allakhverdiev SI, Klimov VV and Van Gorkom HJ (1996) Bicarbonate-reversible formate inhibition at the donor side of photosystem II. Biochim Biophys Acta 1273: 1–3

Wydrzynski T and Govindjee (1975) A new site of bicarbonate effect in photosystem II of photosynthesis: evidence from Chl fluorescence transients in spinach chloroplasts. Biochim Biophys Acta 387: 403–408

Wydrzynski T and Satoh K (eds) (2005) Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 22. Springer, Dordrecht

Xiong J, Subramaniam S and Govindjee (1996) Modeling of the D1/D2 proteins and cofactors of the photosystem II reaction center: Implications for herbicide and bicarbonate binding. Protein Sci 5: 2054–2073

Xiong J, Subramaniam S and Govindjee (1998a) A knowledge-based three dimensional model of the photosystem II reaction center of *Chlamydomonas reinhardtii*. Photosynth Res 56: 229–254

Xiong J, Minagawa J, Crofts A and Govindjee (1998b) Loss of inhibition by formate in newly constructed photosystem II D1 mutants, D1-R257E and D1-R257M, of *Chlamydomonas reinhardtii.* Biochim Biophys Acta 1365: 473–491

Xu C, Taoka S, Crofts AR and Govindjee (1991) Kinetic characteristics of formate/formic acid binding at the plastoquinone reductase site in spinach thylakoids. Biochim Biophys Acta 1098: 32–40

Xyländer M and Hagen C (2002) 'Low-waves' in chlorophyll fluorescence kinetics indicate deprivation of bicarbonate. Photosynth Res 72: 255–262

Yruela I, Allakhverdiev SI, Ibarra JV and Klimov VV (1998) Bicarbonate binding to the water-oxidizing complex in the photosystem II. A Fourier transform infrared spectroscopy study. FEBS Lett 425: 396–400

Zaltsman L, Bruntrager E, Ananyev G and Dismukes GC (1997) A quantitative kinetic model for photo-assembly of the photosynthetic water oxidase from its inorganic constituents: requirements for manganese and calcium in the kinetically resolved steps. Biochemistry 36: 8914–8922

Chapter 21

Cytochrome b_6f Complex at the Heart of Energy Transduction and Redox Signaling

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Summary

The cytochrome (Cyt) b_6f complex (plastoquinol–plastocyanin or Cyt c_6 oxidoreductase) lies at the intersection of photosynthetic and respiratory electron transfer pathways in cyanobacteria and chloroplasts. It functions in both linear electron flow between the Photosystem II and I reaction centers and cyclic flow around Photosystem I, serving as an efficient coupling site that generates a majority of the proton gradient for ATP synthesis. The Cyt b_6f complex is related to mitochondrial-bacterial Cyt bc_1 complexes and belongs to a larger family of energy transducing 'Rieske cytochrome *b*' complexes that are widespread among bacterial and archael lineages. In addition to energy conversion, Cyt b_6f complexes serve as sensors of plastoquinone pool redox status and in signaling both short-term adjustments in photosynthesis and longer-term gene expression events. The past several years have witnessed remarkable advances in understanding of structures and reaction mechanisms. This chapter attempts to convey the excitement of these discoveries and the central role of the Cyt b_6f complex in energy conversion and environmental responses of photosynthetic organisms. Important advances include: the high-resolution structures of Cyt b_6f and Cyt bc_1 complexes; evidence for domain movement of the Rieske iron-sulfur protein subunit during catalysis; discovery of a previously undetected c-type heme required for transmembrane electron flow and activity of the plastoquinone-reductase site of Cyt b_6f complexes; evidence for supercomplex formation in direct cyclic electron flow from Photosystem I to the Cyt b_6f complex; roles for small subunits and peripherally associated proteins, and evidence for biologically significant intermonomer electron transfer in the dimeric Cyt bc_1-b_6f complexes. Many intriguing questions remain unanswered. I apologize to those whose contributions were not included or overlooked.

Abbreviations: COX – Cytochrome oxidase; CtaI – Cytochrome oxidase type I; CtaII – Cytochrome oxidase type II; Cyd – Cytochrome *bd*-type quinol-oxidase Cyt – Cytochrome; DBMIB – 2,5 Dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU – 3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea; DNP-INT – 2,4-Dinitrophenylether of 2-iodo-4-nitrothymol; DOPC – Dioleoylphosphatidyl-choline; EPR – Electron paramagnetic resonance; Fd – Ferredoxin; FNR – Ferredoxin-NADP$^+$-oxidoreductase; heme b_n – *b* heme near the *n*-side of the membrane; heme b_p – *b* heme near the *p*-side of the membrane; heme c_n – High-spin *c*-type heme near the *n*-side of the membrane; HiPIP – 'High-potential' iron-sulfur protein; HQNO – 2n-Heptyl-4-hydroxy-quinoline-N-oxide; ISP – Iron-sulfur protein; LHCI – Light-harvesting complex I of oxygenic phototrophs; LHCII – Light-harvesting complex II of oxygenic phototrophs; MGDG – Monogalactosyldiacylglycerol; NADPH – Nicotinamide adenine dinucleotide phosphate; NDH-1 – NAD(P)H dehydrogenase type I; NDH-2 – NAD(P)H dehydrogenase type II; NPQ – Non-photochemical quenching; NQNO – 2N-Nonyl-4-hydroxy-quinoline-N-oxide; PBS–Phycobilisome; PQ – Plastoquinone; PQ•$^-$ – Platosemiquinone anion radical; PQH_2 – Plastoquinol or plastohydroquinone; PS I – Photosystem I; PS II – Photosystem II; PTOX – Plastoquinol terminal oxidase; Q_n – Quinone-reduction (Q_i) site near the *n*-side of the membrane; Q_p – Quinol-oxidation (Q_o) site near the *p*-side of the membrane; QOX – Quinol oxidase; ROS – Reactive oxygen species; SDH – Succinate dehydrogenase; SQDG – Sulphoquinovosyldiacylglycerol; SQR – Sulfide-quinone reductase; TDS – Tridecyl-stigmatellin

I. Introduction

The cytochrome (Cyt) b_6f complex (plastoquinol–plastocyanin or Cyt c_6 oxidoreductase) is one of four essential, major thylakoid membrane protein complexes required for conversion of sunlight energy into ATP and NADPH that drives carbon dioxide (CO_2) fixation and sustains life on Earth. Functionally, the Cyt b_6f complex resides between the Photosystem II (PS II) and I (PS I) photochemical reaction centers of oxygenic photosynthesis as illustrated in Fig. 21.1. The complex functions in linear electron flow between the two photosystems, cyclic electron flow around PS I, and in formation of the transmembrane proton gradient for ATP synthesis. In addition, the Cyt b_6f complex serves as a sensor of redox potential in electron transport and in signaling that adjust light-harvesting antenna protein distribution between the photosystems (the so-called 'state transitions') (Wollman, 2001; Eberhard et al., 2008) as well as longer-term gene expression events (Pfannschmidt et al., 1999a, 2008). Cyt b_6f complexes are found in all cyanobacteria and chloroplasts of algae and plants. In cyanobacteria, Cyt b_6f complexes lie at the intersection of photosynthetic and respiratory electron transfer pathways and are essential for both (Scherer,

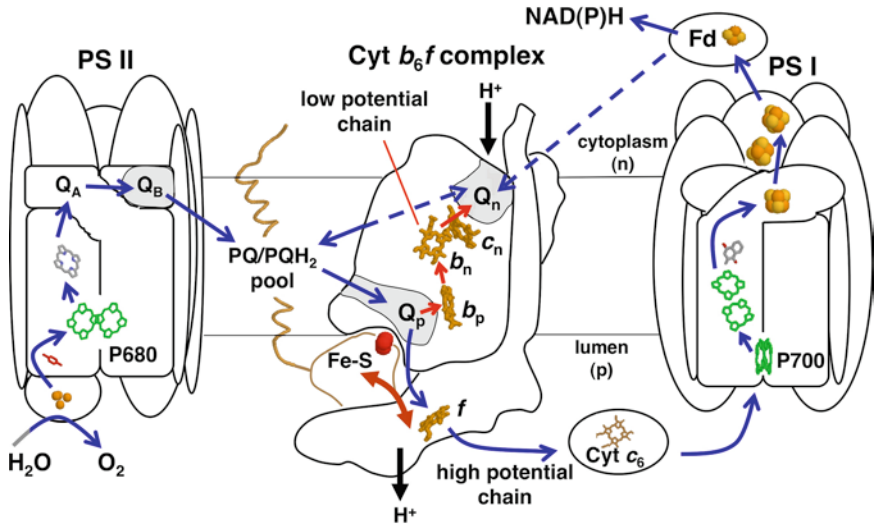

Fig. 21.1. Photosynthetic electron transport and high- and low-potential electron transfer chains of the cytochrome b_6f complex. *PS II* and *PS I*, photosystems II and I; *P680*, reaction center chlorophylls of PS II; Q_A and Q_B, acceptor-side quinones of PS II; *PQ/PQH$_2$ pool*, plastoquinone/plastoquinol pool; *Fe–S* and *f*, Rieske iron-sulfur cluster and cytochrome *f* of the Cyt b_6f high-potential chain (*blue arrows*); Q_p and Q_n, plastoquinol-oxidation and plastoquinone-reduction sites; hemes b_p, b_n, and c_n of the Cyt b_6f low-potential chain (*red arrows*); *Cyt c_6*, cytochrome c_6 protein; *Fd*, ferredoxin protein; *P700*, reaction center chlorophylls of PS I. Note that plastocyanin (*PC*) replaces Cyt c_6 exclusively in plant chloroplasts and facultatively in some algae and cyanobacteria. The *two-headed arrow* depicts the domain movement of the Rieske protein. *H$^+$ arrows* indicate the direction of proton translocation across the membrane. The cytoplasmic, electronegative (*n*) and lumenal, electropositive (*p*) sides of the membrane are labeled and electron transfer pathways are shown by arrows. The *dashed line* from Fd to the Q_n-site indicates a possible direct electron transfer path from PS I to the Cyt b_6f complex.

1990; Kallas, 1994). Related Cyt bc_1 complexes function in mitochondria and photosynthetic and non-photosynthetic bacteria (Berry et al., 2000; Darrouzet et al., 2004; Kramer et al., 2008). Diverse, hybrid Cyt bc_1-b_6f complexes occur in many bacterial and archael lineages (Schütz et al., 2000; Nitschke et al., 2010). Collectively, these enzymes have been termed 'Rieske Cyt *b*' complexes to reflect their core, catalytic Rieske iron-sulfur and Cyt *b* protein subunits (Kramer et al., 2008; Schütz et al., 2000).

Robert Hill and colleagues (Hill and Scarisbrick, 1951; Davenport and Hill, 1952; Hill, 1954) discovered *c*- (Cyt *f*) and *b*- (Cyt b_6) type cytochromes in plant leaves. Nelson and Neumann (1972) isolated, from chloroplasts, a protein 'particle' that contained Cyt *f*, Cyt b_6, and non-heme iron and exhibited plastoquinol:plastocyanin reductase activity. The high-potential Rieske 2Fe-2S center, a core component of Cyt bc_1 complexes (Rieske et al., 1964), was first detected in chloroplasts by Malkin and Aparicio (1975) and is a defining feature of Cyt b_6f and indeed all 'Rieske-Cyt *b*' complexes. Understanding of Cyt b_6f complexes was advanced greatly by discoveries of: (1) plastoquinol-1-plastocyanin (Wood and Bendall, 1976) and duroquinol-plastocyanin (White et al., 1978) oxidoreductase activities allowing in vitro measurements of Cyt b_6f electron transfer reactions; (2) specific inhibitors of the Cyt b_6f complex such as DBMIB (2,5 dibromo-3-methyl-6-isopropyl-p-benzoquinone) (Trebst et al., 1970), DNP-INT (2,4-dinitrophenylether of 2-iodo-4-nitrothymol) (Trebst et al., 1978), and NQNO (2n-nonyl-4-hydroxy-quinoline-N-oxide) (Selak and Whitmarsh, 1982; Jones and Whitmarsh, 1988) that helped define plastoquinone binding sites in the complex; (3) advances in electron paramagnetic resonance (EPR) (Malkin, 1981, 1982; Nitschke et al., 1992) and optical spectroscopy (Joliot and Joliot, 1984, 1992; Nitschke et al., 1988) for characterization of redox centers and reaction kinetics in isolated complexes, membranes, chloroplasts, and intact plant and algal cells, and (4) methods for isolation of defined, catalytically active Cyt b_6f

complexes (Hurt and Hauska, 1981; Hauska, 2004). The complex first isolated by Hurt and Hauska (1981) from spinach chloroplasts contained five polypeptides (M_r 34, 33, 23.5, 20, and 17.5 kDa), one *f* and two b_6 hemes, and one Rieske 2Fe-2S cluster. The 34 and 33 kDa polypeptides were later found to represent heterogenous forms of Cyt *f* (Hurt and Hauska, 1982).

The Cyt b_6f complex has been the subject of numerous reviews (Hauska et al., 1983; Malkin, 1992; Hope, 1993; Cramer et al., 1996, 2006, 2011; Allen, 2004; Cramer, 2004; Darrouzet et al., 2004; Baniulis et al., 2008; Kramer et al., 2008; Soriano et al., 1999) including one by the author (Kallas, 1994) in a previous volume of this series. Since that previous review, several remarkable discoveries have greatly advanced our understanding of this protein complex. These include: (1) Evidence from crystal structures of Cyt bc_1 complexes for domain movement of the Rieske iron-sulfur protein (ISP) required for catalysis (Zhang et al., 1998); several lines of evidence indicate a corresponding although not identical movement of the Rieske ISP in Cyt b_6f complexes as discussed later in Section VII.A.6.b (also see de Vitry et al., 2004 and references therein); (2) X-ray crystal structures of complete Cyt b_6f complexes from the alga *Chlamydomonas reinhardtii* (Stroebel et al., 2003) and the cyanobacteria *Mastigocladus laminosus* (Kurisu et al., 2003; Yan et al., 2006; Yamashita et al., 2007) and *Nostoc* sp. PCC 7120 (Baniulis et al., 2009). These have provided invaluable insights into the arrangement of protein subunits, redox centers, and substrate and inhibitor binding sites. The Cyt b_6f structures further revealed a previously undetected *c*-type heme (heme c_i, c_x, or c_n; herein designated c_n) located in the quinone-reductase (Q_i or Q_n, herein designated Q_n) site near the electronegative (*n*) side of the membrane. This heme is unique to Cyt b_6f complexes and essential for function as discussed below (Section VII.A.5). A chlorophyll and a carotenoid molecule are additional unique, and still enigmatic, features of Cyt b_6f complexes confirmed by the X-ray structures and not found in Cyt bc_1 complexes. The current review focuses on these and other advances since the previous review that have contributed enormously to our understanding of the biological function and evolution of Cyt b_6f complexes of photosynthesis.

II. Role of the Cytochrome b_6f Complex

A. In Photosynthetic Electron Transfer Chains

The Cyt b_6f complex lies at the heart of photosynthetic electron transport in cyanobacteria and chloroplasts, linking electron flow from PS II via the plastoquinone (PQ) pool to plastocyanin or Cyt c_6 to PS I (Fig. 21.1). Plastoquinol oxidation by the Cyt b_6f complex is the typical rate-limiting step in photosynthetic electron transport (Witt, 1971; Lee and Whitmarsh, 1989) and under some circumstances limits overall CO_2 fixation and growth (Price et al., 1995). The functional complex is a dimer as discussed below (Section VI.B). Each monomer is comprised of four major polypeptides (Cyt b_6, subunit IV, the Rieske ISP, and Cyt *f*) and four, membrane-spanning small subunits, PetG, PetL, PetM, PetN as revealed by the *C. reinhardtii* (Stroebel et al., 2003) and *M. laminosus* (Kurisu et al., 2003) Cyt b_6f structures. Two *b* hemes (b_L or b_p and b_H or b_n in the Cyt b_6 polypeptide), one *c* heme (in the Cyt *f* polypeptide), and a 2Fe-2S cluster (in the Rieske ISP) have long been known as redox cofactors. The b_L and b_H terminology arises from the midpoint potentials of the *b* hemes (Hauska et al., 1983; Kramer and Crofts, 1994; Pierre et al., 1995; Baymann et al., 2001), b_L typically being lower than b_H. However, there is continuing debate as to whether these potentials actually differ in thylakoid membranes in vivo or in situ (see Sacksteder et al., 2000; Cape et al., 2006; Baniulis et al., 2008; Cramer et al., 2011 for additional discussion). The designations b_p (for b_L) and b_n (for b_H) will be used here and refer to the locations of these hemes near the electropositive (*p*) and electronegative (*n*) sides of the membrane, respectively. As mentioned, the Cyt b_6f structures revealed a previously undetected and unique heme designated heme c_n located in the quinone-reductase (Q_n) pocket near the cytoplasmic, *n* side of the membrane (Kurisu et al., 2003; Stroebel et al., 2003).

The Cyt b_6f complex functions in both linear and cyclic photosynthetic electron transfer pathways. Linear electron flow from PS II through the PQ pool, Cyt b_6f complex, and PS I generates ATP and reduced ferredoxin (Fd), which reduces $NADP^+$ to NADPH via a Fd-$NAD(P)^+$ oxidoreductase (FNR) enzyme. Cyclic electron

transfer is driven by PS I alone and involves the return flow of electrons through the Cyt b_6f complex back to the PS I, P700 reaction center. Cyclic flow generates ATP only by pathways that are still controversial (see Section VIII.D). In both linear and cyclic flow, the Cyt b_6f complex transfers electrons from a lipophilic, two-electron donor (plastoquinol, PQH_2 from the PQ pool) to a hydrophilic, one-electron acceptor (plastocyanin or Cyt c_6) as depicted in Fig. 21.1. These reactions are efficiently coupled to proton translocation across the membrane by a 'Q-cycle' mechanism (Mitchell, 1976; Crofts et al., 1983), or possible variations thereof (Osyczka et al., 2004), all of which involve two binding sites for plastoquinone. Plastoquinol oxidation occurs at a quinol-oxidation site (Q_o or Q_p, herein designated Q_p) near the lumenal, electropositive (p) side of the thylakoid membrane. PQH_2 oxidation at this site occurs via a bifurcated pathway where one electron from PQH_2 is injected into a high-potential chain leading to PS I and the second electron into a low potential, transmembrane chain leading to the plastoquinone-reduction (Q_n) site located near the opposite, stromal (in chloroplasts) or cytoplasmic (in cyanobacteria), electronegative (*n*) side of the thylakoid membrane. Deposition of protons (H^+) from PQH_2 on the *p* side of the membrane and their acquisition by plastoquinone (PQ) on the *n* side generates a transmembrane proton gradient. These reactions and continuing controversies that surround them are further discussed below in Section VIII.

B. At the Intersection of Photosynthesis and Respiration in Cyanobacteria

In cyanobacteria the Cyt b_6f complex functions not only in photosynthetic electron flow but also in respiratory and other electron pathways that converge on the PQ pool and Cyt b_6f complex as illustrated in Fig. 21.2. These pathways are complex, not fully understood, and common as well as unique components reside in thylakoid and

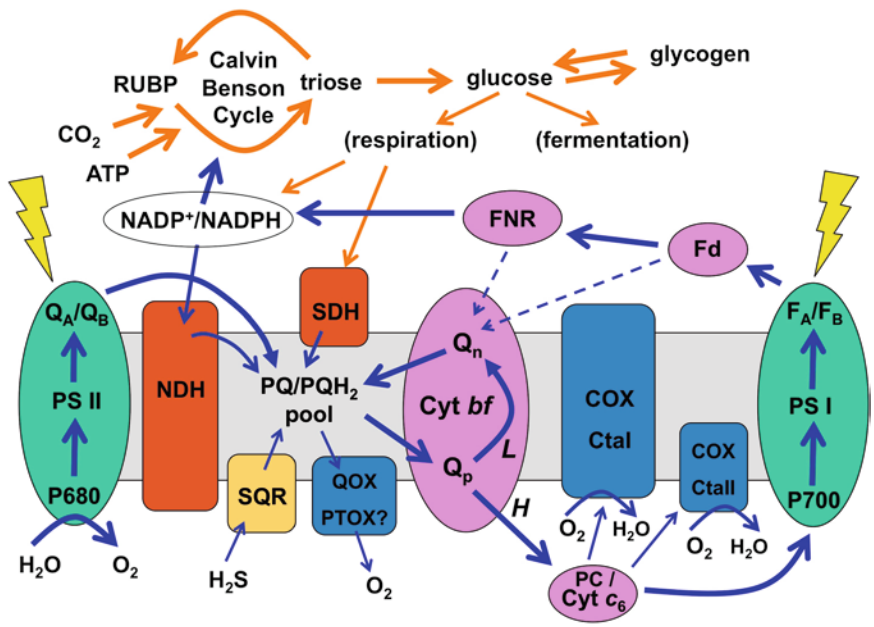

Fig. 21.2. Cytochrome b_6f complex at the crossroads of electron transport in cyanobacteria. *PS II and PS I*, photosystems II and I (*green*); *PQ/PQH₂ pool*, plastoquinone/plastoquinol pool; and Cyt b_6f complex as in Fig. 21.1. *H* and *L* mark the Cyt b_6f high- and low-potential chains. F_A/F_B are the final 4Fe-4S electron acceptors within the PS I complex. *NDH*, NAD(P)H dehydrogenase; *SDH*, succinate dehydrogenase; *SQR*, sulfide-quinone oxidoreductase; *QOX*, quinol oxidase; *PTOX*, plastoquinone terminal oxidase; *COX (Cta)*, cytochrome oxidase I or II; *PC/Cyt* c_6, plastocyanin or cytochrome c_6; *Fd*, ferredoxin; *FNR*, ferredoxin-$NADP^+$-oxidoreductase. The *grey region* depicts the thylakoid membrane. *Dark arrows* represent electron transfer pathways with *wider arrows* showing the major path of photosynthetic electron flow. *Dashed lines* from Fd or FNR to the Q_n-site depict possible direct, cyclic electron flow from PS I to the Cyt b_6f complex. CO_2, ATP, and NAD(P)H inputs into the Calvin-Benson cycle are shown and *orange arrows* represent major pathways of carbon or chemical energy flow.

cytoplasmic membranes (Schmetterer, 1994; Kerfeld and Krogmann, 1998; Cooley and Vermaas, 2001; Pils et al., 2004; Nomura et al., 2006a). Pathways for electron flow into the PQ pool include NAD(P)H dehydrogenases NDH-1 and NDH-2 (Pils et al., 2004; Zhang et al., 2004b), succinate dehydrogenase (SDH) (Cooley et al., 2000; Cooley and Vermaas, 2001), and sulfide-quinol reductase (SQR) (Garlick et al., 1977; Arieli et al., 1994). Under dark, aerobic conditions, electrons flow from carbon reserves such as glycogen to NADPH, then via NAD(P)H dehydrogenase into the PQ pool, and via the Cyt b_6f complex and plastocyanin or Cyt c_6 to cytochrome oxidase (COX or Cta1, CtaII) with oxygen as the terminal electron acceptor (Schmetterer, 1994; Hart et al., 2005; Navarro et al., 2005). Because cytochrome oxidases are not very active in cyanobacteria, the plastoquinone (PQ) pool generally becomes highly reduced in most cyanobacteria during darkness. In *Synechocystis* sp. PCC 6803, SDH appears to be the major route of dark, PQ pool reduction (Cooley and Vermaas, 2001). However, mutations in NDH-1, NDH-2, and SDH all decrease NAD(P)H oxidation and thereby increase NAD(P)H levels because of interwoven electron transfer pathways and regulatory events. NAD(P)H levels in turn critically influence electron and metabolite fluxes in diverse pathways, including CO_2 fixation (Fig. 21.2) as well as dark respiratory and fermentation pathways such as that for hydrogen production, which depend on NAD(P)H for electrons (Cournac et al., 2004).

The Cyt b_6f complex is the major and essential route for PQ pool oxidation, although some cyanobacteria such as *Synechocystis* sp. PCC 6803 posses a Cyt *bd*-type quinol-oxidase (Cyd or QOX) (Howitt and Vermaas, 1998; Berry et al., 2002) that can directly oxidize the PQ pool and bypass the Cyt b_6f complex. Other cyanobacteria, including marine, planktonic *Synechococcus* strains, have a plastoquinone terminal oxidase (PTOX) (Bailey et al., 2008; Mackey et al., 2008) that is unrelated to *bd*-type oxidases but related to chloroplast PQ terminal oxidases (Streb et al., 2005). PTOX, like the *bd* oxidases, affords protection from PQ pool over-reduction during periods of high light and nutrient deprivation. Interestingly, marine, coastal algae and cyanobacteria that are less likely to experience nutrient deprivation appear to have neither a Cyd nor a PTOX oxidase (Cardol et al., 2008; Mackey et al., 2008). For example, the marine, coastal cyanobacterium, *Synechococcus* sp. PCC 7002 (Van Baalen, 1962), which tolerates extreme light intensities of greater than twice full sunlight (Nomura et al., 2006b), lacks any apparent quinol oxidase other than the Cyt b_6f complex. Its basis for extreme, high-light tolerance remains largely unknown although the allophycocyanin-B alpha subunit, ApcD protein, for efficient energy transfer from the phycobilisome (PBS) to PS I and 'state transitions' (see Section IX.A), appears to be important (Dong et al., 2009).

Electrons from the Cyt b_6f complex can flow to either PS I or a terminal CtaI or CtaII cytochrome oxidase (Navarro et al., 2005). Elegant membrane inlet mass spectrometry and isotopic labeling experiments demonstrate that cyanobacteria may simultaneously evolve oxygen from PS II and consume it via PS I Mehler ('pseudo-cyclic') (Helman et al., 2003) or plastoquinol-oxidase (Cyd-QOX or PTOX) reactions, both of which reduce O_2 to H_2O. Mehler (Mehler, 1951) and 'pseudo-cyclic' electron flow reactions (Allen, 2003) both refer to the photoreduction of oxygen by electrons from PS I. The Mehler reaction in *Synechocystis* sp. PCC 6803, described by Helman and co-workers (2003), is a 'water-water' (PS II water oxidation to PS I oxygen reduction) reaction that does not generate oxygen radicals. These simultaneous pathways presumably allow cells to maintain PQ pool redox poise and 'open,' uninhibited PS II reaction centers while maintaining a high ATP/NAD(P)H ratio for optimal CO_2 fixation (Bailey et al., 2008; Cardol et al., 2008). Cyanobacterial terminal oxidases, of which there are several (Pils et al., 2004; Navarro et al., 2005), also serve crucial roles in redox homeostasis as illustrated by the lethality of a CtaI (CoxI) mutation in *Synechocystis* sp. PCC 6803 grown at low light intensity (Kufryk and Vermaas, 2006). The CtaI mutation is fatal under this condition presumably because PS I activity is too low and cytochrome oxidase (CtaI) activity absent to prevent over-reduction of the PQ pool and lethal generation of oxygen radicals. In *Synechococcus* sp. PCC 7002 as well, the CtaI and CtaII terminal oxidases both serve important roles in regulation of oxidative stress responses (Nomura et al., 2006a, b). With the exception of the *bd* (Cyd or QOX) and PTOX oxidases that can directly

oxidize the PQ pool, all of these alternative electron pathways involve electron flow through the Cyt b_6f complex.

Plants and algae have mitochondria equipped with Cyt bc_1 complexes that perform respiratory electron transport for generating ATP during darkness. The electron transfer pathways of chloroplasts have thus evolved toward greater simplicity than those of cyanobacteria. However, chloroplast thylakoid membranes of phototrophic eukaryotes do possess multi-subunit, type I (NDH 1 (Burrows et al., 1998)) or monomeric, type II (NDH 2 (Jans et al., 2008; Desplats et al., 2009)) NAD(P)H dehydrogenases that are capable of non-photochemical (PS II independent) reduction of the PQ pool. These have roles in 'chlororespiration' (Bennoun, 1982; Nixon, 2000), which refers to the oxygen-dependent oxidation of the PQ pool either via PQ oxidases that do not involve the Cyt b_6f complex or via cyclic electron flow around PS I (Burrows et al., 1998; Jans et al., 2008) that does require the b_6f complex. These pathways are important for energizing the thylakoid membrane during periods of darkness and for maintaining redox homeostasis of the PQ pool. It is important to note that Cyt b_6f complexes do not require light for activity and are capable of oxidizing the PQ pool under any condition as long as oxidized electron acceptors are available.

III. Relation to Bacterial-Mitochondrial Cytochrome bc_1 Complexes

'Cyanobacterial-chloroplast-type' Cyt b_6f and 'bacterial-mitochondrial-type' Cyt bc_1 oxidoreductase complexes are both strikingly similar and uniquely different in aspects of their structure and function. These 'Rieske-Cyt *b*' complexes operate as quinol oxidases and Cyt *c* or plastocyanin reductases that couple the energy released from electron transport to proton translocation across the membrane (reviewed by Hauska et al., 1983; Berry et al., 2000; Schütz et al., 2000; Darrouzet et al., 2004; Smith et al., 2004; Baniulis et al., 2008; Kramer et al., 2008). 'Typical' Cyt bc_1 complexes occur in all mitochondria and in diverse groups of alpha, beta, and gamma proteobacteria that include photosynthetic purple bacterial, rhizobial, and *Paraccocus* strains (see Darrouzet et al., 2004). Mitochondrial Cyt bc_1 complexes have ten or eleven subunits of which three core, membrane-spanning subunits, Cyt *b* (with low and high potential *b*-hemes, b_L and b_H), the Rieske ISP (with a 2Fe-2S cluster), and Cyt c_1 (with heme c_1), perform the catalytic reactions of the complex (Berry et al., 2000; Zara et al., 2009). Indeed, alpha proteobacteria such as *Rhodobacter capsulatus*, *R. sphaeroides*, and *Paracoccus denitrificans* have catalytically active bc_1 complexes comprised of only the three core polypeptides (Berry et al., 2000; Darrouzet et al., 2004). It has long been evident from sequence comparisons that the Cyt b_6 and subunit IV polypeptides of Cyt b_6f complexes correspond to N- and C-terminal segments, respectively, of Cyt *b* polypeptides of Cyt bc_1 complexes (Widger et al., 1984). The four histidine residues that provide axial ligands for the two *b*-hemes align precisely in Cyt *b*-b_6 sequences with the exception of an additional residue between the two His in helix D of Cyt b_6. Similarly the C-terminal, cluster binding domains of Cyt bc_1-b_6f Rieske ISPs are well conserved, in particular the 'box I' and 'box II' 2Fe-2S cluster binding motifs, CTHLGC and CPCHGS in typical Cyt b_6f-bc_1 Rieske ISPs (Carrell et al., 1997; Kallas et al., 1988b). The Cyt *f* and c_1 polypeptides both function as electron acceptors for the Rieske ISP and donors to soluble *c* cytochromes or plastocyanin in the high-potential chains of Cyt b_6f and bc_1 complexes, respectively, but are otherwise completely unrelated with the exception of the *c*-heme binding motif Cys-X-X-Cys-His (where X represents any amino acid) (Chi et al., 2000; Martinez et al., 1994). Note that the '*f*-heme' of Cyt *f* polypeptides is actually a covalently bound, *c*-type heme. The cytochrome *f* terminology derives from 'frons,' Latin for leaf (Allen, 2004).

The excellent structures of Cyt bc_1 (Xia et al., 1997; Iwata et al., 1998; Kim et al., 1998; Zhang et al., 1998) and b_6f (Kurisu et al., 2003; Stroebel et al., 2003) complexes that have emerged since 1997 and 2003, respectively, confirm the overall conservation of protein architecture and geometry of *b*-hemes and quinone binding sites in these complexes (see Figs. 21.3 and 21.4 for views of the Cyt b_6f structure). For example, the distances between the iron atoms at the centers of the two *b*-hemes are identical within experimental error, ~20.8 Å, in both Cyt b_6f and bc_1 complexes. The position of the Rieske 2Fe-2S cluster is variable

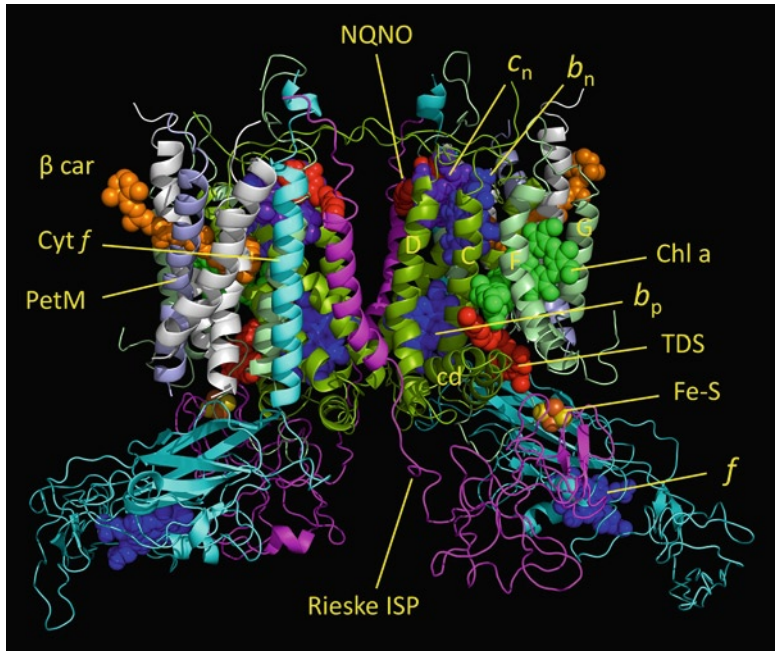

Fig. 21.3. Membrane parallel view of the *Mastigocladus laminosus* cytochrome b_6f dimer. Shown is a ribbon diagram of X-ray crystal structure 1VF5 (Kurisu et al., 2003) with the inhibitors TDS, 'ring-in' conformation, and NQNO superposed from structures 2E76 and 2E75, respectively (Yamashita et al., 2007). The cytoplasmic (negative, *n*) side of the thylakoid membrane is at the *top*, the lumenal (positive, *p*) side at the *bottom*. Membrane-spanning helices C and D and the amphipathic, membrane-parallel helices cd of Cyt b_6 (*olive green*) and helices F and G of the subunit IV polypeptide (*light grey-green*) are visible and labeled in the monomer at the right. The Cyt *f* helix (*turquoise*) and the helices of the four small subunits (PetM, *grey blue, the others white*) are visible in the monomer at the *left*. Note that the Rieske iron-sulfur protein (*ISP, magenta*) 'crosses-over' between monomers of the Cyt b_6f complex. Its transmembrane helix lies in one monomer and its lumenal, iron-sulfur cluster (Fe-S) binding domain lies in the other monomer. Hemes b_p, b_n, and *f* are shown as *blue*, space-filling structures. Heme c_n is *purple*. TDS and NQNO are shown as space-filling, red molecules and define the quinol-oxidation (Q_p) and quinone-reduction (Q_n) domains, respectively. The chlorophyll *a* (*Chl a, green*) and β-carotene (*β car, orange*) are shown in space-filling form. More recent refinements of the original 1VF5 dimer structure show the N-terminal, *n*-side regions of Cyt b_6 and subunit IV as membrane parallel alpha helices (see Cramer et al., 2011). Structure images in this and other figures were created with PyMol (http://www.pymol.org/).

in many of these structures and this difference in mitochondrial bc_1 complexes crystallized in the presence or absence of inhibitors provided the first compelling evidence for domain movement of the Rieske ISP during catalysis (Kim et al., 1998; Zhang et al., 1998). A general consensus has emerged that Cyt b_6f complexes, like Cyt bc_1 complexes, typically operate according to a 'Q-cycle' model of electron transfer as originally proposed by Mitchell (1976) with subsequent modifications (Crofts et al., 1983). Questions, however, remain as to the details of reaction mechanisms and whether the Q-cycle is engaged continuously in Cyt b_6f complexes. These points are discussed below (Section VIII). Overall, the structures of quinol-oxidation (Q_p) sites of Cyt b_6f and bc_1 complexes and the reaction mechanisms of quinol oxidation are quite similar. In contrast, although the respective quinone-reductase (Q_n) sites of b_6f-bc_1 complexes perform similar functions, the structures of these sites and their reaction mechanisms differ markedly. One early indication of divergence of Q_n-sites in b_6f-bc_1 complexes came from the observation of very different inhibitor specificities as discussed below (Section VIII.A). For example, the classical Q_n-site inhibitor of Cyt *bc* complexes, antimycin-A (Hauska et al., 1983), has no activity in Cyt b_6f complexes, at least not at the Q_n site (Cramer et al., 2006). The unique c_n heme of Cyt b_6f complexes, that resides in the Q_n-pocket (Kurisu et al., 2003; Stroebel et al., 2003), represents a major

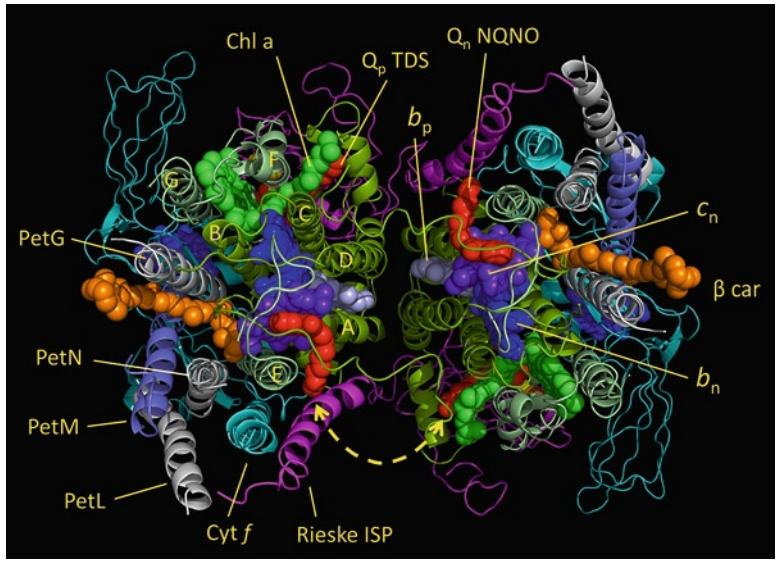

Fig. 21.4. Cytoplasmic, *n*-side view of the cytochrome b_6f dimer. *Mastigocladus laminosus* Cyt b_6f dimer structure IVF5 with the inhibitors TDS and NQNO as in Fig. 21.3, viewed perpendicular to the membrane from the cytoplasmic (electronegative, *n*) side of the thylakoid membrane. Helices A, B, C, and D of the Cyt b_6 polypeptide (*olive green*) and helices E, F, and G of subunit IV (*light grey-green*) are labeled and protein subunits are colored as in Fig. 21.3. Note again that the transmembrane helix of each Rieske ISP (*magenta*) crosses over and is anchored in the opposite monomer. The four small protein subunits (PetM, *grey blue*, and PetG, PetL, and PetN, *light grey*) form a four-helix, transmembrane bundle at the periphery of each Cyt b_6f monomer. These have been re-assigned since (Kurisu et al., 2003) and are labeled here according to the assignments in (Stroebel et al., 2003) and (Yamashita et al., 2007) as discussed in the text. The inhibitors, TDS and NQNO again define the quinol-oxidation (Q_p) and quinone-reductase (Q_n) substrate binding pockets of the Cyt b_6f complex. The close juxtapositions of NQNO, heme c_n, and heme b_n are clearly evident and these components are largely exposed near the *n*-side of the membrane. Hemes b_p (*pale blue*) are visible near the opposite, *p*-side of the membrane. Quinones at the Q_p and Q_n sites can diffuse into the plastoquinone pool or exchange via a 'quinone-exchange cavity' or groove at the periphery of the dimer interface as illustrated by the two-headed, *dashed arrow*. A chlorophyll *a* (Chl *a*) molecule is sandwiched between Cyt b_6 helix C and subunit IV helices F and G of each monomer with its phytyl tail extending into the quinol-oxidation (Q_p) pocket. One β-carotene (β car) per monomer appears as a skewer located between the PetG and PetM helices: – of each monomer.

distinction that may explain most of the observed differences in quinone-reductase, Q_n-site functions of Cyt b_6f and bc_1 complexes.

The four small subunits, PetG, PetL, PetM, and PetN, that form a membrane-spanning, 'picket fence' on the periphery of the Cyt b_6f structures (Kurisu et al., 2003; Stroebel et al., 2003), also have no counterpart in Cyt bc_1 complexes. These, the c_n heme, a chlorophyll, and a β-carotene molecule are among the unique features that distinguish Cyt b_6f from Cyt bc_1 complexes (Berry et al., 2000; Baniulis et al., 2008). Finally, the chloroplast and cyanobacterial b_6f complexes serve important roles in redox sensing of PQ pool oxidation-reduction potential and in signal transduction that mediates the distribution of harvested light energy between the two photosystems, the so-called, 'state transitions,' (Wollman, 2001; Eberhard et al., 2008), as well as adaptive gene expression events (Pfannschmidt et al., 1999a, 2008). Comparable redox sensing-signaling functions have not been identified in Cyt bc_1 complexes.

IV. Relation to Menaquinol-Oxidizing and Other 'Rieske-Cytochrome *b*' Complexes

In addition to the cyanobacterial-chloroplast Cyt b_6f and bacterial-mitochondrial Cyt bc_1 quinol-cytochrome *c* oxidoreductases, diverse forms of bc_1-b_6f complexes are prevalent in numerous eubacterial and archaeabacterial lineages (see Schütz et al., 2000; Darrouzet et al., 2004; Kramer et al., 2008 for reviews). 'Rieske-Cyt *b*' has been coined as a collective designation for these Cyt bc_1-b_6f-type complexes, all of which use a Rieske ISP as the initial electron acceptor and transfer

electrons across a membrane through *b* hemes (Schütz et al., 2000; Kramer et al., 2008). Plastoquinol and ubiquinol serve as the typical electron donors for Cyt b_6f and bc_1 complexes, respectively. However, menaquinone is the most widely distributed quinone among diverse phylogenetic groups and it or naphthoquinone occur in all of the early, deeply branching archaeal and eubacterial lineages (Schütz et al., 2000, 2003). Menaquinol (i.e., the menaquinol/menaquinone, MQH_2/MQ couple) has a midpoint potential of about −60 mV, or ~150 mV lower than that of plastoquinol, ubiquinol, or the caldariellaquinone of archaea (Schütz et al., 2000). Schoepp-Cothenet et al. (2009) argue compellingly that menaquinone was the ancestral quinone of anaerobic Earth environments and that the higher potential quinones evolved in response to the advent of molecular oxygen in the atmosphere some 2.5 billion years ago. The midpoint potentials of Cyt bc_1-b_6f complexes, and in particular those of the crucial Rieske 2Fe-2S center, the initial electron acceptor for quinol oxidation, have adjusted to the midpoint potential and thus driving force of their quinol donors. Rieske centers of Cyt bc_1-b_6f complexes have midpoint potentials ($E_{m,7}$) in the range of +260 to +320 mV (see Kallas, 1994; Holton et al., 1996; Carrell et al., 1997) whereas those of menaquinol-oxidizing complexes have E_m values as low as +110 mV (Schoepp-Cothenet et al., 2009). The apparent disadvantage of menaquinol as an electron donor for Cyt bc_1-b_6f complexes is the hazard of oxygen exposure: (1) MQ becomes rapidly oxidized in the presence of oxygen, and (2) the large thermodynamic driving force of a MQ donor at +110 mV relative to a high-potential Rieske ISP acceptor at ~+300 mV greatly increases the probability of oxygen radical formation in bc_1-b_6f complexes (see Muller et al., 2002; Sun and Trumpower, 2003; Forquer et al., 2006; Cape et al., 2007 and Section VIII.B.4 for further discussion).

Organisms have been identified that may represent species in evolutionary transition from MQ to UQ or PQ-oxidizing Cyt bc_1-b_6f complexes. Indeed, some have more than one type of quinone donor. The gamma proteobacterium *Halorhodospira halophila* uses MQ as its pool quinone rather than the typical UQ of this group but also possesses UQ (Schoepp-Cothenet et al., 2009). The common gamma-proteobacterium *Escherichia coli* is another example. *E. coli* possesses both MQ and UQ although its electron transfer chains lack any 'Rieske-Cyt *b*' complex (Schütz et al., 2000). These seem to reflect examples of evolutionary 'bricolage' resulting in modified protein structures and redox centers with altered midpoint potentials for adaptation to different electron transport chains and evolving environmental requirements.

V. Genes and Evolutionary Relationships

Genes for core subunits of Cyt b_6f complexes in most cyanobacteria are arranged in two operons, *petB-petD* and *petC1-petA*, encoding the Cyt b_6–subunit IV and PetC1 Rieske ISP–Cyt *f* polypeptides, respectively (Kallas et al., 1988a, b; see also CyanoBIKE, http://cyanobike-community.csbc.vcu.edu/). The *petBD* operon structure is conserved in all forty-some currently available cyanobacterial genome sequences. Flanking genes and their arrangements differ considerably. Similarly the *petC1-A* gene cluster is highly conserved in cyanobacteria with the possible exception of *Synechococcus* sp. BL107 and *Crocosphaera watsonii* WH8501. These apparent exceptions may represent interpretations based on incomplete sequence data. The genes surrounding the *petC1-A* operon also diverge greatly. The *petG, petL, petM,* and *petN* genes for the small subunits of the Cyt b_6f complex are largely scattered across cyanobacterial genomes and appear to be monocistronic. In many strains, *petG* is adjacent to the *cytM* gene for the cryptic cytochrome c_M but transcribed divergently. The significance of this, if any, is not clear. Many, but not all cyanobacteria carry genes for additional forms of PetC Rieske ISPs (Schneider et al., 2002, 2004). The *petC2* and *petC3* genes as well appear to be monocistronic or at least not part of any consistent operon structure. In several cyanobacteria, including *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, a small open reading frame (G0078 in *Synechococcus* sp. PCC 7002) encoding a hypothetical protein precedes *petC2* and may be coordinately regulated with it (G. Weir and T. Kallas, unpublished). In *Synechococcus* sp. PCC 7002, *petC2* resides on a plasmid and, as in several other cyanobacteria, in proximity to the *sqr* gene for sulfide-quinone

oxidoreductase. The significance of this is not clear but might imply the remnants of a possible 'fitness island' (Ochman et al., 2000), perhaps for sulfide-dependent electron transport.

In eukaryotic algae and plants, the *petB, D*, and *A* genes for core Cyt b_6, subunit IV, and Cyt *f* subunits of Cyt b_6f complexes reside in chloroplast genomes although their arrangements may differ considerably. The *petC* gene for the major Rieske ISP resides in the nucleus (see Kallas, 1994; de Vitry et al., 1999). Locations of genes for the small subunits (*petG, L, M, and N*) vary. For example, in the alga *C. reinhardtii*, *petN* and *petM* are in the nucleus and *petG* and *petL* in the plastid genome (Zito et al., 2002; Smith and Lee, 2009). In contrast, in *Arabidopsis thaliana* (Maiwald et al., 2003), and typically in green plants (www.ncbi.nlm.nih.gov/genomes), only *petM* is in the nucleus and genes for the other small subunits are in the chloroplast. With the exception of separate nuclear genes for chloroplast and mitochondrial Rieske proteins (Atteia et al., 2003), plants and algae do not have known genes for additional, alternative Rieske ISP proteins. However, two modestly different cDNAs for chloroplast Rieske ISPs have been reported in tobacco (Madueno et al., 1992) and two forms with different isoelectric points identified in spinach chloroplasts (Yu et al., 1994).

In a larger context, the cyanobacterial-chloroplast Cyt b_6f complexes are most closely related to those of the firmicutes, which are low GC% content gram positive bacteria that include the heliobacteria (Schütz et al., 2000; Lebrun et al., 2006; Kramer et al., 2008). The Rieske-Cyt *b* complexes of this so-called 'green clade' (Nitschke et al., 2010) have split Cyt b_6 and subunit IV polypeptides and a c_n heme bound to a Cyt b_6 polypeptide. Also included in this clade, based on protein sequence similarities but ancestral to the others, are the green sulfur bacteria (Chlorobiaciae). These do not have a split Cyt *b* protein nor heme c_n, but do possess a Cyt *b* with seven transmembrane helices as in Cyt b_6-subunit IV proteins. Considerable and interesting divergence of 'Rieske-Cyt *b*' structures and gene arrangements occurs within the 'green clade.' The green sulfur bacteria (e.g., *Chlorobium limicola*) have clustered Rieske ISP–Cyt *b* genes but no gene for any Cyt c_1 or *f* protein (Schütz et al., 1994). The electron acceptor for this Rieske-Cyt *b* complex is unknown but may be the reaction center or an oxidase complex as in the supercomplex found in *Bacillus* PS3 (Tanaka et al., 1996). Firmicutes, such as *Bacillus subtilis* (Yu et al., 1995), have an operon encoding Rieske ISP, Cyt b_6, and a fused subunit IV – mono-heme Cyt *c* protein. Heliobacteria (e.g., *Heliobacillus mobilis*) have a similar operon structure except that the *c*-cytochrome component is a di-heme cytochrome instead of the mono-heme Cyt *c* fused to subunit IV (see Baymann and Nitschke, 2010).

Genes for the Cyt bc_1-type, 'Rieske-Cyt *b*' complexes of purple photosynthetic bacteria and other proteobacteria are typically in a single operon (designated *fbcFBC* or *petABC*) encoding, respectively, the core Rieske ISP, Cyt *b*, and Cyt c_1 subunits (Gabellini and Sebald, 1986; Davidson and Daldal, 1987). However, gene arrangements and compositions differ as well in these groups. For example, the alpha proteobacterium, *Bradyrhizobium japonicum*, has a single fused gene for Cyt *b* and Cyt c_1 whose product is post-translationally processed to yield separate polypepdtides (Thony-Meyer et al., 1991). *Helicobacter pylori*, a member of the epsilon proteobacteria, has the Rieske-Cyt *b* gene cluster followed by a gene for a di-heme, type-4 cytochrome (Schütz et al., 2000; Kramer et al., 2008). Intriguingly, the deeply branching eubacterial thermophile, *Aquifex aeolicus*, which is not a proteobacterium based on ribosomal RNA phylogeny, has Rieske-Cyt *b* genes much like those of *H. pylori* but the cytochrome gene encodes a monoheme, type-1, *c*-type cytochrome (Schütz et al., 2003). *Aquifex aeolicus* has apparently acquired a fairly typical Cyt bc_1 complex via lateral gene transfer from epsilon proteobacteria (Schütz et al., 2000) but with downshifted midpoint potentials to accommodate electron transfer from naphthoquinone (Schütz et al., 2003). The mono-heme *c*-cytochrome of *A. aeolicus* shares homology with regions of the epsilon proteobacterial di-heme cytochrome and is proposed to have arisen from it (Baymann et al., 2004). Known members of the delta proteobacteria (e.g., species belonging to the genus *Desulfovibrio*) have the typical Rieske-Cyt *b*-Cyt *c* gene cluster but the *c* cytochrome is a type-3, tetra-heme cytochrome typical of this group (Louro et al., 2001).

A theme that emerges is that the core Rieske-Cyt *b* genes and by implication the encoded proteins are almost ubiquitous and relatively well conserved among the eubacterial and archaeal lineages. In contrast, the genes/proteins for the 'third' subunit, the electron acceptor, diverge wildly. A variety of mono-, di-, and tetra-heme *c*-type cytochromes, as well as *b*-type cytochromes and 'high-potential' ISPs (HiPIP), both soluble and anchored to the membrane, may serve this function. For example, *Thermus/Deinococcus* bacteria have a gene for a mono-heme cytochrome *c* and actinobacteria (high GC gram positive bacteria) a gene for a di-heme cytochrome *c*, in both cases located upstream of Rieske-Cyt *b* genes (Schütz et al., 2000; Kramer et al., 2008). Instead of any *c*-type cytochrome, the crenarchaeon *Sulfolobus acidocaldarius* apparently uses a *b*-cytochrome (Cyt $b_{558/566}$) as the electron acceptor for a Rieske-Cyt *b* complex (Hiller et al., 2003). A HiPIP is implicated as the electron carrier between the Cyt bc_1 complex and photosynthetic reaction center in species such as the beta proteobacterium *Rubrivivax gelatinosus* (Schoepp et al., 1995) and the gamma proteobacterium, *Halorhodospira halophila* (Lieutaud et al., 2005). In contrast to the diversity of electron acceptors proteins of these other 'Rieske-Cyt *b*' complexes, cytochrome *f* is the only known acceptor for Rieske proteins of Cyt b_6f complexes.

Interestingly, some bacteria have a complete set of genes for more than one Rieske-Cyt *b* complex. The gamma proteobacterium, *Acidothiobacillus ferroxidans* (Brasseur et al., 2002; Bruscella et al., 2007) and possibly the archaean *Sulfolobus acidocaldarius* (Hiller et al., 2003) are two examples. Genes for alternative or additional Rieske ISPs were discovered in cyanobacteria from the genome sequence of *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996) and it has become evident that multiple genes for Rieske ISPs exist in many eubacteria and archaea as well. *Rubrivivax gelatinosus* carries genes (*petA1* and *petA2*) for two functionally interchangeable, Cyt bc_1 Rieske ISPs as well as a *petA3* pseudogene (Ouchane et al., 2005). *Aquifex aeolicus* (Schütz et al., 2003), *Deinococcus,* and the archaea *Aeopyrus pernix* and *Sulfolobus* sp. strain 7 all have at least one additional Rieske ISP gene (see Schütz et al., 2000; Schneider and Schmidt, 2005). One of the ISPs of *Sulfolobus* sp. strain 7 is an unusual, soluble Rieske ISP termed 'sulredoxin' (Iwasaki et al., 1996). Sequence comparisons suggest that many of these ISP genes encode ISPs of Rieske-Cyt *b* complexes rather than Rieske proteins of bacterial dioxygenases (Schmidt and Shaw, 2001) or arsenite oxygenases (Lebrun et al., 2003). The activity and possible functions of these additional Rieske-Cyt *b* genes/proteins is generally not known but they likely operate in specific electron transfer chains or under different environmental conditions. From differential gene expression studies, Bruscella et al. (2007) propose that the *petI* operon of *A. ferroxidans* encodes a Cyt bc_1 complex that functions in the energetically uphill electron flow from reduced iron to NAD(P) whereas the complex encoded by the *petII* operon transfers electrons from sulfur to oxygen.

VI. Overall Structure and Organization

A. X-ray Crystal Structures

Figures 21.3 and 21.4 show the structure of the Cyt b_6f dimer derived from X-ray diffraction analysis of protein crystals from the cyanobacterium *M. laminosus* (Kurisu et al., 2003; Yan et al., 2006; Yamashita et al., 2007). The *C. reinhardtii* Cyt b_6f structure is similar (Stroebel et al., 2003). Each monomer contains 13 transmembrane helices for a total of 26 helices in the ~220 kDa dimer complex. Eight transmembrane helices belong to the core 'Rieske-Cyt *b*' components. The split Cyt b_6 and subunit IV proteins contain four and three transmembrane helices, respectively, and the Rieske ISP has another. These subunits together with Cyt *f* comprise the catalytic core of the Cyt b_6f complex. Note again that the transmembrane helix of each Rieske ISP 'crosses-over' and associates with the other monomer. In Figs. 21.3 and 21.4, the Rieske ISPs of the Cyt b_6f dimer are colored magenta, and the Cyt b_6, subunit IV, and Cyt *f* polypeptides are olive green, light grey-green, and turquoise, respectively. The four additional, membrane-spanning small subunits, PetG, PetL, PetM, PetN, each have one transmembrane helix and together form a 'picket fence' (Cramer et al., 2005) at the periphery of the Cyt b_6f dimer. Table 21.1 summarizes the subunit and prosthetic group composition of typical cyanobacterial-chloroplast

Table 21.1. Subunits, prosthetic groups, and redox centers of cytochrome b_6f complexes

Subunit	Encoding gene	Prosthetic group or binding site [a]	Mass (Da) [b]	*p*I	$E_{m,7}$ (mV) [c]
Rieske ISP	*petC1*	2Fe-2S cluster	19,295	6.8	+300 to +320
Cyt b_6	*petB*	Heme b_p (b_L)	24,710	9.0	–120 to −150
		Heme b_n (b_H)			–30 to −50
		Heme c_n (c_i)			< −50 to +100
Subunit IV	*petD*	–	17,528	8.1	–
		Q_p site			–
		Q_n site			–
		Chlorophyll *a*			–
		β carotene			–
Cyt *f*	*petA*	Heme *f*	32,270	6.7	+270 to +380
PetG	*petG*	–	4,057	4.5	–
PetL	*petL*	–	3,530	10.2	–
PetM	*petM*	–	3,841	4.3	–
PetN	*petN*	–	3,304	5.7	–

[a]The plastoquinone binding sites Q_p and Q_n on the positive and negative sides of the thylakoid membrane, respectively, are formed largely by the Cyt b_6 and subunit IV proteins as described in the text. Chlorophyll *a* is also associated with Cyt b_6 and subunit IV. The β carotene is associated primarily with PetG and PetM

[b]Masses measured by mass spectrometry (LC-MS) are for Cyt b_6f subunits from *Mastigocladus laminosus* (Whitelegge et al., 2002) and include the masses of covalently bound prosthetic groups

[c]Redox midpoint potentials are those reported for the *Mastigocladus laminosus* (Baniulis et al., 2008; Cramer et al., 2011), *Chlamydomonas reinhardtii* (Ponamarev and Cramer, 1998; Alric et al., 2005), and *Synechococcus sp.* PCC 7002 (Baymann et al., 2001; Nelson et al., 2005) Cyt b_6f complexes. Heme c_n midpoint potentials are from (Lavergne, 1983; Zhang et al., 2004b; Alric et al., 2005). Rieske ISPs have a pH-dependence of −30 to −80 mV per pH unit in the pH 6.5 to 9.0 range (Soriano et al., 2002 and references therein)

Cyt b_6f complexes as well as the midpoint potentials of the redox centers. The core Rieske ISP and Cyt b_6 polypeptides contain most of the redox centers (Figs. 21.3. and 21.4). The ISP carries the high-potential 2Fe-2S cluster ($E_{m,7}$ ~+300 to +320 mV in typical Cyt b_6f complexes) of the high-potential chain. The Cyt b_6 polypeptide carries the two non-covalently bound *b* hemes ($E_{m,7}$ −130 to −150 mV and −35 to −50 mV for hemes b_p and b_n, respectively) as well as the unique heme c_n ($E_{m,7}$ +100 mV in *C. reinhardtii*) that comprise the low-potential electron transfer chain (see Table 21.1 and references therein). The Cyt *f* polypeptide carries the final redox center, the covalently bound *c*-type heme (Cyt *f*, $E_{m,7}$ +355 mV in *M. laminosus*) that together with the Rieske ISP comprises the high-potential electron transfer chain of the Cyt b_6f complex.

In addition to the redox-active prosthetic groups that function in electron transfer, the Cyt b_6f complex has two binding sites for quinones, the Q_p (plastoquinol oxidation) and Q_n (plastoquinol reduction) sites formed primarily within the Cyt b_6 and subunit IV polypeptides, and the chlorophyll *a* and β-carotene molecules that are not involved in electron transfer. The general locations of the Q_p and Q_n quinone pockets are defined by the tridecyl-stigmatellin (TDS) and NQNO binding sites (Figs. 21.3 and 21.4). These inhibitors, which are quinone analogs, bind within the Q_p and Q_n pockets, respectively. A chlorophyll *a* molecule is nestled in each monomer between helices B and D of Cyt b_6 and F and G of subunit IV and its phytyl tail protrudes into the Q_p site of each Cyt b_6f monomer. A β-carotene lies perpendicular to the subunit IV transmembrane helix E and those of PetG, PetM, and PetN. The edge-to-edge distance between the chlorophyll *a* and β-carotene molecule in each monomer is ~14 Å and the chlorophyll *a* chlorin ring is only ~5.5 Å from heme b_n near the Q_n site. Hence a possible role for the chlorophyll *a* molecule in redox signaling and/or communication between the Q_p and Q_n sites has been suggested (Cramer et al., 2005;

Stroebel et al., 2003). A structural or assembly role of the hydrophobic β-carotenes, which skewer the hydrophobic, transmembrane small subunit bundle of each monomer, has been suggested in a 'toothpick and hors d'ouevres' model (Cramer et al., 2005). Overall, there are two 2Fe-2S clusters, four *b*-hemes, two unique c_n hemes, two typical *c*-hemes (Cyt *f*), four quinone binding sites, two chlorophyll *a* molecules, and two carotenoid molecules per Cyt b_6f dimer. Note that the cyanobacterium *Synechocystis* sp. PCC 6803 contains echinenone rather than β-carotene in its Cyt b_6f structure (Boronowsky et al., 2001).

Prior to the complete Cyt b_6f structures, high-resolution X-ray crystal structures were solved at ~1.9 Å for soluble domains of Cyt *f* from turnip (Martinez et al., 1994, 1996) and *C. reinhardtii* (Chi et al., 2000; Sainz et al., 2000) chloroplasts and from the cyanobacterium *Phormidium laminosum* (Carrell et al., 1999) and at 1.83 Å for a iron-sulfur cluster bearing fragment of the spinach chloroplast Rieske ISP (Carrell et al., 1997). These high-resolution structures revealed several unique features including the axial ligation of heme *f* by the N-terminal α-amino group of the mature Cyt *f* protein (Martinez et al., 1994).

Currently, structures have been solved for complete Cyt b_6f complexes from *M. laminosus* (Kurisu et al., 2003) and *C. reinhardtii* (Stroebel et al., 2003) as mentioned above and for the *M. laminosus* complex crystallized in the presence of the Q_p-site inhibitors DBMIB (Kurisu et al., 2003; Yan et al., 2006) and TDS (Kurisu et al., 2003; Stroebel et al., 2003; Yan and Cramer, 2004; Yamashita et al., 2007) and the Q_n-site inhibitor NQNO (Yamashita et al., 2007). Recently, the structure of an active, dimeric Cyt b_6f complex has been solved at 3 Å from the cyanobacterium *Nostoc* sp. PCC 7120 (Baniulis et al., 2009), which is amenable to genetic manipulation. For reasons that are not clear, the isolation of active, dimeric Cyt b_6f complexes has not been possible from cyanobacteria such as *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7002 that have been well developed for genetic analysis. The *Nostoc* sp. PCC 7120 result now opens the way for targeted mutagenesis and functional as well as structural analysis of the Cyt b_6f complex in a genetically amenable cyanobacterium. Directed mutagenesis and structure analysis of the *C. reinhardtii* chloroplast-encoded Cyt b_6, subunit IV, and Cyt *f* proteins (e.g., Kuras and Wollman, 1994; Zhou et al., 1996; Kuras et al., 1997; Zito et al., 1998) and the nucleus encoded Rieske ISP, via complementation of an ISP deficient mutant (de Vitry et al., 1999, 2004), is also possible and has provided many insights.

Thus far, the structures of complete Cyt b_6f complexes have been limited to a resolution of about 3 Å. While these structures have provided enormous insight into the structure-function of Cyt b_6f complexes, their resolution does not allow accurate determination of amino acid side chains. Thus lowering of the '3 Å barrier' will be important for further progress as discussed in Baniulis et al. (2008). Proteolysis and delipidation are major problems for isolation of active Cyt b_6f complexes and high-resolution crystal formation. Successful crystallization of the *C. reinhardtii* b_6f complex was accomplished by His-tagging of the Cyt *f* C-terminus (Choquet et al., 2003) and rapid isolation of the complex (Stroebel et al., 2003). Depletion of lipids by extensive purification of the Cyt b_6f complex was solved by re-addition of lipids during purification of the *M. laminosus* complex (Zhang et al., 2003). Interesting discussions of the proteolysis problem and possible solutions are provided by Baniulis and co-workers (Baniulis et al., 2008, 2009). Comparative analysis of cyanobacterial genome sequences and predicted protease genes (via the MEROPS peptidase data base) by these authors revealed hundreds of possible proteases but only four that are shared among cyanobacteria from which native, dimeric Cyt b_6f complexes could not be isolated. Inactivation of these proteases may be the key to obtaining active Cyt b_6f complexes and high-resolution structures from cyanobacteria that are widely used for genetic, biochemical, and biophysical studies. This concept has been demonstrated in *C. reinhardtii* where a mutant deficient in the ClpP protease (Majeran et al., 2000) allowed the assembly and characterization of several genetically modified Cyt b_6f complexes (de Vitry et al., 1999).

B. The Functional Dimer and Quinone-Exchange Cavities

As mentioned, the transmembrane helix of each Rieske ISP 'crosses-over' and associates with the other monomer of the Cyt b_6f complex. This

observation resolved earlier controversies as to whether monomers or dimers of Cyt b_6f complexes are catalytically active (reviewed by Kallas, 1994) and clearly demonstrates that the functional complex is the dimer. In other words, electron flow from plastoquinol (PQH_2) bound to the Q_p site of one monomer depends on its oxidation by a 2Fe-2S cluster that belongs to the Rieske ISP anchored to the other monomer. This is evident from inspection of the dimer structure shown in Fig. 21.3. The Rieske ISP 'cross-over' and peripheral location of the Rieske transmembrane helices further explains how the ISP subunit may be easily lost during purification resulting in inactive Cyt b_6f monomers. Loss of the Rieske ISP has been a major problem for isolation and characterization of active Cyt b_6f complexes from a variety of sources including cyanobacteria such as *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 that are widely used for genetic and biochemical investigations (e.g., Bald et al., 1992; Baniulis et al., 2008, 2009).

A second reason for the dimer requirement for catalysis is the so called 'quinone exchange cavity' (Kurisu et al., 2003), two of which are formed at the periphery of the monomer interfaces in the dimer complex. The cavity on one side of the Cyt b_6f dimer is illustrated by the yellow dashed arrow in Fig. 21.4 and is formed by the Cyt b_6f – subunit IV C, D, and F transmembrane helices of one monomer and the A, E, and Rieske ISP transmembrane helices of the other monomer. The *n*-side 'roof' of the cavity is formed by N-terminal residues of Cyt b_6 and its *p*-side 'floor' is enclosed by the membrane-parallel, amphipathic cd helices of Cyt b_6. Note that the orientation of the Cyt b_6f complex shown here (stromal-cytoplasmic, *n*-side at the top and lumenal, *p*-side at the bottom) is the opposite (*p*-side at the top) of that typically shown by the Cramer group (Kurisu et al., 2003; Yamashita et al., 2007). Catalytic activity requires the exchange of oxidized plastoquinone (PQ) from the Q_p-site and reduced plastoquinone (plastoquinol, PQH_2) from the Q_n-site with the PQ pool and the opposite Q-binding sites of the b_6f complex. The shortest distance between these PQ-binding sites is between the Q_p-site of one monomer and the Q_n-site of the other monomer. Structures of Cyt bc_1 complexes reveal a similar dimer organization and quinone exchange cavities where quinone reduction can occur at the Q_n-site of one monomer and its oxidation at the Q_p-site of the other monomer without the quinone molecule leaving the quinone exchange cavity (Xia et al., 1997).

C. Role of Lipids in the Cytochrome b_6f Structure

Endogenous lipids co-purify with Cyt b_6f complexes, are present in the crystal structures, and are necessary for integrity and function of the complex in vitro and in vivo. The sulpholipid (SQDG, sulphoquinovosyldiacylglycerol) resides near the *n*-side of the *C. reinhardtii* Cyt b_6f complex and its sulphonate head group interacts via hydrogen bonding with residues in the transmembrane helices of Cyt *f* and the Rieske ISP (Stroebel et al., 2003). Modifications of either Cyt *f* Lys272 (Choquet et al., 2003) or Rieske ISP Asn17 (de Vitry et al., 2004) in these transmembrane helices disrupts the interaction with SQDG and the assembly-mediated regulation of Cyt *f* biogenesis (Choquet et al., 1998) resulting in a decreased accumulation of some of the Cyt b_6f subunits. The lumenal side of the *C. reinhardtii* Cyt b_6f complex incorporates two other lipids, probably MGDG (monogalactosyldiacylglycerol), whose acyl chains are buried in the protein and have contacts with the chlorophyll *a* and β-carotene molecules (Stroebel et al., 2003).

As mentioned, the addition of an exogenous, synthetic lipid (DOPC, dioleoylphosphatidylcholine) was necessary to obtain stable crystals of the *M. laminosus* Cyt b_6f complex. Two molecules of this lipid are bound to each quinone-exchange cavity in the *M. laminosus* crystals and appear to act as struts that stabilize the cavity (Kurisu et al., 2003). It is assumed that native lipids, and perhaps specific lipids, fill these cavities in vivo. The importance of specific lipids in membrane protein structure-function has become increasingly apparent (see Hunte and Richers, 2008, for a review). Specific phospholipids (phosphatidylcholine and phosphatidylglycerol) are necessary for restoration of activity to plastoquinone-depleted Cyt b_6f complexes (Willms et al., 1988) and to stimulate binding of the inhibitor DBMIB to the Q_p site (Malkin et al., 1988). Several tightly bound phospholipids are present in X-ray structures of Cyt bc_1 complexes and important for structural integrity as shown by directed mutagenesis of their

binding sites (Lange et al., 2001). The phospholipid cardiolipin was recently shown to be essential for catalytic activity of the Cyt bc_1 complex from yeast and supercomplex formation with cytochrome *c* oxidase (Wenz et al., 2009). Similarly important roles of specific lipids may be anticipated for function and protein-protein interactions of Cyt b_6f complexes.

D. Supercomplexes?

It has become apparent that many membrane protein complexes function not as isolated islands in strictly fluid environment but rather as components of dynamic 'supercomplexes' whose composition varies according to cellular physiology demands. Supercomplexes in mitochondrial respiratory membranes have been studied extensively (see Dudkina et al., 2008; Stuart, 2008 for recent reviews). Single particle electron microscopy (Dudkina et al., 2010) together with blue native polyacrylamide gel electrophoresis (Schagger and Pfeiffer, 2000), which allows the separation of intact protein complexes, and mass spectrometry (Zhang et al., 2001; Whitelegge et al., 2002; Ryan et al., 2010) have been particularly useful in these studies. Mitochondrial Cyt bc_1 complexes (complex III) may form supercomplexes with NADH dehydrogenase (complex I) or cytochrome *c* oxidase (complex IV) alone or a 'megacomplex' with both. The role of cardiolipin was mentioned above in formation of a Cyt bc_1–cytochrome *c* oxidase supercomplex (Wenz et al., 2009).

Chloroplast thylakoid membranes are heterogeneous consisting of stacked (or appressed, granal) regions to which PS II and light-harvesting II (LHCII) complexes are mostly confined and unstacked (or unappressed, stromal) regions to which PS I, LHCI, and ATP synthase complexes are predominantly localized (Albertsson, 2001). Cyt b_6f complexes occur in both regions and the functional unit is a dimer as discussed above. PS II complexes are dimers associated with a variable number of LHCII complexes. At high light intensity some of the LHCII dissociate to decrease light absorption and allow access to the PS II core by proteins needed for energy dissipation by non-photochemical quenching (NPQ) (Betterle et al., 2009). 'Plastoquinone (PQ) diffusion domains' have been proposed in which a few PS II complexes and a Cyt b_6f complex associate within granal membranes allowing rapid diffusion of PQ between these complexes (Lavergne and Joliot, 1991), but direct experimental evidence for this is lacking. The PS I complex is a monomer in chloroplasts but exists in both trimer and monomer forms in cyanobacteria (Fromme and Grotjohann, 2008). The trimer predominates at low light intensities whereas monomerization occurs at high light intensities. In chloroplasts, a variable number of light-harvesting antenna complexes (LHCI and LHCII) may associate with PS I, depending on the species and environmental and cellular status. In 'state 2' conditions (PQ pool largely reduced, see Section IX.A below), some LHCII complexes become phosphorylated and associate with PS I forming transient PS I–LHCI–LHCII complexes (see Wollman, 2001; Eberhard et al., 2008; Lemeille and Rochaix, 2010 for reviews of state transitions). In typical cyanobacteria, membrane extrinsic phycobilisome complexes serve as the major light-harvesting antenna and various combinations of phycobiliproteins, depending on species, environmental conditions, and redox states of the PQ pool, can associate dynamically with PS I as well as PS II complexes (see Mullineaux, 2008; Dong et al., 2009; Kondo et al., 2009 and references therein). Cyanobacteria further have a unique IsiA, chlorophyll-containing light-harvesting complex that forms one or more rings of IsiA monomers around the PS I trimer during iron-starvation and other stresses (Bibby et al., 2001; Boekema et al., 2001; Singh and Sherman, 2007).

Vallon and co-workers (1991) showed some time ago that in addition to LHCII complexes, some of the Cyt b_6f complexes also migrate to the non-appressed, stromal membranes during 'state 2' conditions. This results in elevated cyclic electron flow around PS I (Finazzi et al., 2002) and formation of Cyt b_6f–PS I supercomplexes has been proposed to account for this. Cyt b_6f–PS I supercomplexes were also predicted from mathematical modeling of electron transfer processes (Laisk, 1993). P. Joliot and A. Joliot (2002, 2005) provided compelling kinetic evidence for highly active, transient PS I cyclic electron transfer following illumination of dark-adapted spinach leaves. These authors proposed that the active cyclic flow arises from formation of supercomplexes containing Cyt b_6f, PS I, plastocyanin, and

Fd; that these complexes form in response to low ATP levels triggering the lateral migration of Cyt b_6f complexes; and that the cyclic flow rapidly provides the ATP required to initiate CO_2 fixation by the Calvin-Benson cycle. The Cyt b_6f structures further suggested that Fd or FNR may interact with the largely exposed Cyt b_6f Q_n-pocket on the *n*-side of the membrane in supercomplex formation and direct electron transfer from PS I to the Cyt b_6f complex (Kurisu et al., 2003; Stroebel et al., 2003). Consistent with this, FNR has been found in association with Cyt b_6f complexes isolated from chloroplasts (Clark et al., 1984; Zhang et al., 2001).

Two other chloroplast proteins, (PGR5) proton gradient regulation (Munekage et al., 2002, 2004) and PGRL1 (DalCorso et al., 2008), play important roles in Cyt b_6f–PS I supercomplexes in plants. PGR5 was identified by fluorescence screening of *A. thaliana* for mutants defective in NPQ and thus in cyclic electron flow required to generate the high ΔpH that induces thermal energy dissipation by PS II (see Holt et al., 2004; Li et al., 2009 for reviews of NPQ). The thylakoid PGRL1 protein, encoded by homologous *PGRL1A* and *PGRL1B* genes, was identified from a set of nuclear genes as having a '*PGRL5*-like photosynthetic phenotype' (DalCorso et al., 2008). PGR5 was initially shown to be essential for cyclic electron flow (Munekage et al., 2004). However, further analysis revealed that PGR5 is not absolutely needed for cyclic electron transport but rather has a regulatory role required to elicit elevated cyclic flow under high-light stress conditions (Nandha et al., 2007). DalCorso et al. (2008) subsequently showed that the transmembrane PGRL1 protein, like PGR5, is required for efficient cyclic electron transfer and growth under the conditions tested. These authors further demonstrated in two-hybrid assays that PGRL1 interacts with the stromal PGR5 protein and with Fd, the PS I PsaD subunit, the Cyt b_6f complex, and with two forms of FNR. Based on these findings, a variety of scenarios were proposed in which PGRL1-PGR5 might either regulate the formation of or serve as essential structural components of Cyt b_6f–PS I supercomplexes. Recently, Iwai et al. (2010) have isolated from *C. reinhardtii* the elusive Cyt b_6f–PS I supercomplex responsible for active cyclic electron transfer in chloroplasts. These authors used the uncoupler FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) to deplete ATP levels and thereby induce a 'state 2' (largely reduced PQ pool) condition (see Bulte et al., 1990) and cyclic electron flow. Membrane protein complexes extracted with n-tridecyl-β-D-maltoside detergent from such cells and separated by sucrose density gradient centrifugation included a supercomplex consisting of PS I–LHCI antenna–LHCII antenna–Fd–FNR together with the Cyt b_6f complex and PGRL1. This complex catalyzed light-driven Cyt *f* oxidation and Fd-dependent *b*-heme reduction thus providing direct evidence for electron transfer from PS I to the Cyt b_6f complex. Interestingly, this Cyt b_6f–PS I supercomplex did not contain PGR5 but did contain the chloroplast Cyt b_6f associated, PetO subunit that undergoes phosphorylation in 'state 2' conditions and has a postulated role in redox signal transduction and state transitions (Hamel et al., 2000).

Cyanobacteria do not possess apparent homologs of the PGRL1 or PetO proteins. However, several unicellular cyanobacteria carry a gene (e.g., *Synechocystis* sp. PCC 6803_ssr2016, *Synechococcus* sp. PCC 7002_A1477) for a hypothetical protein that shares considerable sequence identity with the N-terminus of PGR5. Inactivation of Ssr2016 in *Synechocystis* sp. PCC 6803 resulted in slower growth at high light intensity and a small increase in sensitivity to antimycin A (AA) in an Ssr2016–NdhB double mutant (Yeremenko et al., 2005). The authors suggested that Ssr2016, as originally suggested for PGR5 in plants (Munekage et al., 2002), is involved as a regulator or component of a cyclic pathway that transfer electrons from PS I into the plastoquinone pool via an antimycin-A-sensitive Fd quinone reductase (FQR). The FQR enzyme proposal is interesting, and has been postulated for some time (Moss and Bendall, 1984), but remains hypothetical because no FQR enzyme has yet been isolated from any source. Cyanobacteria do not have stacked and unstacked thylakoid membrane regions, but they do undergo 'state transitions' and modulate cyclic versus linear electron flow in response to the redox status of the PQ pool. Dynamic Cyt b_6f–PS I associations are very likely important in cyanobacterial cyclic electron flow but because of the trimer organization of PS I and the absence of PGRL1 and PetO proteins, these supercomplexes will necessarily be different from those of chloroplasts. Because cyanobacterial PS I trimers dissociate to monomers

under high light or stress conditions (Fromme and Grotjohann, 2008) that result in elevated reduction of the PQ pool and increased cyclic flow, it is tempting to speculate that supercomplexes comprised of Cyt b_6f dimers – PS I monomers – and Fd or Fd with FNR, might form under these conditions.

VII. Subunits, Redox Centers and Prosthetic Groups

A. Major Subunits and Redox Centers

1. Cytochrome b_6, Subunit IV and the b Hemes

As mentioned, the Cyt b_6 and subunit IV proteins of chloroplast-cyanobacterial Cyt b_6f complexes are equivalent to the longer Cyt *b* protein of Cyt *bc* complexes (Widger et al., 1984). These and a Rieske ISP form the catalytic core of 'Rieske-Cyt *b*' complexes. The seven transmembrane helices of Cyt b_6 and subunit IV (labeled A–G in Figs. 21.3 and 21.4) correspond to the first seven helices of Cyt *b* in Cyt *bc* complexes and have very similar orientations in the membrane (Breyton, 2000; Kurisu et al., 2003; Stroebel et al., 2003). The transmembrane helices of the Cyt b_6 proteins form much of the interface between the two monomers in the Cyt b_6f dimer. Interactions between the aromatic Phe52, Phe56, and Phe189 residues of each monomer help stabilize the dimer structure (Cramer et al., 2006).

The two *b* hemes of each Cyt b_6 polypeptide are tethered between transmembrane helices B and D via non-covalent, bis-imidazole axial ligation to His residues that are conserved in all known Cyt b_6 and Cyt *b* sequences (see Cramer and Zhang, 2006 for representative alignments). His86 and 187 bind heme b_p, whereas His100 and 202 bind heme b_n, thereby cross-linking helices B and D in the Cyt b_6f structure (residue numbers as in the *C. reinhardtii* and *M. laminosus* Cyt b_6 sequences). The two *b* hemes have virtually identical orientations in Cyt bc_1 (Xia et al., 1997; Kim et al., 1998; Zhang et al., 1998) and Cyt b_6f (Kurisu et al., 2003; Stroebel et al., 2003) structures despite an additional amino acid between the heme-liganding His residues in helix D of Cyt b_6 proteins (Widger et al., 1984). A kink in the Cyt b_6 D helix in the region of Phe189 accommodates the extra amino acid and maintains the conserved *b* heme orientations implying that this organization may be important for heme alignment and efficient electron transfer relative to the other redox centers.

Figure 21.5 illustrates the axial, *b* heme ligation and the juxtapositions of the *b* hemes and heme c_n in the Cyt b_6f low potential chain. Heme c_n is bound to the Cyt b_6 protein via a single, covalent thioether linkage to the conserved Cys35 residue of helix A (Kurisu et al., 2003; Stroebel et al., 2003). Hemes b_p and b_n are largely perpendicular to the membrane plane and heme c_n is nearly perpendicular to b_n and closely coupled to it via a water molecule. An oxygen of one of the heme b_n propionates, the water oxygen, and the

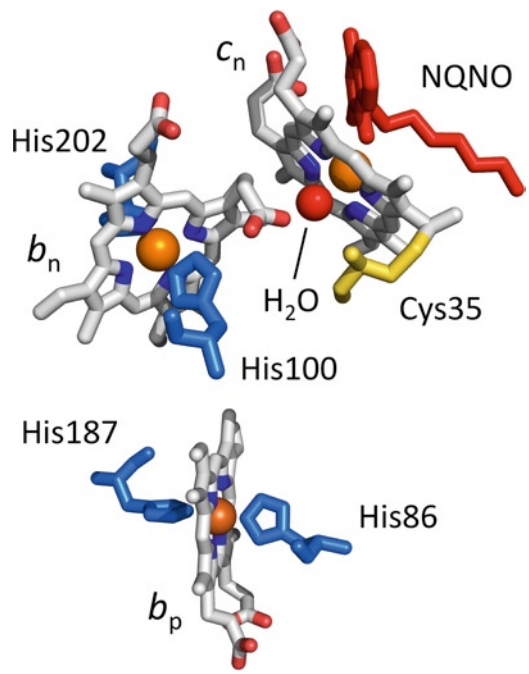

Fig. 21.5. Hemes and NQNO in the cytochrome b_6f low-potential chain. Hemes b_p, b_n, and c_n are shown from the *Mastigocladus laminosus* 2E75 structure crystallized in the presence of NQNO (Yamashita et al., 2007). The axial bis-histidine ligands of the *b* hemes are shown as is the Cys35 residue that provides a thioether linkage to heme c_n. Both *b* hemes are essentially perpendicular to the membrane and heme c_n is essentially perpendicular and closely coupled to heme b_n via a water molecule or hydroxide. A propionate oxygen of heme b_n, the water oxygen, and the iron atom of heme c_n are all within a few Ångstroms of each other. The inhibitor NQNO binds close to the heme c_n face with its hydroxyl group a few Ångstroms from the heme iron.

heme c_n iron atom are separated from each other by no more than ~2.5 Å between centers. The inhibitor NQNO binds near the heme c_n face with its ring hydroxyl within a few Å of the heme iron (Fig. 21.5). NQNO is a plastoquinone analog and its binding site approximates the native plastoquinone binding site in the Q_n pocket. Thus the Cyt b_6f structures provide striking evidence that heme c_n must be an integral, coupled component of the low-potential electron transfer pathway from the quinol-oxidation (Q_p) to quinone-reduction (Q_n) sites that lies largely within the Cyt b_6 and subunit IV subunits. The redox properties of the b_6 and c_n hemes were described in Table 21.1 above.

2. The Quinone-Exchange Cavity and Quinone-Binding Niches

The Cyt b_6 and subunit IV polypeptides provide most of the surfaces for the quinone-exchange cavities of the Cyt b_6f dimer as well as the plastoquinol-oxidation (Q_p) and plastoquinone-reduction (Q_n) pockets associated with each cavity. Figure 21.6a shows a space-filling representation of the quinone-exchange cavity on one side of the Cyt b_6f dimer and the entry portals at either end leading to the Q_n-pocket of one monomer and the Q_p-pocket of the other monomer. Panels b and c show enlarged views of the entry passages to the Q_n and Q_p pockets. Inhibitors of quinol-oxidation and quinone-reduction have been extremely useful in defining these Q_p and Q_n sites within Cyt bc_1 and b_6f complexes. Prior to the emergence of the Cyt bc_1-b_6f structures, mapping of inhibitor resistance mutations to protein 2D models and studies of their impacts on electron transfer reactions were instrumental for revealing two separate sites, Q_o (Q_p) and Q_i (Q_n), for quinol-oxidation and quinol-reduction on opposite electro-positive (*p*) and -negative (*n*) sides of the membrane, respectively. Many of the mutational studies in particular were first done in purple photosynthetic bacteria and yeast and subsequently in *C. reinhardtii* and cyanobacteria (see Kallas, 1994; Brasseur et al., 1996; Berry et al., 2000; Darrouzet et al., 2004 for reviews of the earlier works). A stable ubiquinone occurs in the Cyt bc_1 Q_n-site and it has been possible to solve structures of Cyt bc_1 complexes with the native ubiquinone bound to the Q_n-pocket (Gao et al., 2003; Huang et al., 2005). Inhibitors have been invaluable for defining the quinone-binding sites in Cyt bc_1 complexes. Structural and other studies show that the inhibitor's sites in the Cyt bc_1 quinone pockets overlap the native ubiquinone-ubiquinol binding sites (reviewed in Berry et al., 2000; Darrouzet et al., 2004).

Plastoquinone bound to the Q_n-site was reported in the original Cyt b_6f structure from *M. laminosus* (Kurisu et al., 2003) but electron densities in the Q_n pocket of the *C. reinhardtii* structure (Stroebel et al., 2003) were interpreted somewhat differently (see Nelson et al., 2005 for a discussion). Subsequent structures of the *M. laminosus* Cyt b_6f complex (e.g., Yamashita et al., 2007) no longer show PQ in the Q_n-pocket and thus the original assignment may be open to question. Accordingly, the positions of the quinone analog inhibitors TDS and NQNO bound to the Q_p- and Q_n-pockets may provide the best approximations of the actual PQ binding sites. The inhibitors bind much more tightly and are thus more readily recovered in crystals than the native quinones whose residency time in these sites may be only a few ms. Note that the turnover rate, and thus PQ binding cycle, of Cyt b_6f complexes is on the order of 250–400 s^{-1} (e.g., Hope et al., 1992; Pierre et al., 1995; Baniulis et al., 2009). Thus Cyt b_6f structures with bound Q_p- and Q_n-site inhibitors (Stroebel et al., 2003; Yamashita et al., 2007) together with functional and mutagenesis data (reviewed in Allen, 2004; Baniulis et al., 2008; Kramer et al., 2008; Cramer et al., 2011) allow reasonable definitions of the native plastoquinone-plastoquinol (PQ/PQH_2) binding sites. Note again, however, that electron densities in structures resolved to ~3 Å, and thus inhibitor binding positions, are subject to interpretation. For example, an electron density in the original *M. laminosus* structure was interpreted as TDS bound in an unusual 'ring out' position near the entry portal of the Q_p pocket (Kurisu et al., 2003; Cramer et al., 2006). In light of more refined data sets, it became apparent that this density likely arose from added lipid or detergent rather than TDS. Addition of TDS prior to lipid addition during crystallization and refined data have led to the revised interpretation that TDS binds within the Q_p pocket in the expected 'ring in' orientation (Yamashita et al., 2007) consistent with its orientation in the *C. reinhardtii* (Stroebel et al., 2003) and Cyt bc_1

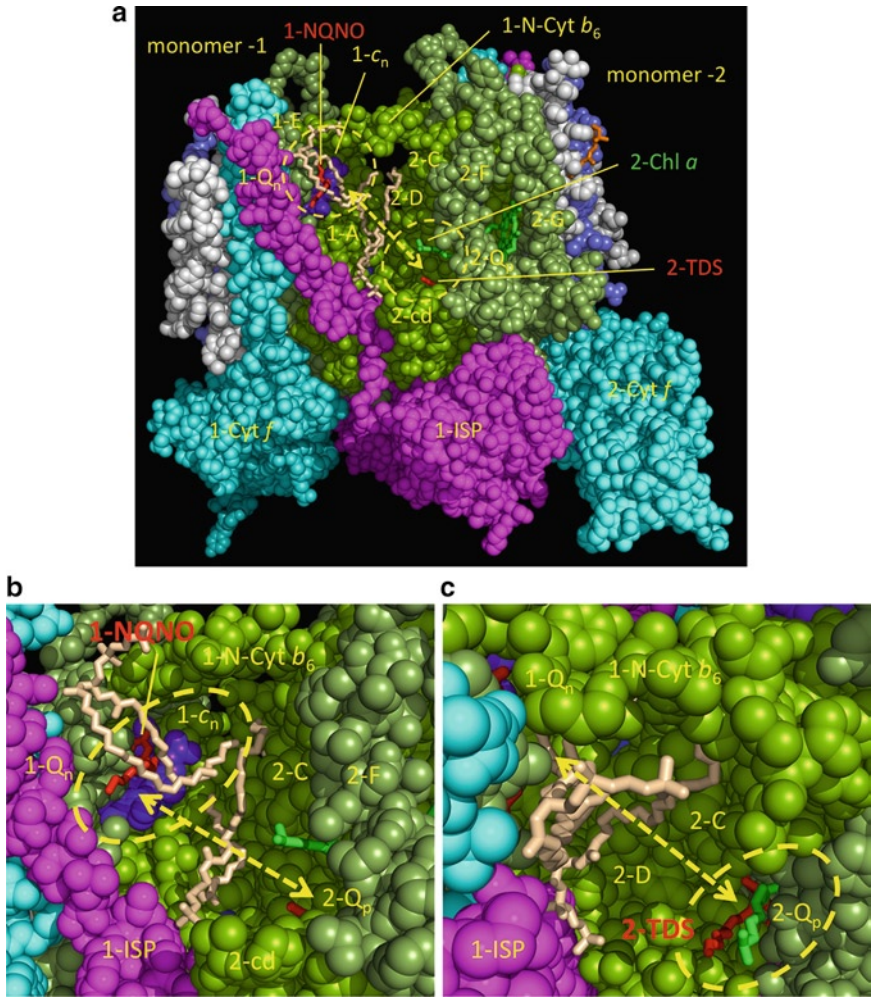

Fig. 21.6. Plastoquinone binding sites and quinone-exchange cavity of the cytochrome b_6f dimer. (**a**) Space filling model illustrating one of two quinone-exchange cavities formed at the periphery of the two monomers of the Cyt b_6f complex. Shown is the *Mastigocladus laminosus* Cyt b_6f structure 1VF5 (Kurisu et al., 2003) with the inhibitors TDS, 'ring-in' and 'tail-out' conformation, and NQNO superposed from structures 2E76 and 2E75, respectively (Yamashita et al., 2007). The view is similar to that of the ribbon diagram in Fig. 21.3 and the coloring is the same. Components belonging to monomers 1 or 2 are designated with the prefixes 1 or 2. Capital letters refer to the Cyt b_6 and subunit IV helices that largely form the cavity. The general locations of the quinone-reduction (Q_n) and quinol-oxidation (Q_p) sites at either end of the cavity are marked with *dashed circles*. NQNO and heme c_n are partially visible and define the Q_n pocket of monomer 1. The tails of TDS and chlorophyll *a* (*Chl a*) protrude into the cavity and define the narrow entry portal to the Q_n pocket of monomer 2. Two molecules of the added, synthetic lipid, DOPC, are visible in the cavity. (**b**) Enlarged view tilted to show the entry to the Q_n pocket. The c_n heme is clearly visible and NQNO is expected to approximate the binding site of native plastoquinone (*PQ*). The TDS and chlorophyll *a* tails are barely visible from the Q_p portal. (**c**) Enlarged view tilted to show the narrow entry portal to the Q_p pocket. The TDS and chlorophyll *a* tails are more clearly visible. The TDS head group is largely buried within the pocket and approximates the expected binding position of native plastoquinol (PQH_2).

structures (Kim et al., 1998; Zhang et al., 1998). The current structural interpretations should now reasonably reflect the binding sites for inhibitors, and thus approximate the native plastoquinone-plastoquinol binding sites in the Cyt b_6f complex.

Thus NQNO bound to the Q_n pocket of one monomer and TDS bound 'ring in' to the Q_p pocket of the other monomer define the plastoquinone-plastoquinol (PQ/PQH_2) binding niches at either end of the quinone-exchange cavities in the Cyt b_6f complex (Fig. 21.6). As mentioned

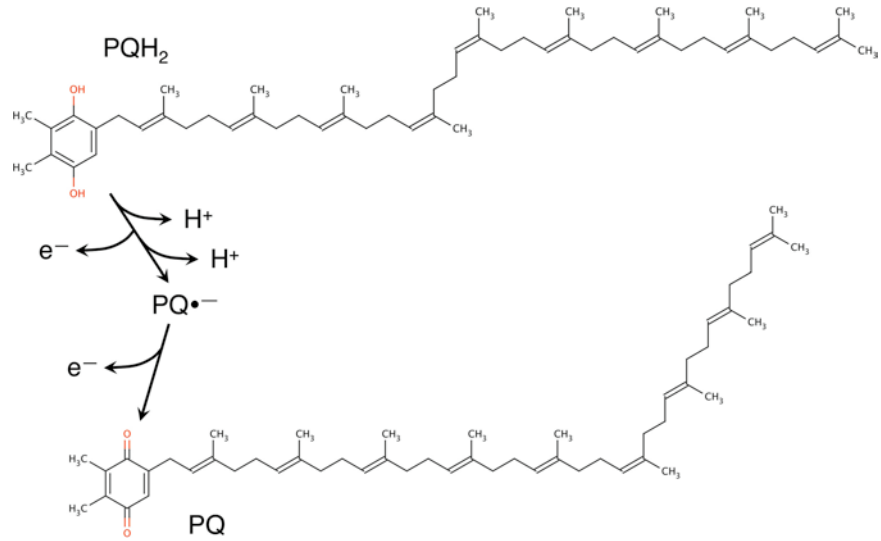

Fig. 21.7. Reduced and oxidized forms of plastoquinone. The reduced, plastoquinol or plastohydroquinone (*PQH_2*), and oxidized, plastoquinone (*PQ*), forms of plastoquinone-9 (*PQ-9*) are shown. The hydrocarbon tail consists of nine, C5 isoprenoid units for a total of 45 carbons. Oxidation of PQH_2 occurs via loss of two protons and one electron to generate the platosemiquinone anion radical (*PQ•⁻*) and then loss of a second electon to generate PQ. Structure coordinates were obtained from NCBI PubChem (http://pubchem.ncbi.nlm.nih.gov) and images created with MarvinView software from ChemAxon (http://www.chemaxon.com).

and seen in a space-filling representation (Fig. 21.6), the cavity is formed by the Cyt b_6-subunit IV transmembrane helices A and E (1-A and 1-E in Fig. 21.6) and the Rieske ISP helix of one monomer and the C, D, and F helices (2-C, 2-D, and 2-F in Fig. 21.6) of the other monomer. The *n*-side 'roof' of the cavity is formed by N-terminal region of Cyt b_6 from monomer 1 and the *p*-side 'floor' by the cd helical loop connecting helices C and D of Cyt b_6 in monomer 2. The entry portals to the Q_n and Q_p pockets are marked by dashed circles and defined by the edges of the NQNO molecule and heme c_n visible from the Q_n site and the TDS and chlorophyll *a* tails barely visible from the Q_p portal. Panels b and c (Fig. 21.6) show enlarged and tilted views to better display the entries to the quinone-binding pockets. Note in particular that the entry portal to the Q_p pocket is very narrow, ~11 Å × 12 Å (Cramer and Zhang, 2006), that this may constrain movement in of plastoquinol (PQH_2) and movement out of plastoquinone (PQ). This has been suggested as a possible factor limiting the overall PQH_2 oxidation reaction (Baniulis et al., 2008), which is the rate limiting step in photosynthetic electron transport (Witt, 1971). Note that the plastoquinol-plastoquinone molecules (PQH_2/PQ) of cyanobacterial-chloroplast photosynthesis have a very long hydrocarbon tail of nine isoprenoid units or 45 carbons attached to the redox-active quinone ring (Fig. 21.7). These large tails may pose a severe topological impediment to the rapid movement of PQH_2/PQ in and out of the Q_p niche for exchange with the Q_n site and PQ pool. The rate-limiting PQH_2 oxidation step and electron and proton transfer reactions at the Q_p and Q_n sites are discussed further below (Section VIII.B.2) (Breyton, 2000).

3. The Quinol-Oxidation (Q_p) Pocket

The plastoquinol-oxidation (Q_p) pocket itself is formed by the Cyt b_6 transmembrane helices C and D, the connecting helices cd, subunit IV transmembrane helix F, the long connecting loop ef, heme b_p, and 2Fe-2S cluster-binding loops of the Rieske ISP (Fig. 21.8). The ef loop contains the famous, conserved 'PEWY' residues that were postulated long ago to be involved in plastoquinol (PQH_2) binding (Hauska et al., 1988). A role for these residues, and specifically for Glu78 (*C. reinhardtii* numbering) in quinol binding and proton translocation during quinol oxidation, has since been demonstrated by mutagenesis studies

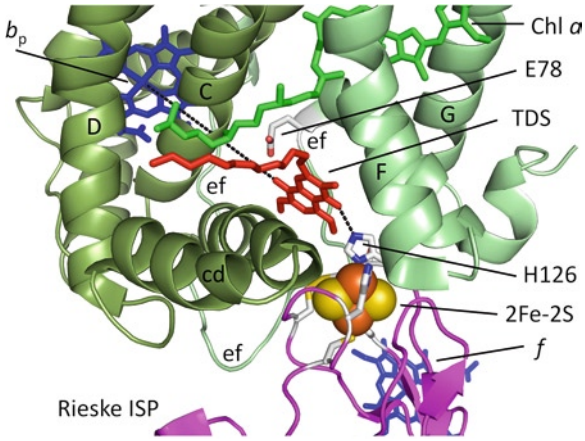

Fig. 21.8. Quinol-oxidation (Q_p) pocket of the cytochrome b_6f complex. Features shown are from the *Chlamydomonas reinhardtii* 1Q90 Cyt b_6f structure (Stroebel et al., 2003). The Q_p pocket is formed by transmembrane helices C and D of Cyt b_6 (*olive green*), the connecting helices cd, heme b_p, transmembrane helix F of subunit IV (*light grey-green*), the long ef loop, and 2Fe-2S cluster-binding loops (*magenta*) of the Rieske iron-sulfur protein (*ISP*). The Cyt *f* heme is visible in the distance ~30 Å from the 2Fe-2S cluster (*iron, orange and sulfur, yellow*). TDS is bound to the Q_p pocket via a hydrogen bond (~2.9 Å) between the TDS ring carbonyl oxygen to an imidazole nitrogen of His126 of the Rieske ISP. His107, His126, Cys105, and Cys123 provide ligands for the 2Fe-2S cluster (residues numbers are from the mature Rieske ISP of *Chlamydomonas reinhardtii*, de Vitry et al., 1999). The ring hydroxyl of TDS is ~15 Å away from the heme b_p iron. The chlorophyll *a* phytyl tail protrudes into the Q_p pocket and extends out through the entry portal. Subunit IV Glu78 is labeled and implicated in proton transfer from plastoquinol (Zito et al., 1998).

both in Cyt bc_1 (Crofts et al., 1999) and b_6f (Zito et al., 1998) complexes. Numerous other mutations that alter reaction kinetics and/or confer resistance to inhibitors have been mapped as well to the Q_p pockets of Cyt bc_1 and b_6f complexes (reviewed in Kallas, 1994; Brasseur et al., 1996; Berry et al., 2000; Crofts, 2004a; Darrouzet et al., 2004; Kramer et al., 2008). These and the structures obtained with and without inhibitors have helped define the Q_p pocket and mechanism of quinol oxidation. Figure 21.8, for example, shows TDS in the Q_p pocket bound via a hydrogen bond (2.9 Å) linking its ring carbonyl oxygen to an imidazole nitrogen of His155 (His126 in Fig. 8), one of the 2Fe-2S cluster binding histidines of the Rieske ISP (Stroebel et al., 2003). A similar hydrogen bond is believed to form between the native plastoquinol and Rieske ISP in the first step of quinol-oxidation in Cyt bc_1 and b_6f complexes (Berry et al., 2000; Crofts, 2004b). Questions remain, but the picture that has emerged is that the overall structure of the Cyt b_6f–bc_1 Q_p–(Q_o) domains and mechanism of quinol oxidation by these complexes has remained highly conserved.

4. The Quinone-Reduction (Q_n) Pocket

In contrast, although the overall quinone-reduction reaction performed in Cyt b_6f and bc_1 quinone-reduction pockets is the same – i.e., the two electron reduction and protonation of a quinone (Q) to generate a quinol (QH_2) molecule – the structures and reaction mechanisms of Cyt b_6f and bc_1 Q_n–(Q_i) quinone-reduction domains differ radically. These differences arise from the highly divergent structures of the Q_n sites. The Cyt b_6f Q_n-pocket is formed by the *n*-side exposed N- and C-terminal regions of Cyt b_6; the N-terminal extension of subunit IV; the A, D, and E transmembrane helices of these proteins; and the *n*-side exposed face of the unique c_n heme (Fig. 21.9). The classical Cyt bc_1 quinone-reduction, Q_n-site inhibitor, antimycin A, does not bind to Cyt b_6f complexes. Rather, heme c_n resides in the Q_n-pocket in a position that corresponds to the ubiquinone or antimycin A binding site in Cyt bc_1 complexes (Kurisu et al., 2003; Stroebel et al., 2003). One face of heme c_n is axially liganded via hydrogen-bonding through a water molecule or hydroxyl group to a propionate of heme b_n as illustrated in Fig. 21.5 (above) and Fig. 21.9. The other face of heme c_n is largely exposed to the quinone-exchange cavity (Fig. 21.6) and is the site of NQNO and plastoquinone (PQ) binding. The electron density interpreted as PQ in the original *M. laminosus* structure lies close to the exposed heme c_n face but is ~10 Å away from the heme iron and ~4 Å from the edge of the closest heme propionate (Kurisu et al., 2003, see Fig. 21.9). PQ was not unambiguously defined in the *C. reinhardtii* structure but an electron density near the exposed heme c_n face was indicated that could belong to the PQ ring (Stroebel et al., 2003). This electron density occupies a position close to that of NQNO bound to heme c_n in the *M. laminosus* structure (Yamashita et al., 2007) and may represent the actual PQ binding site in the Q_n-pocket (Fig. 21.9).

Photosynthetic reaction center and Cyt bc_1 complexes crystallized with bound quinones have

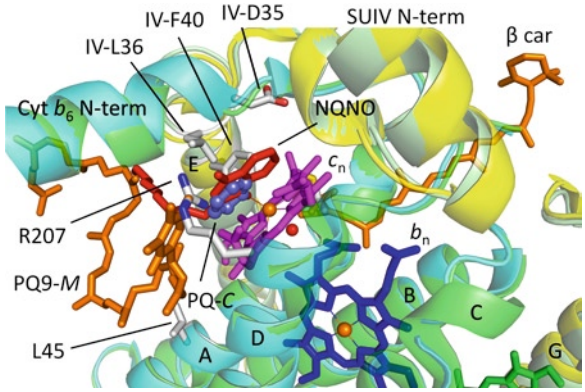

Fig. 21.9. Quinone-reduction (Q_n) pocket of the cytochrome b_6f complex. Features shown are from the *Mastigocladus laminosus* 1VF5 native (Kurisu et al., 2003), 2E75 NQNO (Yamashita et al., 2007), and the *Chlamydomonas reinhardtii* 1Q90 (Stroebel et al., 2003) Cyt b_6f structures. All of these structures were aligned. The Cyt b_6 and subunit IV backbone ribbons are displayed for both *Mastigocladus laminosus* (*green and light grey-green*) and *Chlamydomonas reinhardtii* (*turquoise and yellow*). The Q_n pocket is formed by the *n*-side exposed N-termini of the Cyt b_6 and subunit IV proteins; the A, D, and E transmembrane helices of these proteins; and the *n*-side exposed face of heme c_n (*purple*). The ring hydroxyl of NQNO (*red*) is within hydrogen bonding distance (~2.2 Å) of the heme c_n iron. Plastoquinone (*PQ*) from the *Mastigocladus laminosus* 1VF5 (*PQ-M*) and the tentatively assigned PQ ring (PQ-*C*, Daniel Picot, personal communication) from the *Chlamydomonas reinhardtii* 1Q90 structures are *orange* and *lavender*, respectively. β-carotene (*β-car*) is *orange* also. Cyt b_6 Leu45 and subunit IV Leu36 and Phe40 are labeled and implicated in PQ binding (Kurisu et al., 2003; Stroebel et al., 2003; Cramer et al., 2006; de Lacroix de Lavalette et al., 2008). Cyt b_6 Arg207 and subunit IV Asp35 have possible roles in PQ binding and/or modulating the properties of heme c_n (Nelson et al., 2005; de Lacroix de Lavalette et al., 2008). PQ-*M* and Arg207 originate from different structures and their apparent clash reflects differences in local conformations or data interpretation of these structures.

revealed general features of quinone binding where stabilization occurs through interactions with neighboring hydrophobic groups and hydrogen bonding of the quinone oxygens (Baciou and Michel, 1995; Hunte, 2001). The hydrogen bonds may be direct or via intervening water molecules as in the yeast Cyt bc_1 complex (Hunte, 2001). Residues surrounding the putative PQ binding site in the Cyt-b_6f Q_n-pocket that may provide hydrophobic contacts include the invariant subunit IV (PetD) Phe40 and Leu36 near the heme c_n face (Fig. 21.9). Cyt b_6 Tyr45, Leu45, and Phe203 were indicated as possible sites for hydrophobic interactions with the PQ isoprenoid tail based on the *M. laminosus* 1VF5 structure (Cramer et al., 2006). The conserved Cyt b_6 Arg207 residue is crucial for Q_n-site function. The corresponding PetB-R214H mutation (Arg214 to His) in the cyanobacterium *Synechococcus* sp. PCC 7002 severely impairs Q_n-site function resulting in accumulation of electrons in the Cyt b_6f low-potential chain, slower turnover of the b_6f complex, and slower growth (Nelson et al., 2005). Arg207 may function in the Q_n pocket in several ways. Its amide nitrogens are within 2.5–3.0 Å of the NQNO ring carbonyl oxygen, about the same distance from the suggested PQ ring in the *C. reinhardtii* Cyt b_6f structure (Stroebel et al., 2003), and within 3.0 Å of propionate oxygens of heme c_n (Fig. 21.9). Thus Arg207 may provide a key hydrogen bond for PQ binding and quinone reduction in the Q_n site. Arg207 also quite likely helps establish the relatively high midpoint potential of heme c_n by providing a nearby positive charge to help stabilize an electron on heme c_n. Note, however, that midpoint potential estimates of heme c_n vary considerably from $E_{m,7}$ <−50 mV to +100 mV (Zhang et al., 2004a; Alric et al., 2005) but that this variance may be significant for Q_n-site reaction mechanism as discussed below. Subunit IV Asp35 might also modulate the midpoint potential of heme c_n by providing a full negative charge within 6 Å of the heme edge (Fig. 21.9).

Genetic modifications of the *C. reinhardtii* Cyt b_6f Q_n-pocket have yielded several mutants that either fail to assemble the Cyt b_6f complex (e.g., mutations of Cyt b_6 Gly37 or Arg207 and subunit IV Asp35 or Phe120) or that are phenotypically wild type (e.g., mutations of Cyt b_6 Phe41 or Phe44 and subunit IV Leu36, Phe40, or Pro41) (de Lacroix de Lavalette et al., 2008). An interesting exception is a subunit IV Phe40>Tyr replacement of the conserved phenylalanine that lies ~3.2 Å from the heme c_n iron and partially occludes the heme face (de Lacroix de Lavalette et al., 2008). Upon short flash excitation, this mutant has a phenotype like that of the cyanobacterial PetB-R214H mutant (Nelson et al., 2005) where the Cyt b_6f low-potential chain becomes reduced and subsequent *b* heme oxidation is greatly slowed. Paradoxically, however, the Q_n-site and Cyt b_6f complex of the Phe40 to Tyr mutant strain functions largely normally during extended, high-intensity illumination. The authors

suggest that the phenolate ring of tyrosine provides a weak axial ligand to the exposed face of heme c_n (imagine a Tyr phenolate ring in place of the Phe40 benzene ring, see Fig. 21.9), which competes with PQ for binding to heme c_n. They further propose that the binding affinity of the Tyr ligand or of the native PQ to heme c_n is determined by the redox state of the heme. The Phe40 to Tyr substitution precipitously lowers the midpoint potential ($E_{m,7}$) of heme c_n from +100 mV to −200 mV, which apparently impedes PQ binding and clearly impedes *b* heme oxidation upon short-flash illumination. However, upon extended illumination and increased reduction pressure from the Cyt b_6f low-potential chain, heme c_n becomes reduced, and thus apparently weakens the Tyr interaction and allows access to the native PQ. This is an interesting finding that reveals a crucial role for subunit IV Phe40 in the native Cyt b_6f complex in modulating the midpoint potential of heme c_n and PQ binding in a way that might allow the proposed, concerted two electron reduction of PQ at the Q_n-site (Allen, 2004; Baymann et al., 2007); as further discussed below (Sections VII.A.5 and VIII.B.3).

Note that the Cyt c_n heme and the Q_n pocket are also largely exposed to the *n* (cytoplasmic or stromal) exterior face of the thylakoid membrane (Figs. 21.3, 21.4, and 21.9). This led to the proposal that the *n*-side exposed Q_n-domain, surrounded by largely positive surface charges, could serve as a binding site for anionic Fd or FNR in a direct cyclic electron transfer pathway from PS I (Kurisu et al., 2003; Stroebel et al., 2003; Cramer and Zhang, 2006). Indeed, FNR has been found in association with isolated Cyt b_6f complexes from spinach (Whitelegge et al., 2002; Zhang et al., 2001) and a Cyt b_6f–FNR–Fd–PS I supercomplex has now been isolated that catalyzes PS I driven cyclic electron flow in vitro (Iwai et al., 2010).

5. The Long Undiscovered c_n Heme

Several features of heme c_n and its crucial role in the Cyt b_6f Q_n-pocket were discussed above. Heme c_n is a *c*-type heme covalently linked via only a single thioether bond to Cys35 of Cyt b_6 and the only known heme with no axial ligand provided by an amino acid side chain (Stroebel et al., 2003). Typical *c*-type hemes are attached to proteins by thioether linkages to two Cys residues and by one or more amino acid axial ligands (the fifth and sixth nonheme ligands) to the heme iron (Meyer and Kamen, 1982). Such heme irons are thus penta- or hexa-coordinated. A water molecule or more likely an OH^- (Twigg et al., 2009) provides one axial ligand to heme c_n (Fig. 21.5) and thus heme c_n iron is penta-coordinated. Accordingly, heme c_n belongs to the family of penta-coordinated, high spin c' hemes (Meyer and Kamen, 1982) that have very weak or absent absorbance in the α-band (~550–565 nm) region typically used as a convenient window for heme detection in photosynthetic organisms. This explains how heme c_n evaded detection despite years of intensive biophysical and biochemical investigations of Cyt b_6f complexes. Alric and coworkers (2005) have demonstrated that heme c_n corresponds to the cryptic, *n*-side 'G' signal first observed by Lavergne (Lavergne, 1983; Joliot and Joliot, 1988) by high-resolution, difference spectroscopy of the alga *Chlorella sorokiniana* in the Soret band (~400–500 nm) region. In retrospect, other previous indications of heme c_n were characteristic, high-spin $g = 6$ EPR signals from isolated Cyt b_6f complexes (Nitschke and Hauska, 1987), indications from heme-staining of polyacrylamide gels of a heme covalently bound to Cyt b_6 (Gabellini et al., 1982; Kuras et al., 1997), and extra mass associated with Cyt b_6 (Zhang et al., 2001; Whitelegge et al., 2002).

As a result of having only a single axial ligand, one face of heme c_n is largely exposed to the quinone-exchange cavity and the *n*-side of the membrane (see Figs. 21.4, 21.6, and 21.9). The exposed face is protected by Phe40 mentioned above (de Lacroix de Lavalette et al., 2008) and more distantly by N-terminal regions of Cyt b_6 and subunit IV as well as *n*-side lipids (Kurisu et al., 2003; Stroebel et al., 2003). NQNO binds to the heme c_n face as mentioned above (Figs. 21.5 and 21.9) as does TDS (Yamashita et al., 2007). TDS is a classical Q_p-site inhibitor and it binds effectively to the Q_p-pocket of Cyt b_6f (Stroebel et al., 2003; Yamashita et al., 2007) and bc_1 (Kim et al., 1998; Zhang et al., 1998) structures as expected (Fig. 21.8). Its binding to heme c_n in the Cyt b_6f Q_n-pocket was unexpected but not entirely surprising because TDS is a quinone analog and binding may have been promoted by the high molar ratio of inhibitor to protein (~5:1) used during crystallization. TDS binding to Q_n- as well

as Q_p-sites had been previously indicated by TDS-induced perturbations of heme b_n and b_p spectra in both Cyt b_6f and bc_1 complexes (Hauska et al., 1989). Hydrogen bonding of both the NQNO and TDS quinone analogs to heme c_n suggests that the native PQ can similarly form a ligand with heme c_n. Interestingly, a range of midpoint potentials has been reported for heme c_n (Table 21.1). The original, in vivo spectroscopic studies of the heme c_n, 'G' signal (Lavergne, 1983; Joliot and Joliot, 1988) inferred an $E_{m,7}$ ~20–30 mV lower than that of heme b_n, thus ~−60 mV. Zhang and coworkers (2004a) reported an $E_{m,7}$ <−50 mV based on the inability of ascorbate to abolish EPR signals in the $g<6$ region attributed to the oxidized form of heme c_n. Alric and coworkers (2005) via optical spectroscopic measurements of isolated *C. reinhardtii* Cyt b_6f complexes, reported an $E_{m,7}$ of +100 mV and a pH dependence of −60 mV/pH unit from pH 6–9. This suggested involvement of heme c_n in protonation events, perhaps arising from hydrogen-bonding of PQ or proton transfer at the Q_n site. In addition, NQNO binding lowers the midpoint potential on heme c_n by ~225 mV indicating that substrate binding and ligand formation with heme c_n can dramatically alter its redox properties.

Studies of EPR signals associated with hemes c_n and b_n indicate a strong electronic coupling of these nearby hemes (Zhang et al., 2004a; Baymann et al., 2007) as expected from the Cyt b_6f structures (see again Figs. 21.5 and 21.9). Moreover, the addition of NQNO to isolated Cyt b_6f complexes not only lowers the midpoint potential of heme c_n (Alric et al., 2005) but considerably simplifies the high-spin region ($g=13$ to $g=4$) of the EPR spectrum (Baymann et al., 2007). The simplified EPR spectrum still revealed a 'high-spin state' indicating that heme c_n remained pentacoordinated despite the addition of the 'sixth' axial ligand from NQNO. This was interpreted in terms of the new NQNO-heme-c_n ligand displacing the H_2O/OH^- ligand that normally bridges hemes c_n and b_n (see Figs. 21.5 and 21.9 for reference) and thus loss of the EPR signals arising from heme c_n–b_n interaction. These findings and the observed variable potentials of heme c_n suggest that heme c_n has a 'tunable' midpoint potential dependent on substrate (inhibitor, axial ligand, or PQ) binding and redox state of the adjacent b_n heme. The Phe40 to Tyr mutation near heme c_n, where Tyr40 very likely provides a ligand and dramatically lowers the heme midpoint potential as discussed above (de Lacroix de Lavalette et al., 2008, Section VII.A.4), further supports this hypothesis. Interestingly, the Rieske-Cyt *b* complex of *Heliobacterium modesticaldum* does have an apparent amino acid axial ligand to the exposed heme c_n face where a glutamic acid (Glu) residue has replaced the usual subunit IV Phe40 in the protein structure (Ducluzeau et al., 2008). The Heliobacterial Rieske-Cyt *b* complex shows a simplified heme c_n EPR spectrum like that of NQNO-treated Cyt b_6f complexes (Baymann et al., 2007) consistent with a sixth axial ligand. These authors propose that protonation of the Glu residue upon quinone binding releases the Glu ligand to heme c_n making it accessible to quinone binding as in the Cyt b_6f complex (Ducluzeau et al., 2008; Baymann and Nitschke, 2010). The above studies point to dynamic interactions between hemes b_n–c_n and the PQ substrate to favor a concerted two-electron reduction of PQ at the Q_n site. The Cyt b_6f Q_n-domain structure and mechanism has apparently evolved to minimize the one-electron reduction of PQ to the reactive semiquinone ($PQ^{\bullet-}$) capable of reducing O_2 to generate the damaging superoxide radical ($O_2^{\bullet-}$) as previously suggested (Allen, 2004; Zatsman et al., 2006; Baymann et al., 2007). According to this model: (i) PQ (the oxidized form) binds more tightly than $PQ^{\bullet-}$ or PQH_2 to oxidized heme c_n; (ii) PQ binding lowers the midpoint potential of c_n; (iii) this makes unlikely the one-electron reduction of heme c_n by heme b_n and thus makes unlikely the one-electron reduction of PQ by c_n to form the semiquinone, and (iv) when electron pressure builds up in the Cyt b_6f low-potential chain, this allows two electrons to flow more or less simultaneously through hemes b_n–c_n to reduce PQ to PQH_2 without formation of the semiquinone radical.

The danger of superoxide formation at the Q_n site is evident from the surface exposure of heme c_n. Moreover, recent EPR data show that NO, a surrogate for O_2, can also form a ligand to heme c_n (Twigg et al., 2009). Surface exposure of heme c_n may be important for allowing cyclic electron flow from PS I as discussed below (Section VIII.D) and thus the evolution of a unique mechanism to prevent superoxide formation at the Q_n-site may have been particularly compelling. In that context,

it is interesting to consider how Cyt bc_1 complexes have solved this problem. Note that mitochondrial-bacterial Cyt bc_1 complexes do not catalyze any cyclic electron flow that would require direct access from the *n*-side of the membrane. Indeed, the Cyt bc_1 Q_n (or Q_i) niche is more hidden from the *n*-side surface but clearly water molecules, and by implication oxygen, have access to the Q_n-pocket as evidenced by the bc_1 structures (e.g., Lange et al., 2001; Esser et al., 2008). The presence of a stable semiquinone in the Cyt bc_1 Q_n-site is well established (see Crofts, 2004b; Crofts et al., 2008 for reviews). Why then does this semiquinone not pose the risk of superoxide formation? Although questions remain, the reason lies in the structure of the Cyt bc_1 quinone-reductase (Q_n) site. The semiquinone ($Q^{\bullet-}$) is stabilized by the surrounding protein environment, which raises the midpoint potential of the quinone/semiquinone ($Q/Q^{\bullet-}$) couple to ~+110 to +150 mV or about that of the quinone/quinol (Q/QH_2) couple at ~ +90 mV (Kramer et al., 2008). Semiquinone in solution or bound to the Cyt bc_1 Q_p-site has an estimated $E_{m,7}$ of −300 mV to −400 mV (Crofts and Wang, 1989; Muller, 2000), which can readily reduce molecular oxygen (O_2) to superoxide ($O_2^{\bullet-}$). Stabilization of the Cyt bc_1 Q_n-semiquinone occurs through hydrogen bonding either directly to amino acid side chains (Berry et al., 1999; Esser et al., 2008) or via water molecules (Hunte et al., 2000; Lange and Hunte, 2002; Gao et al., 2003). High-resolution Cyt bc_1 structures (Huang et al., 2005) and EPR analyses (e.g., Kolling et al., 2003) have identified His217, Asn221 (or Ser206 in yeast and chicken), and Asp229 as residues that provide stabilizing hydrogen bonds to the semiquinone (*Rhodobacter sphaeroides* residue numbers). See Crofts (2004a) for an illustration of Q_n-semiquinone hydrogen-bonding to these residues and further discussion. Mutagenesis of these residues has provided strong evidence for their involvement in catalysis (Kolling et al., 2003).

6. The Rieske Iron-Sulfur Protein

a. Structure and Physical-Chemical Properties

As stated, the Rieske ISP is an essential, core component of Rieske-Cyt *b* complexes strictly required for the initial, rate-limiting quinol-oxidation step in the Q_p-pocket. Rieske 2Fe-2S clusters display characteristic, low temperature (15°K) EPR spectra with *g* values at $g_x = 1.74–1.81$, $g_y = 1.89–1.90$, $g_z = 2.01–2.03$ (Rieske et al., 1964; Malkin and Aparicio, 1975). Perturbation of these signals by Q_p-site inhibitors provided early evidence for interactions of quinone substrates with the ISP cluster (Malkin, 1982). Rieske ISP clusters from Cyt b_6f-bc_1 complexes as well as bacterial oxygenase enxymes also display characteristic, redox-dependent optical circular dichroism spectra with a distinctive negative signal near 500 nm that allows redox titrations at ambient temperature (Fee et al., 1986; Degli Esposti et al., 1987; Link et al., 1996). Cyt b_6f-bc_1 Rieske proteins have high midpoint potentials of ~+280 to +320 mV (reviewed by Kallas, 1994; Snyder et al., 1999) with a pH dependence of −30 to −80 mV/pH unit in the pH 6.5 to 9.0 range for Cyt b_6f Rieske ISPs (Soriano et al., 2002 and references therein) and −60 to −120 mV/pH unit for Cyt bc_1 Rieske ISPs (Link et al., 1992; Ugulava and Crofts, 1998). The pH-dependence of the midpoint potential arises from increased ionization (deprotonation) with increasing pH of the cluster-binding His residues and amino acids in the vicinity of the Rieske ISP cluster. These features are essential for quinol-oxidation activity and mutations that either lower the midpoint potential or alter the pK values of ionizable residues near the ISP cluster substantially lower catalytic activity as demonstrated by mutational studies of Cyt bc_1 complexes (Denke et al., 1998; Guergova-Kuras et al., 2000; Merbitz-Zahradnik et al., 2003; Lhee et al., 2010).

Spectroscopic studies first revealed histidine imidazole nitrogens as well as cysteine sulfurs as ligands for Rieske clusters in Cyt b_6f-bc_1 complexes (Britt et al., 1991; Gurbiel et al., 1991) as well as bacterial mono- and di-oxygenases (Fee et al., 1986; Kuila et al., 1992) in contrast to the four Cys ligation of typical 2Fe-2S ferredoxins, which have much lower midpoint potentials (Tsukihara et al., 1986). Structures of bovine (Iwata et al., 1996) and spinach (Carrell et al., 1997) Rieske ISP soluble fragments and the subsequent structures of Cyt bc_1 (Xia et al., 1997; Iwata et al., 1998; Kim et al., 1998; Zhang et al., 1998) and b_6f (Kurisu et al., 2003; Stroebel et al., 2003) complexes confirmed the unique 2-His, 2-Cys coordination of the 2Fe-2S cluster. Cys105 and

His107 in the CTHLGC 'box I' and Cys123 and His126 in the CPCHGS 'box II' cluster-binding motifs provide the ligands for the 2Fe-2S cluster (Fig. 21.8, see also Carrell et al., 1997 for Rieske ISP sequence alignments). Cys110 and Cys125 (*C. reinhardtii* mature Rieske ISP numbering (de Vitry et al., 1999)) form a disulfide bridge that is conserved among Cyt b_6f-bc_1 Rieske ISPs and stabilizes the cluster domain. The high midpoint potential of Cyt b_6f-bc_1 Rieske clusters has been attributed to: (i) the electronegativity of the His ligands; (ii) solvent exposure of the His-liganded Fe(II) of the 2Fe-2S cluster; (iii) the disulfide near the cluster, and (iv) a network of hydrogen bonds to the sulfur atoms of the cluster and the Cys ligands (Iwata et al., 1996; Link et al., 1996). Some of these ideas are supported by mutational studies. Disruption of the disulfide bridge in the Cyt bc_1 Rieske ISP (Merbitz-Zahradnik et al., 2003) and in an expressed Cyt b_6f Rieske ISP of *C. reinhardtii* (Cys110 to Met substitution; L.-W. Guo, R. Grebe and T. Kallas, unpublished) lowered the midpoint potential of the cluster by 70 to 115 mV and otherwise disrupted the cluster environment and dramatically lowered catalytic activity. Mutations that disrupt the H-bonding network around the cluster similarly lower the ISP midpoint potential by 40 to 130 mV (Denke et al., 1998; Guergova-Kuras et al., 2000; Lhee et al., 2010).

The idea that solvent exposure of Cyt b_6f-bc_1 Rieske ISP clusters contributes substantially to their high midpoint potentials has been largely contradicted by the structures of bacterial ring-hydroxylating oxygenases (Colbert et al., 2000; Elsen et al., 2007). These enzymes carry Rieske 2Fe-2S clusters liganded via 2-His, 2-Cys linkages but have markedly lower Em's of ~ −150 mV to −170 mV that are largely invariant as a function of pH (Couture et al., 2001). They also lack the disulfide bridge of Cyt b_6f-bc_1 Rieske ISPs. Their clusters were thought to be buried within the protein structure. However, the structures of these enzymes reveal that their iron-sulfur clusters are actually as much exposed to the surface as those of Cyt b_6f-bc_1 Rieske ISPs. The much lower midpoint potentials of the oxygenase 2Fe-2S clusters are now, rather attributed to fewer hydrogen-bonding or polar interactions in the cluster environment that could counterbalance the negative charge of the reduced cluster and thereby raise its midpoint potential (Colbert et al., 2000; Hunsicker-Wang et al., 2003; Elsen et al., 2007).

b. Domain Movement During Catalysis

X-ray crystal structures of Cyt bc_1 complexes show that the Rieske ISP comprises a soluble domain tethered via a flexible hinge to a transmembrane, α-helical anchor (Xia et al., 1997; Iwata et al., 1998; Kim et al., 1998; Zhang et al., 1998). Cyt b_6f Rieske ISPs have a similar arrangement within structures of Cyt b_6f complexes (Kurisu et al., 2003; Stroebel et al., 2003) as illustrated in Fig. 21.3. The first Cyt bc_1 structure (Xia et al., 1997) as well as the Cyt b_6f structures (Kurisu et al., 2003; Stroebel et al., 2003) presented a profound enigma in terms of the ~30 Å, center-to-center distance observed between the Rieske ISP cluster and Cyt c_1 (in bc_1 complexes) or Cyt *f* heme (in b_6f complexes) in the high-potential electron transfer chain. Figure 21.10 shows nearest edge-to-edge distances between redox centers in the *M. laminosus* Cyt b_6f dimer (Kurisu et al., 2003). The minimum distance between the 2Fe-2S cluster and heme *f* is still at least 26.4 Å. Empiricial analysis of electron transfer proteins and the resulting Moser-Dutton equation (Moser et al., 1992, 1995; Page et al., 1999) demonstrate that the rate of electron transfer between redox centers depends largely on the edge-to-edge distance between the centers. Rates fall off sharply from a maximum of ~10^{15} to 10^{13} s^{-1} at van der Waals distances (3.6 Å) with a 10-fold decline in rate per each 1.7 Å increase in distance. Accordingly, most electron transfer complexes have evolved such that distances between redox centers rarely exceed 10–15 Å, thus allowing rapid and physiologically meaningful electron transfer rates (Page et al., 1999). In contrast, the predicted electron transfer rate between the Rieske 2Fe-2S cluster and Cyt *f* heme, even at the most optimistic distance of 26.4 Å, would be on the order of 2.5 x 10^{-4} s^{-1} or only ~1 electron per hour! This calculation assumes a reorganization energy (λ) of 1.0 eV for inter-protein electron transfer (Moser et al., 1992) and a driving force of 0.03 eV, reflecting a typical difference of 30 mV between the midpoint potentials of the Rieske ISP cluster and Cyt *f* heme. If actual electron transfer in Cyt b_6f-bc_1 complexes occurred only

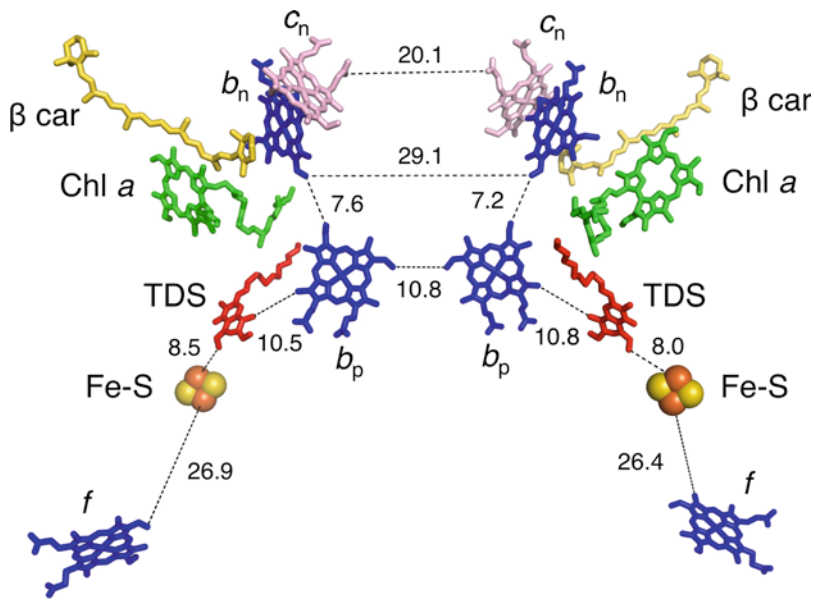

Fig. 21.10. Edge-to-edge distances of redox centers in the cytochrome b_6f dimer. Nearest edge-to-edge distances in Ångstroms are shown between redox centers in the *Mastigocladus laminosus* 1VF5 Cyt b_6f dimer structure (Kurisu et al., 2003). TDS, 'ring-in' conformation, is superposed from structure 2E76 (Yamashita et al., 2007) and serves as a surrogate to indicate the approximate position of plastoquinol (PQH_2) relative to the Rieske 2Fe-2S cluster (*Fe-S*) and heme b_p. Note that TDS, and by implication PQH_2, is actually more closely linked to the Rieske 2Fe-2S cluster than shown because of evidence for hydrogen bonding to one of the ISP cluster-liganding His residues (Stroebel et al., 2003; Yamashita et al., 2007) as illustrated in Fig. 21.8. Note also that the distance between the two b_p hemes of the two monomers is quite small.

once per hour, much of life on Earth as we know it, and any higher forms of life, would be impossible! Fortunately, electron transfer rates in Cyt b_6f complexes (measured as Cyt *f* re-reduction after a single-turnover flash) are considerably faster, on the order of 200–500 s^{-1} with a half-time ($t_{1/2}$) of ~2–5 ms for the rate-limiting plastoquinol-oxidation step (Selak and Whitmarsh, 1982; Hope, 1993; Pierre et al., 1995; Baniulis et al., 2009).

The solution to the distance enigma came from the data of Zhang and coworkers (1998) and the subsequent structures of mitochondrial Cyt bc_1 complexes crystallized in the presence and absence of the Q_p (Q_o)-site inhibitor, TDS and other inhibitors (Iwata et al., 1998; Kim et al., 1998). As discussed already (Section VII.A.3), TDS forms a tight hydrogen bond with one of the cluster-liganding His residues of the Rieske ISP in Cyt b_6f as well as Cyt bc_1 complexes and thereby locks the cluster in a 'proximal' position within a few Ångstroms of the quinol-binding niche in the Q_p pocket (see Fig. 21.8). The structures by Zhang and coworkers (1998) revealed that the Rieske ISP cluster can occupy different positions in the bc_1 complex. Thus the tethered ISP cluster domain undergoes a profound translational and rotational movement of 16 Å and 57° between the 'proximal,' TDS-bound position and a 'distal' position minimally within 8 Å of the Cyt c_1 heme (functionally equivalent to the Cyt b_6f, *f* heme) in the high-potential chain of the Cyt bc_1 complex. Together, the Cyt bc_1 structures from different sources, in the presence and absence of inhibitors, revealed that the Riekse ISP cluster occupies different positions along the trajectory from the Q_p (Q_o) pocket to the acceptor Cyt c_1 heme (Xia et al., 1997; Iwata et al., 1998; Kim et al., 1998; Zhang et al., 1998). These findings suggested a domain movement of the Rieske ISP cluster domain as a mechanism that has evolved to allow physiologically relevant electron transfer rates and bifurcated electron flow into the high- and low-potential chains of 'Rieske-Cyt *b*' complexes. Subsequent mutational (Tian et al., 1998, 1999; Darrouzet et al., 2000b, 2001) and EPR spectroscopic (Brugna et al., 2000) studies of bacterial and mitochondrial Cyt bc_1 complexes

provided kinetic and functional data supporting the Rieske ISP domain movement and its requirement for efficient electron transfer. The flexible hinge domain of the Rieske ISP is essential for the ISP movement and catalytic activity as demonstrated by mutations that modify the length or structure of this region (Darrouzet et al., 2001).

Perhaps surprisingly, crystal structures of Cyt b_6f complexes have not yet been obtained that show the Rieske ISP cluster in radically different positions, regardless of the presence of inhibitors. However, movement of the Rieske ISP cluster domain, or some other re-positioning of redox centers, is clearly as necessary and anticipated in Cyt b_6f as in bc_1 complexes. Indeed, several other lines of evidence support a Rieske ISP movement in Cyt b_6f complexes. EPR studies of two-dimensionally ordered b_6f complexes or thylakoid membranes treated with the Q_p-site inhibitor DBMIB indicate that the Riekse ISP cluster occupies positions close to the Q_p-site in the treated samples and more distant positions in untreated samples (Schoepp et al., 1999; Roberts et al., 2004). EPR analysis of oriented samples treated with inhibitory concentrations of Zn^{2+} or Cu^{2+} ions suggested an intermediate position of the ISP cluster (Roberts et al., 2002). Investigation of low-resolution (10–15 Å) 2D crystals of the *C. reinhardtii* Cyt b_6f complex in the presence and absence of the Q_p-site inhibitor TDS revealed conformational changes of the Rieske ISP as well as extensive conformational changes of other transmembrane helices (Breyton, 2000). Increased lumenal viscosity in thylakoid membrane suspensions reversibly slows Cyt *f* oxidation-reduction kinetics consistent with a necessary movement of the Rieske ISP cluster domain (Heimann et al., 2000). Electron transfer between Rieske ISP and Cyt *f* fragments in vitro is independent of electrostatic binding interactions (Soriano et al., 2002). The measured second-order (concentration dependent) rate constant for this electron transfer in vitro implied unproductively slow electron transfer in vivo unless docking between these partners is constrained as in tethered movement of the ISP cluster along a guided trajectory. Finally, mutational studies of *Synechococcus* sp. PCC 7002 (Yan and Cramer, 2003) and *C. reinhardtii* (de Vitry et al., 1999, 2004) showed that the Cyt b_6f Rieske ISP flexible hinge domain is more permissive of modifications, for example those that increase length or rigidity, than the Cyt bc_1 ISP hinge (reviewed by Darrouzet et al., 2001), but clearly essential for efficient turnover of the Cyt b_6f complex. Deletion of two glycines in the *Synechococcus* sp. PCC 7002 ISP hinge resulted in ~4-fold decrease in flash-induced Cyt f/c_6 reduction kinetics and increased resistance to the Q_p-site inhibitors DBMIB and stigmatellin (Yan and Cramer, 2003). Deletion of three glycines from the ISP hinge in *C. reinhardtii* resulted in a dramatic 10-fold decline in electron flow and severely impaired phototrophic growth (de Vitry et al., 2004).

Taken together, these findings and implications from the Cyt b_6f structures provide compelling evidence for a domain movement of the Cyt b_6f Rieske ISP required for viable energy transduction in photosynthesis. It should be noted, however, that the Cyt b_6f Rieske ISP hinge domains differ considerably from their Cyt bc_1 counterparts. In *Synechococcus* sp. PCC 7002 and *C. reinhardtii*, the 9-residue ISP hinge is comprised of the amino acids SSGGAGGG and SSGGGGGG, respectively. The glycines provide extreme flexibility because they lack any side chain on the α-carbon. Mutagenesis studies show that a minimum of seven residues in this region is required for adequate electron flow and phototrophic growth (Yan and Cramer, 2003; de Vitry et al., 2004). Resistance of the *Synechococcus* sp. PCC 7002 Δ2-residue ISP hinge mutants to Q_p-site inhibitors is thought to arise from constrained access of the 2Fe-2S cluster to the Q_p-pocket for effective hydrogen-bonding with the inhibitors or with PQH_2 for efficient plastoquinol oxidation. In contrast, the corresponding ISP hinge region in Cyt bc_1 Rieske ISPs is comprised of residues SADVKAMA and SADVLAMS in the *R. capsulatus* and bovine complexes, respectively (see Yan and Cramer, 2003, for alignments). These bc_1 ISP hinge domains are much less flexible and more ordered. Indeed, the Cyt bc_1 structures show the ISP hinge domain in discrete configurations. When the Rieske 2Fe-2S cluster is bound to TDS in the Q_p-pocket, the ISP hinge is extended to allow this. When the cluster is in a 'distal' position closer to Cyt c_1 heme, the hinge has shortened to form a helix such that the ISP cluster domain has pivoted away from the Q_p-pocket bringing the 2Fe-2S cluster close to the c_1 heme (see Fig. 1 in Darrouzet et al., 2001). What

controls these conformational changes of the hinge and cluster domain, whether simple diffusion or an active and regulated process, remains an open question. The Cyt b_6f Rieske ISP hinge domain is thus much less ordered than the Cyt bc_1 ISP hinge. Indeed, mutations that alter these regions have quite the opposite effects in these complexes. Shortening the hinge had little impact in bc_1 complexes (Tian et al., 1998; Darrouzet et al., 2000a) but dramatically slowed electron flow in b_6f complexes (Yan and Cramer, 2003; de Vitry et al., 2004). Conversely, increased or decreased flexibility or lengthening the hinge had little impact in b_6f complexes but serious consequences in bc_1 complexes (Tian et al., 1999; Darrouzet et al., 2000a, 2000b; Nett et al., 2000; Xiao et al., 2000). These findings indicate that the Rieske ISP hinge requires a specific combination of length and structure for efficient function of the Cyt bc_1 complex; whereas the Cyt b_6f complex ISP hinge requires only a minimum length and is otherwise largely forgiving of modifications (de Vitry et al., 2004). These differences may arise from constraints imposed by the respective Cyt c_1 or Cyt *f* electron acceptors of the Rieske ISPs (Baniulis et al., 2008). As noted, the Cyt c_1 and Cyt *f* proteins perform analogous functions but are otherwise completely unrelated with the exception of their *c* heme binding motifs. The lumenal domain of Cyt *f* is a largely flat structure comprised of extensive β-sheets regions (Martinez et al., 1994; Chi et al., 2000) that partially wrap around the Rieske ISP cluster domain (see Figs. 21.3 and 21.4). A much smaller rotational movement may be adequate to bring the 2Fe-2S cluster within striking distance of the *f* heme as suggested previously (Cramer et al., 2004).

Domain movement of the Rieske ISP and translation of this movement across the thylakoid membrane to the *n*-side has also been implicated in signaling of PQ pool redox status and antenna protein redistribution via 'state transitions' (Keren and Ohad, 1998; Wollman, 2001; de Vitry et al., 2004) as discussed further below (Section IX.A). The recent, unprecedented discovery by electrospray ionization mass spectrometry of acetylation of the Rieske ISP N-terminus in *Nostoc* sp. PCC 7120 Rieske ISP (Baniulis et al., 2009) raises the interesting question of whether this modification may be part of the Cyt b_6f-mediated redox signaling mechanism in cyanobacteria.

7. Cytochrome f

Cytochrome *f* is the remaining component of the Cyt b_6f high-potential chain. The Cyt *f* heme accepts electrons for the Rieske 2Fe-2S center as discussed above and transfers them to a soluble electron carrier, the blue-copper plastocyanin protein in plant chloroplasts and to either a Cyt c_6 protein or plastocyanin in algae and cyanobacteria (see Kerfeld and Krogmann, 1998; Hope, 2000; Diaz-Quintana et al., 2003 for reviews). As mentioned, the *f* heme, despite its name, is a typical *c*-type heme covalently bound via thioether linkages to a canonical *c*-heme binding motif, generically Cys-X-X-Cys-His and Cys-Ala-Asn-Cys-His in most cyanobacteria and chloroplasts (see Gray, 1992; Ponamarev et al., 2000 for alignments). Cyt *f* hemes have high midpoint potentials that are independent of pH from pH 4–8 (Martinez et al., 1996). Typical potentials ($E_{m,7}$) of Cyt *f* in plant and algal chloroplasts are +340 to +380 mV (Table 21.1) with somewhat lower potentials of +270 to +340 mV in membranes and isolated Cyt *f* proteins of cyanobacteria (Ho and Krogmann, 1980; Ponamarev et al., 2000; Baymann et al., 2001). The cyanobacterial E_m values are in the +230 to +340 mV range of c_1 hemes from bacterial and mitochondrial Cyt bc_1 complexes (see Kallas, 1994). Cyt *f* proteins are targeted to the thylakoid membrane via a cleavable pre-sequence and anchored to the membrane via a single, C-terminal transmembrane helix with the C-terminus of the protein on the *n*-side of the membrane (Choquet et al., 2003). The soluble, heme-binding domain of Cyt *f* is thus anchored to the membrane with the heme bound near the N-terminus of the mature protein (see Figs. 21.3 and 21.4). The other major subunits of the Cyt b_6f have their N-termini on the *n*-side of the membrane.

A soluble fragment of turnip Cyt *f* was the first Cyt b_6f subunit to be resolved by X-ray crystallography (Martinez et al., 1994). This and subsequent high-resolution Cyt *f* structures at 1.9–2.0 Å revealed several unique features (Carrell et al., 1999; Chi et al., 2000). First, the structures displayed a novel ligation of the heme iron. A typical *c*-heme axial ligand is provided by His25 (*C. reinhardtii* numbering) of the heme-binding motif, but the other, highly unusual, axial ligand is the N-terminus of the protein, specifically the

α-amino group of Tyr1 (see Martinez et al., 1994; Chi et al., 2000 for illustrations). Second, the lumenal, extrinsic region consists of two domains, a large heme-bearing domain, and a smaller domain that contains a positive patch thought to be important for plastocyanin docking. Indeed, these positive residues are important for Cyt *f*–plastocyanin interaction in vitro, but surprisingly have no impact on electron transfer in vivo as shown by mutagenesis (Soriano et al., 1998). Together, both domains form an elongated, shallow bowl-like structure comprised predominantly of β-sheets that partially wraps around the Rieske ISP (Figs. 21.3 and 21.4). This protein fold occurs in few other proteins and the N-terminal heme ligation has no known counterparts. The unique Cyt *f* structure exemplifies the theme of 'Rieske-Cyt *b*' complexes where the core Cyt *b* and Rieske ISP components are conserved to differing degrees but the subsequent electron acceptor proteins, for example Cyt *f* and Cyt c_1, may be completely different (Kramer et al., 2008). Third, the Cyt *f* structures revealed a unique, conserved, buried chain of five water molecules hydrogen-bonded to polar amino acid residues. This chain extends from the Cyt *f* heme to a lysine on the protein surface and was postulated as a possible route for release of the second proton from plastoquinol oxidation (Martinez et al., 1994, 1996). The structures of the complete Cyt b_6f complexes (Kurisu et al., 2003; Stroebel et al., 2003), however, render this idea less likely. For thermodynamic reasons discussed below (Section VIII.B.1), the PQH_2 oxidation at the Q_p-site must be initiated by at least a one-proton deprotonation of PQH_2 to $PQH^{\bullet-}$ prior to electron transfer to the Rieske 2Fe-2S cluster. The solvent exposed ISP cluster should be accessible to the lumenal *p*-side aqueous phase, providing a path for release of the first proton. For release of the second proton, the Cyt water chain appears to be too far away. Moreover, the Rieske ISP cluster is rotated away from the Cyt *f* heme. Conceivably a proton could remain on the cluster-liganding His126 (*C. reinhardtii* numbering, de Vitry et al., 2004) and travel with the ISP cluster during its rotational movement for deposition into the Cyt *f* water chain. However, structural support for this hypothesis is currently lacking. Mutagenesis data indicate that the second proton (from PQH•, after one proton deprotonation and one electron oxidation of PQH_2) travels via the carboxylate of Glu78 (*C. reinhardtii* numbering) in the conserved PEWY (Pro-Glu-Trp-Tyr) sequence of the subunit IV ef loop near the Q_p-pocket (Zito et al., 1998). Again there is no obvious connection to the Cyt *f* water chain.

However, mutations in the Cyt *f* water chain do have dramatic phenotypes. Modifications of the *C. reinhardtii* Gln and Asn residues that hydrogen bond with these water molecules resulted in up to 2-fold slower growth and up to 5-fold lower Cyt *f* re-reduction rates after flash excitation (Ponamarev and Cramer, 1998). One mutant, N168F, was non-phototrophic. Most interestingly, reduction rates of the *b* hemes were hardly altered resulting in a de-coupling of the usually tightly coupled electron transfers from PQH_2 into the Cyt b_6f high- (Rieske ISP → Cyt *f*) and low-potential (hemes $b_p \rightarrow b_n \rightarrow c_n$) chains. Expressed Cyt *f* proteins bearing some of these mutations have been crystallized and two with the most severe phenotypes, N168F and Q158L, showed loss or displacement of water molecules (Sainz et al., 2000). The Q158L modification showed an $E_{m,7}$ of +294 mV, ~80 mV lower than the +373 mV potential of the native Cyt *f*. Since a role for the water chain in coupled electron-proton transfer is no longer evident, the reasons for the severe phenotypes of the N168F and Q158L mutants are not clear. However, both resulted in loss of hydrogen bonding and polarity in the water-chain region near heme *f* (Sainz et al., 2000). This might explain the lower E_m and phenotype of the Q158L mutant. There is no obvious explanation for the observed de-coupling of electron flow into the high- and low-potential chains. An intriguing possibility is that impeded electron flow to Cyt *f* in the high-potential chain might allow 'bypass' reactions where part of the initial electron flow from PQH_2 goes directly to plastocyanin and more slowly to Cyt *f*. This could then allow a still rapid overall reduction of the *b* hemes (in the low-potential chain) from the rapidly formed fraction of $PQH^{\bullet-}$ semiquinone. A more rapid reduction of the *b* hemes relative to Cyt *f* has been observed under other conditions that impede the high-potential chain (Selak and Whitmarsh, 1982; Heimann et al., 2000) and some evidence suggesting a direct 'Cyt *f* bypass' reduction of plastocyanin has been presented (Fernandez-Velasco et al., 2001). It is not clear, however, from available Cyt b_6f

structures or anticipated ISP domain movement that a direct Rieske ISP 2Fe-2S cluster–plastocyanin Cu-center interaction could occur at a distance that might allow a reasonable rate of electron transfer.

Notably, Cyt *f* from chloroplasts and cyanobacteria typically have somewhat different midpoint potentials and Soret (γ) and α-band absorbance maxima (reviewed in Soriano et al., 2001). Cyt *f* of most plants and algae have midpoint potentials of +340 to +380 mV and α-band maxima at ~554 nm whereas those of most cyanobacteria have $E_{m,7}$ of +270 to +330 mV and red-shifted α-band maxima at ~556 nm (Ho and Krogmann, 1980; Soriano et al., 2001) (and see also Ponamarev et al., 2000 and references therein). The molecular basis for this fine-tuning of midpoint potential and spectral properties is now quite well understood. As mentioned, the α-amino group of the N-terminal amino acid, Tyr or Phe, provides an axial ligand for the *f* heme (Martinez et al., 1994). However, an N-terminal aromatic amino acid is not essential as shown by a Tyr1 to Ser substitution in *C. reinhardtii* that had only a modest impact on Cyt *f* reduction kinetics and none on growth (Zhou et al., 1996). Another aromatic side chain, Tyr or Phe at position 4 in chloroplasts, and Trp in cyanobacteria, lies perpendicular to the heme plane ~3.5 Å away. A Phe4 to Trp mutation in *C. reinhardtii* made the chloroplast Cyt *f* more cyanobacterial with an α-band maximum at 556 nm and a drop in potential of ~40 mV. The converse Trp4 to Phe mutation in *Phormidium laminosum* made the cyanobacterial protein more like the chloroplast Cyt *f* with an α-band maximum at 553.7 nm and a higher midpoint potential (Ponamarev et al., 2000). Changes in the expected directions also occurred in the Soret (γ) band absorbance spectra of the cytochromes. These differences are explained in terms of greater interactions of the σ and π orbitals of the larger, Trp indole side-chain with the heme π orbitals resulting in lower excited state energy reflected as a spectral red shift and stronger electrostatic repulsive interactions resulting in a lower midpoint potential (Ponamarev et al., 2000). The conserved Pro at position 2 in the Cyt *f* structure serves a crucial role, presumably by forming a hydrophobic pocket for the heme together with Phe4, Pro117, and Pro161. Mutation of Pro2 to Val in *C. reinhardtii* resulted in a ten-fold decline in Cyt *f* reduction kinetics and two-fold slower growth (Zhou et al., 1996). The impact of this mutation on Cyt *f* spectral and redox properties has not been examined.

B. Small Subunits and Associated Proteins

As mentioned, each monomer of the Cyt b_6f complex contains four membrane-spanning small subunits, PetG, PetL, PetM, and PetN in addition to the core Rieske ISP, Cyt b_6–subunit-IV, and Cyt *f* subunits. Historically, various small polypeptides have copurified with Cyt b_6f preparations (reviewed in Kallas, 1994). The first one convincingly associated with the b_6f complex was PetG (originally designated PetE), a 4 kDa protein that copurified with the maize chloroplast b_6f complex together with other small proteins (Haley and Bogorad, 1989). Antibodies against PetG cross-reacted with polypeptides from several plant species and the *petG* gene was identified in several chloroplast genomes and the cyanelle genome of *Cyanophora paradoxa* (Stirewalt and Bryant, 1989). Additional small subunits were confirmed in Cyt b_6f complexes isolated from plant and algal sources (Schmidt and Malkin, 1993; Pierre et al., 1995). Three small subunits of ~4 kDa each, designated PetG, PetL, and 'PetX,' were identified in a highly resolved and active, *C. reinhardtii* Cyt b_6f preparation (Pierre et al., 1995). PetX was subsequently designated PetM to conform to the "*pet*" (photosynthetic electron transport) nomenclature for Cyt b_6f genes and proteins and is encoded by a nuclear gene (de Vitry et al., 1996). Disruption of the *C. reinhardtii* chloroplast *petG* gene led to markedly diminished levels of the major Cyt b_6f subunits and loss of phototrophic growth demonstrating a role for PetG in assembly or stability of the b_6f complex (Berthold et al., 1995). Takahashi et al. (1996) subsequently demonstrated by mutagenesis of the chloroplast *petL* gene that PetL is a bonafide subunit of the Cyt b_6f complex. Disruption of *petL* (*ycf7*) resulted in lower accumulation and destabilization of Cyt b_6f complexes, particularly in stationary phase cells (shown also by Zito et al., 2002), with a 3–4 fold lower rate of transmembrane charge transfer in vivo. PetL deletion in tobacco chloroplasts had a less severe phenotype with no impact on biogenesis but loss of Cyt b_6f stability in mature leaves (Fiebig et al., 2004; Schottler et al., 2007). Recent evidence from

tobacco indicates that PetL stabilizes the Rieske iron-sulfur protein and thus the Cyt b_6f dimer (Schwenkert et al., 2007). The work of Hager and colleagues (Hager et al., 1999) led to identification of the 29 amino acid PetN subunit. These authors inactivated the tobacco chloroplast *ycf6* gene, which resulted in complete loss of the Cyt b_6f complex and photosynthetic electron transport. Purification of Cyt b_6f complexes from spinach and characterization by MALDI-TOF mass spectrometry identified a mass of 3169.87 Da, essentially identical to the predicted mass of the *ycf6* gene product. This gene was renamed *petN* and thus encodes PetN, the eighth subunit of the Cyt b_6f complex.

The occurrence of the four low-molecular-mass subunits, PetG, PetL, PetM, and PetN, in Cyt b_6f complexes of both chloroplasts and cyanobacteria was confirmed by electrospray ionization (ESI) mass spectrometry of highly-active complexes isolated from spinach and *M. laminosus* (Whitelegge et al., 2002) and by the Cyt b_6f structures from *C. reinhardtii* (Stroebel et al., 2003) and *M. laminosus* (Kurisu et al., 2003). The masses of these subunits range from ~3,200 to ~4,200 Da with masses determined by ESI mass spectrometry within 1 mass unit of masses predicted from sequence data with the exception of PetG from *M. laminosus* for which sequence data were not available (Whitelegge et al., 2002). The mass spectrometry data and Cyt b_6f structures resolved the question of whether b_6f complexes of cyanobacteria and chloroplasts have the same small subunits or some that are unique. A *petL* gene was not annotated in the first cyanobacterial genome sequence (that of *Synechocystis* sp. PCC 6803) because of low similarity with chloroplast PetL subunits (Kaneko et al., 1996). As noted, PetG and PetN, and to a lesser extent PetL, have roles in Cyt b_6f complex assembly or stability in chloroplasts (Berthold et al., 1995; Takahashi et al., 1996; Hager et al., 1999; Schwenkert et al., 2007). Similarly, PetG and PetN could not be deleted from *Synechocystis* sp. PCC 6803 whereas PetL could, indicating essential roles most likely in assembly or stability of the Cyt b_6f complex for PetG and PetN but not PetL (Schneider et al., 2007). Inactivation of the *Synechocystis* sp. PCC 6803 PetM subunit had little impact on assembly or catalytic activity of the Cyt b_6f complex (Schneider et al., 2001). However, the PetM deletion did result in a more oxidized PQ pool and somewhat lower PS I and phycobilisome content. These findings suggest an involvement of PetM in Cyt b_6f-mediated sensing of PQ pool redox status and regulation of photosynthesis. PetM has apparently not yet been inactivated in plants or algae. Thus the available data indicate that PetG and PetN are essential for assembly or stability of Cyt b_6f complexes both in chloroplasts and cyanobacteria, whereas PetL and PetM are less important. Note that the initial Cyt b_6f structures assigned different locations to the small subunits within the peripheral 4-helix bundle. Because functional studies show that PetL and PetM are not essential, they most likely lie at the outside of the bundle as in the *C. reinhardtii* structure (Stroebel et al., 2003). More recent *M. laminosus* structures (e.g., Yamashita et al., 2007) as well as the *Nostoc* sp. PCC 7120 structure (Baniulis et al., 2009) are compatible with these assignments, which are displayed in Figs. 21.3 and 21.4.

Concerning possible roles in regulation, fusion of the N-terminus of PetL to the C-terminus of subunit-IV on the lumenal, *p*-side of the *C. reinhardtii* b_6f complex did not impair assembly or catalysis but did result in loss of 'state transitions,' the redistribution of light-harvesting proteins in response to the level of reduction of the PQ pool (Zito et al., 2002). Deletion of PetL itself did not impair state transitions in *C. reinhardtii* (Zito et al., 2002) nor tobacco chloroplasts (Schottler et al., 2007). The molecular mechanism of redox signaling of state transitions is not understood in detail but requires the Cyt b_6f complex with PQH_2 binding to Q_p-site (Vener et al., 1997; Zito et al., 1999) and transmembrane signal transduction that in chloroplasts results in activation of an LHCII kinase and phosphorylation of LCHII antenna proteins (see Wollman, 2001; Eberhard et al., 2008; Lemeille and Rochaix, 2010 for reviews). The *n*-side, Q_n-domain of the Cyt b_6f complex is the postulated binding site for the LHCII kinase (Stroebel et al., 2003). Direct involvement of PetL in *n*-side kinase binding and activation is unlikely because ΔPetL mutants retain state transitions as mentioned above. More plausibly, the *p*-side fusion of PetL with subunit-IV inhibited transmembrane conformational changes required for LHCII kinase activation. Subunit-IV itself has been implicated in kinase binding and activation (Zito et al., 2002).

This hypothesis requires further testing, but clearly the PetL-subunit-IV fusion prevented phosphorylation of LHCII as well as of PetO (Zito et al., 2002). PetO is another small protein found peripherally associated with the *C. reinhardtii* Cyt b_6f complex (Hamel et al., 2000). PetO becomes phosphorylated upon PQ pool reduction (so-called 'state 2' conditions) and is implicated in phosphorylation and activation of the LHCII state-transition kinase. PetO is not found in Cyt b_6f complexes, nor encoded in cyanobacterial genomes, and its absence from current Cyt b_6f structures suggests a loose association with the chloroplast b_6f complex. However, an important regulatory or structural role of PetO is indicated by its presence in the cyclic electron transfer PS I–FNR–Cyt b_6f supercomplex recently isolated from *C. reinhardtii* under 'state 2' conditions (Iwai et al., 2010). The Cyt b_6f complex and redox-mediated signaling of state transitions is discussed further below (Section IX.A).

Mass spectrometry has also revealed the co-purification of FNR with the spinach Cyt b_6f complex but not with the cyanobacterial *M. laminosus* complex (Whitelegge et al., 2002; Zhang et al., 2001). This, and inferences from the Cyt b_6f structures for a possible Fd or FNR binding site on the *n*-side of the b_6f complex, suggested a direct cyclic electron flow path from the reducing-side of PS I via Fd and FNR to the Q_n-pocket of the Cyt b_6f complex as previously postulated (see Cramer et al., 1991; Vallon et al., 1991; Kallas, 1994; Zhang et al., 2001 for discussions). In contrast, the absence of FNR from crystal structures of the *M. laminosus* and *C. reinhardtii* Cyt b_6f complexes and its absence from any other isolated Cyt b_6f complex cast doubt on the nature of the FNR–Cyt b_6f association. However, the recent isolation of a PS I–FNR–Cyt b_6f–PGRL1 supercomplex capable of PS I driven cyclic electron flow now confirms the existence of a direct PS I–Cyt b_6f pathway and functional association of FNR with the Cyt b_6f complex under at least some conditions (Iwai et al., 2010). Interesting questions remain as to the nature and regulation of the PS I–FNR–Cyt b_6f association, whether these complexes are transient or stable, and whether the FNR–Cyt b_6f association occurs in cyanobacteria.

Another loosely-associated protein, the 7.2 kDa Ssr2998, has been discovered by co-purification with the Cyt b_6f complex of *Synechocystis* sp. PCC 6803 under mild extraction conditions (Volkmer et al., 2007). The gene for Ssr2998 is highly conserved among cyanobacteria but has not been found in plants or algae. Disruption of Ssr2998 resulted in a lower PS II/PS I ratio, increased reduction of the PQ pool, ~2-fold slower Cyt *f* and P700 (PS I) re-reduction after flash illumination, and loss of state transitions. Ssr2998 is not essential for assembly and activity of cyanobacterial Cyt b_6f complexes as demonstrated by its absence from the highly active, dimeric *M. laminosus* (Zhang et al., 2003) and *Nostoc* sp. PCC 7120 (Baniulis et al., 2009) complexes used for X-ray crystallography. However, the ΔSsr2998 mutant could not grow mixotrophically (photosynthetic illumination in the presence of 10 mM glucose) under any condition nor photoheterotrophically (10 mM glucose and PS II blocked by the addition of DCMU) at a 'high' light intensity (80 μmol photons m^{-2} s^{-1}). These findings suggest that the Ssr2998 mutant cannot respond to conditions resulting in over-reduction of the PQ pool – such as when both PS II and dehydrogenases are active during mixotrophy. Thus the work described in Volkmer et al. (2007) points to an important regulatory role of the Ssr2998 protein in redox-sensing and signal transduction in Cyt b_6f complexes of cyanobacteria. The transient association of Cyt b_6f complexes with peripheral proteins under specific growth or environmental conditions for regulatory or catalytic purposes has been relatively little explored but clearly such strategies have begun to yield interesting findings (Hamel et al., 2000; Volkmer et al., 2007; Iwai et al., 2010).

C. The Mysterious Chlorophyll and Carotenoid Molecules

As mentioned, one chlorophyll *a* (Huang et al., 1994; Pierre et al., 1997) and one carotenoid molecule (Zhang et al., 1999) per monomer (see Figs. 21.3 and 21.4) are conserved features of Cyt b_6f complexes. The typically encountered 9-*cis* β-carotene is replaced by the carotenoid echinenone in some cyanobacterial b_6f complexes as in *Synechocystis* sp. PCC 6803 (Boronowsky et al., 2001). Mutagenesis of *Synechocystis* sp. PCC 6803 echinenone biosynthesis genes resulted in replacement of echinenone with another

carotenoid underscoring the necessity for some carotenoid in the Cyt b_6f structure (Wenk et al., 2005). The role of these pigment molecules in an electron transfer complex that does not require light for its activity remains unresolved, although several hypotheses have been put forth. There is no evidence for direct participation of the chlorophyll in Cyt b_6f redox reactions. Both the ground and excited-state potentials of chlorophyll *a* are very different from those of the Cyt b_6f complex redox centers, the chlorophyll spectrum is not sensitive to the redox state of the complex, and chlorophyll bleaching does not impair Cyt b_6f turnover in vitro (Pierre et al., 1997). The chlorin ring of the chlorophyll rests close to the periphery of the Cyt b_6f complex between helices F and G of subunit-IV and its phytyl tail extends into the Q_p-pocket and out from the Q_p-entry portal into the quinone-exchange cavity (see Figs. 21.3, 21.4, 21.6, and 21.8). Because of this arrangement, it has been suggested that the chlorophyll may 'respond' to quinone binding in the Q_p-pocket and serve in transmembrane signaling of PQ pool redox status (Stroebel et al., 2003). Alternatively, the chlorophyll may, through the serendipity of evolution, perhaps as a relic of an ancestral photo-active complex, simply perform a structural role that might otherwise be served by a lipid (Pierre et al., 1997; Kurisu et al., 2003). Perhaps the chlorophyll still performs a photo-responsive function, perhaps in light-dark conformational changes that tune catalytic or signaling activities, for example in cyanobacteria where the Cyt b_6f complex functions in both photosynthesis and dark respiration. Spectroscopic characterizations of the *Synechocystis* sp. PCC 6803 Cyt b_6f complex indicate a coupling of chlorophyll *a* spectral responses with heme redox changes consistent with a dynamic role for the pigment (Wenk et al., 2005).

The chlorophyll porphyrin (chlorin) ring is surrounded by several aromatic amino acids of helices F and G of subunit-IV and lies ~5.5 Å edge-to-edge from heme b_n. Yan et al. (2008) have mutated some of these residues in *Synechococcus* sp. PCC 7002. Some of these modifications resulted in a lower content of chorophyll *a* and heme b_n in isolated, monomeric Cyt b_6f complexes consistent with a role for chlorophyll in stabilizing the complex and heme b_n. Surprisingly, none of the mutations resulted in a significant decline in electron transfer in vivo nor growth rates under the conditions tested (Yan et al., 2008). Surprisingly as well, mutation of Cyt b_6-Trp125 to Leu, which lies closest to heme b_n, increased the rate of Cyt f/c_6 reduction by ~4 fold. The impact of these mutations on signal transduction has not been tested.

Chlorophyll molecules in protein complexes are typically associated with nearby carotenoid molecules that lie within ~4 Å and quench the excited triplet state of chlorophyll which could otherwise interact with molecular oxygen to generate damaging singlet oxygen species (Dashdorj et al., 2005 and references therein). Indeed, each monomer of the Cyt b_6f complex contains a carotenoid but at a distance of ~14 Å from the chlorophyll (Kurisu et al., 2003; Stroebel et al., 2003), seemingly too great for effective triplet quenching. One ring of the carotenoid lies quite close (~6 Å) to heme c_n near the Q_n-pocket and the other end protrudes out of the complex, between the PetG and PetM helices (Figs. 21.3 and 21.4). The carotenoid has been described as the 'toothpick' in an 'hors d'ouevres' model of the Cyt b_6f complex with a possible role in assembly and stability (Cramer et al., 2005). Proximity to the Q_n-pocket and penetration of the other carotenoid ring into the lipid bilayer have also suggested a role in signal transduction (Stroebel et al., 2003). The chlorophyll triplet dilemma is explained in part by the unusually short ~200 ps singlet excited state lifetime of the chlorophyll *a* that greatly minimizes the probability of subsequent chlorophyll triplet formation (Peterman et al., 1998; Dashdorj et al., 2005). The local protein environment and perhaps aromatic residues in the vicinity of the chlorin ring are proposed to facilitate this rapid quenching. Moreover, an unexpectedly rapid energy transfer of <8 ns has been observed from the chlorophyll *a* excited triplet to the distant carotenoid, seemingly in violation of energy transfer theory (Kim et al., 2005). Again, an unusual protein structure may accommodate this. The authors propose that an intervening molecule between the chlorophyll and carotenoid, perhaps a trapped oxygen, serves as a mediator for the triplet energy transfer.

D. Alternative Rieske Iron-Sulfur Proteins

As mentioned, the *Synechocystis* sp. PCC 6803 genome sequence revealed three genes, sll1316

(*petC1*), slr1185 (*petC2*) and sll1182 (*petC3*), for Rieske ISPs each bearing a signature C-T-H-X-G-C and C-P-C-H-G-X Rieske 2Fe-2S cluster binding motif. With the exception of distinct genes for mitochondrial Riekse ISPs (see Schneider and Schmidt, 2005) and possible exceptions of modestly different cDNAs and isoelectric variants of tobacco (Madueno et al., 1992) and spinach (Yu et al., 1994) chloroplasts, plants and algae do not have known, alternative chloroplast Rieske ISPs. Moreover, the isoelectric variants that have been observed might arise from oxidatively damaged PetC1 Rieske ISPs, for example via carbonylation modifications (Ballesteros et al., 2001), or might reflect modifications, such as the recently reported acetylation of the *Nostoc* sp. PCC 7120 PetC1 ISP (Baniulis et al., 2009) with potentially interesting regulatory roles. Isoelectric variants of the *Synechocystis* sp. PCC 6803 PetC1 Riekse ISP have also been detected (our preliminary data, Schettle and Kallas, 2007). In contrast, three or even four genes, e.g., in *Nostoc* sp. PCC 7120, for different Rieske ISP proteins are typical of cyanobacteria although some cyanobacteria, e.g., *Thermosynechococcus elongatus*, have only a single *petC* gene (see Schneider and Schmidt, 2005 for a review). As noted, many other microbes, both photosynthetic and non-photosynthetic, also have multiple genes for high- or low-potential Rieske ISPs (again, see Schneider and Schmidt, 2005). The functions of the different ISPs are usually not understood but specific physiological roles are presumed.

All three Rieske ISP genes of *Synechocystis* sp. PCC 6803 are expressed to varying degrees at the transcriptional level (Houot et al., 2007; Summerfield et al., 2008; Tsunoyama et al., 2009) (also, S. J. Ranade, M. E. Nelson and T. Kallas, unpublished) and the encoded proteins detected by specific antibodies (Schneider et al., 2002; Schultze et al., 2009) and as GFP fusions (Aldridge et al., 2008). The PetC1 and PetC2 Rieske ISPs have 44% amino acid sequence identity and similar overall structures with masses of ~19 kDa. Both have N-terminal, membrane-spanning alpha helices linked via flexible hinge regions to cluster-binding domains that share a high degree of sequence similarity. The PetC3 ISP is smaller (~14 kDa), lacks the N-terminal helix, and has lower sequence similarity to either PetC1 or PetC2. The PetC1 ISP has a midpoint potential of +320 mV typical of Cyt b_6f Rieske ISPs and PetC2 is assumed to have a similar potential based on its cluster-binding and surrounding sequences. PetC3 has a much lower potential of ~+135 mV (Schneider et al., 2002). Thus PetC3 has neither the structure nor appropriate midpoint potential to catalyze effective plastoquinol oxidation in a typical Cyt b_6f complex. A possible role in oxidation of menaquinone or other lower-potential quinones has been suggested (Schneider et al., 2002; Schneider and Schmidt, 2005). However, there is currently no evidence for such quinones as pool quinones in cyanobacteria. Recent GFP fusion (Aldridge et al., 2008) and immunoblot (Schultze et al., 2009) analyses of *Synechocystis* sp. PCC 6803 place the PetC3 Rieske ISP exclusively in the cytoplasmic membrane. The latter study further reports that subunits of the Cyt b_6f complex are found only in the thylakoid membrane suggesting that PetC3 functions independently of the Cyt b_6f complex in an as yet uncharacterized cytoplasmic membrane electron transport chain.

All three Rieske ISPs of *Synechocystis* sp. PCC 6803 have been deleted either singly or in pairs (Schneider et al., 2004). PetC2 or PetC3 deletions individually or in combination resulted in essentially wild type phenotypes under typical growth conditions. Note again that the Cyt b_6f complex is required for both photosynthetic and respiratory growth of cyanobacteria and inactivating mutations result either in loss of viability or non-segregant genotypes (Osiewacz, 1994; Lee et al., 2001). A double PetC1/PetC2 deletion mutant could not be propagated. Inactivation of PetC1 generated a strain impaired in electron transfer kinetics and growth. These findings show that PetC1 is the major Rieske ISP of Cyt b_6f complexes but that PetC2 can replace PetC1 and function to some extent in a PetC2-Cyt b_6f complex (Schneider et al., 2004). Indeed, we find that the ΔPetC1 mutant grows 2–3 fold slower and with Cyt f/c_6 re-reduction kinetics after single-turnover flash excitation ~50 fold slower than the wild-type control (M. E. Nelson, G. Finazzi and T. Kallas, unpublished). However, closer examination has revealed that Cyt *f* gene expression (S. J. Ranade, M. E. Nelson and T. Kallas, unpublished), Cyt *f* protein determined by immunoblot analysis (Tsunoyama et al., 2009), and Cyt *f* content determined by the amplitude of

the flash-induced Cyt *f* oxidation signal in vivo (S. J. Ranade and T. Kallas, unpublished) are all considerably lower in the ΔPetC1 mutant than the wild type. Subunit-IV protein content is also lower but Cyt b_6 similar in the ΔPetC1 mutant relative to wild type (Tsunoyama et al., 2009). The Cyt b_6 reduction signal detected by flash-excitation is similar in amplitude in ΔPetC1 relative to the wild type although the *b*-heme oxidation rate is considerably slowed (S. J. Ranade and T. Kallas, unpublished). These data suggest that the PetC2-Cyt b_6f complex in the ΔPetC1 mutant is highly unusual, consisting primarily of the core Rieske and Cyt b_6 subunits of a primordial 'Rieske-Cyt *b*' complex. That the extent and rate of *b*-heme reduction is similar in the ΔPetC1 mutant and wild type is further surprising. This raises interesting questions as to the pathway of electrons reaching the low-potential chain *b*-hemes in a PetC2-Cyt b_6f complex that is severely depleted in Cyt *f*, an essential electron carrier in the high-potential chain.

The findings of Tsunoyama et al. (2009) and our kinetics data (S. J. Ranade and T. Kallas, unpublished) further suggest that the slow turnover of the PetC2-Cyt b_6f complex and phenotype of the ΔPetC1 mutant arise not from a less effective PetC2 Rieske ISP but rather from a low content of Cyt *f* and correspondingly low content of functional Cyt b_6f complex relative to PS I. Upon flash-induced oxidation of PS I, multiple turnovers of the PetC2-Cyt b_6f complex are thus required to re-reduce the PS I, P700 reaction center, resulting in a slow apparent rate of Cyt *f* re-reduction. We have tested this by lowering the intensity of the actinic excitation light thus minimizing the number of oxidized PS I centers and number of Cyt b_6f turnovers required to re-reduce PS I. Indeed, under these conditions the Cyt *f* re-reduction rate in the *Synechocystis* sp. PCC 6803 ΔPetC1 mutant speeds up and begins to converge with that of the wild-type control (S. J. Ranade and T. Kallas, unpublished). These data suggest that the PetC2 Rieske ISP may be every bit as effective catalytically as the major PetC1 Rieske ISP. This still leaves unanswered the question of the specific role of the PetC2 Rieske ISP. Expression of *petC2* is upregulated ~4 fold in *Synechocystis* sp. PCC 6803 under low oxygen tension (Summerfield et al., 2008) or high light-intensity (Tsunoyama et al., 2009) and ~4-fold and ~8-fold in the presence of zinc and hydrogen peroxide, respectively (Houot et al., 2007). These data suggest a possible role for PetC2 under semi-aerobic or stress conditions that impede turnover of the Cyt b_6f complex and lead to increased reduction of the PQ pool. Zn and Cu are inhibitors of the Cyt b_6f complex proposed to impede the movement of the Rieske ISP (Roberts et al., 2002). Whether the PetC2-Cyt b_6f complex confers increased resitance to Q_p-site inhibitors has not been tested.

A final clue to possible PetC2 and PetC3 functions comes from the work of Tsuyonama et al. (2009). *Synechocystis* sp. PCC 6803 wild type and ΔPetC1 mutant cells stop growing when shifted from a low to a high light-intensity. However, ΔPetC2 and ΔPetC3 mutants continue to grow at the elevated light-intensity at a rate comparable to their growth rate and that of the wild type at low light-intensity. There is not yet a clear explanation for this phenomenon, but the absence of the PetC2 or PetC3 Rieske ISPs may help prevent over-reduction of the PQ pool and suggest that these proteins may serve regulatory roles.

VIII. Reaction Mechanisms

A. Electron Donors, Acceptors and Inhibitors

As discussed above, plastoquinol (PQH_2) from the PQ pool is the typical electron donor to the Cyt b_6f complex. Structures of the reduced (PQH_2) and oxidized (PQ) forms of plastoquinone were shown in Fig. 21.7. In addition, it has now become evident that the much anticipated direct, cyclic electron transfer pathway from PS I to the Cyt b_6f Q_n-domain (Kurisu et al., 2003; Stroebel et al., 2003) exists and operates at least under some conditions (Iwai et al., 2010). In this case electrons are presumably transferred from FNR or Fd to either the c_n heme, PQ, or a plastosemiquinone ($PQH^{\bullet-}$) in the Q_n-pocket.

Known electron acceptors of Cyt b_6f complexes are plastocyanin in plants and cytochrome c_6 and/or plastocyanin in cyanobacteria and algae with midpoint potentials ($E_{m,7}$) of +310 to +360 mV (see Ho and Krogmann, 1980; Kerfeld and Krogmann, 1998; Merchant and Dreyfuss, 1998 for reviews). Genes for additional c_6-like cytochromes

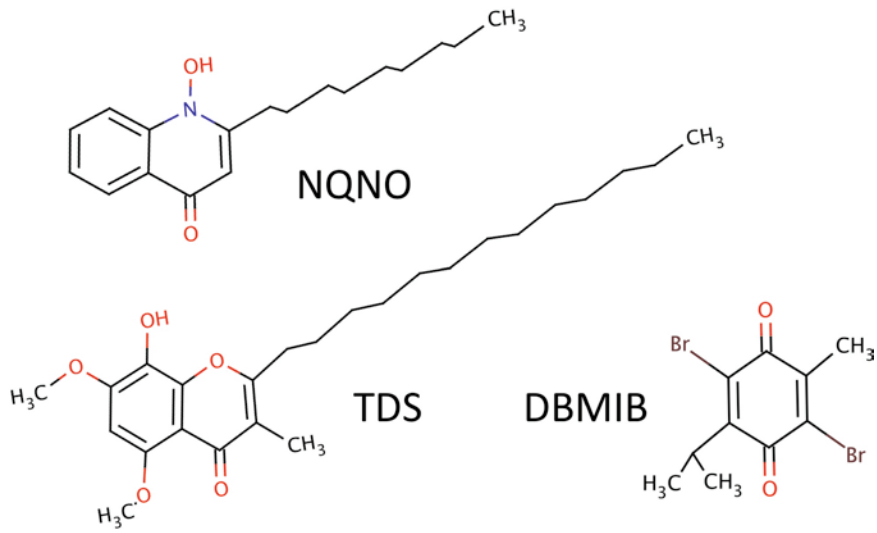

Fig. 21.11. Three widely used inhibitors of the cytochrome b_6f complex. Shown are the plastoquinone reduction (Q_n*-site*) inhibitor NQNO (2n-nonyl-4-hydroxy-quinoline-N-oxide) and the plastoquinol oxidation (Q_p*-site*) inhibitors TDS (tridecyl-stigmatellin) and DBMIB (2,5 dibromo-3-methyl-6-isopropyl-p-benzoquinone). Structure coordinates were obtained from NCBI PubChem (http://pubchem.ncbi.nlm.nih.gov) and images created with MarvinView, ChemAxon (http://www.chemaxon.com) as in Fig. 21.7.

of chloroplasts and cyanobacteria have also been discovered, but these have lower midpoint potentials in the +150 mV range and would thus be poor electron acceptors for the Cyt b_6f complex, and their roles in electron transport or regulation have not yet been defined (see Wastl et al., 2002; Bialek et al., 2008; Worrall et al., 2008 and references therein).

As noted, specific inhibitors have been instrumental in defining the plastoquinone binding pockets and activities of the Cyt b_6f complex. Most of these inhibitors are quinone analogs although certain metal ions, such as Cu^{2+} and Zn^{2+} ions are also effective inhibitors (Roberts et al., 2002). The structures of three widely used inhibitors are shown in Fig. 21.11. NQNO and the related HQNO (2n-heptyl-4-hydroxy-quinoline-N-oxide) are the only known inhibitors that effectively bind in the Q_n-pocket (Fig. 21.9). NQNO impedes plastoquinone reduction and causes electrons to back up in the low-potential chain but only partially slows turnover of the Cyt b_6f complex (Selak and Whitmarsh, 1982; Jones and Whitmarsh, 1988). TDS (Thierbach et al., 1984; Ohnishi et al., 1988) and DBMIB (Trebst et al., 1970) are potent inhibitors of plastoquinol oxidation in the Q_p-pocket. Structures of Cyt b_6f complexes have been solved with these inhibitors bound (Stroebel et al., 2003; Yan et al., 2006; Yamashita et al., 2007) as shown for NQNO and TDS in Figs. 21.8 and 21.9. As noted, TDS at high concentration also binds in the Q_n-pocket (Yamashita et al., 2007). The Q_p- and Q_n-sites both bind quinones but have evolved to preferentially bind reduced or oxidized quinones, and thus different forms of inhibititors based on subtle molecular differences. This is illustrated by a precursor of NQNO synthesis, 4(1H)-quinolone (4-1HQ) that differs from NQNO by only an -H rather than an -OH on the ring nitrogen (see Fig. 21.11). 4-1HQ, in contrast to NQNO, is an effective inhibitor of quinol oxidation at the Q_p-site rather than an inhibitor of quinone reduction at the Q_n-site (A. Ott, S. Zulegar, J. Coplien, V. Johnson, B. Kedrowski, L. Xie and T. Kallas, unpublished). The inhibitor specificities of Cyt b_6f and bc_1 complexes differ markedly and their modes of action have been discussed in several reviews (Hauska et al., 1983; Kallas, 1994; Cramer et al., 1996; Berry et al., 2000; Darrouzet et al., 2004; Trebst, 2007; Yamashita et al., 2007).

B. Plastoquinone Pool, Q-cycle, and High- and Low-Potential Electron Transfer Chains

1. Quinol-Oxidation at the Q_p Site and Bifurcated Electron Flow

A consensus has largely emerged that Cyt b_6f complexes, like Cyt bc_1 complexes, typically operate according to a 'Q-cycle' mechanism as proposed by P. Mitchell (1975, 1976) with some modifications (Crofts et al., 1983; Crofts, 2004c). As noted, two binding sites for plastoquinol are required, a plastoquinol (PQH_2) oxidation or Q_p-site on the *p* side of the thylakoid membrane and a plastoquinone (PQ) reduction or Q_n-site on the *n* side (see Fig. 21.1 for reference). The structure and mechanism of PQH_2 oxidation at the Q_p-site is highly conserved among Cyt b_6f-bc_1 complexes. The overall quinone reduction function of the Q_n-sites of Cyt b_6f-bc_1 complexes is conserved as well, but the structures of the Q_n-sites and quinone-reduction reaction mechanisms of Cyt b_6f and bc_1 complexes differ radically. Mitchell's Q-cycle explains the phenomenon of 'oxidant-induced reduction' of the *b* hemes. According to this model, PQH_2 oxidation at the Q_p-site is triggered by oxidation of the Cyt b_6f high-potential chain and Rieske ISP center, for example by flash-induced oxidation of PS I (see again Fig. 21.1). This initiates a bifurcated oxidation of PQH_2 at the Q_p-site where the first electron from PQH_2 flows into the Cyt b_6f high-potential chain (Riekse ISP $\rightarrow$ Cyt *f* $\rightarrow$ Cyt c_6 or PC $\rightarrow$ PS I) and the second electron flows into the Cyt b_6f low-potential chain (heme $b_p \rightarrow$ heme $b_n \rightarrow$ heme c_n). The first step results in transfer of one electron to PS I via the high-potential chain, the second electron is thought to remain on heme c_n (see Sections VII.A.4 and VII.A.5), and two protons from PQH_2 are released into the thylakoid lumen. A second oxidation of the Cyt b_6f high-potential chain triggers the oxidation of a new PQH_2 molecule at the Q_p-site. Again the first electron flows into the high-potential chain and the second electron into the low-potential chain, but now the second electron joins the electron already on heme c_n in a concerted two electron reduction of a PQ molecule at the Q_n-site. Reduction of PQ to PQH_2 at the Q_n-site releases PQH_2 into the quinone exchange cavity of the Cyt b_6f complex or the PQ pool (see Fig. 21.6). Thus two turnovers of the Cyt b_6f complex result in: (i) oxidation of two PQH_2 molecules at the Q_p-site; (ii) the movement of two electrons through the high-potential chain to PS I; (iii) the movement of two electrons across the membrane to regenerate one PQH_2 molecule at the Q_n-site, and (iv) uptake of two protons from the *n* (negative), stromal or cytoplasmic side of the membrane and deposition of four protons on the *p* (positive), lumenal side of the thylakoid membrane. If we consider that two additional protons were picked up from the *n*-side during reduction of one PQ molecule to PQH_2 at the PS II Q_B-site, then the oxidation of two PQH_2 by the Cyt b_6f complex results in the transfer of two electrons to PS I and the net translocation of four protons from the *n*-side to the *p*-side of the thylakoid membrane (again, refer to Fig. 21.1). Thus the Cyt b_6f complex, and 'Rieske Cyt *b*' complexes more generally, have evolved as highly efficient sites to couple the vectorial movement of two protons across the membrane per electron transferred. This electrochemical charge gradient consisting of transmembrane proton (ΔpH) and electrical charge ($\Delta\mu$H) components, and generated in large part by the Cyt b_6f complex, drives ATP synthesis via a transmembrane, rotary ATPsynthase engine (see Junge et al., 2009 for a review and see Chapter 22).

Some details of the plastoquinol-oxidation reaction at the Cyt b_6f Q_p-site were discussed above in sections on the structure of the Cyt b_6f complex and Q_p-pocket (Sections VII.A.2 and VII.A.3) and domain movement of the Rieske ISP cluster-binding domain (Section VII.A.6.b), which is absolutely essential for biologically meaningful electron flow and energy conversion by the Cyt b_6f complex (see Figs. 21.1, 21.3, 21.6–21.8, and 21.10 for reference). For further discussion of Q-cycle mechanisms and current, detailed understanding of the coupled electron and proton transfer reactions at Q_p- (Q_o) sites of Cyt b_6f-bc_1 complexes, the reader is referred to several excellent, recent reviews on this subject (Allen, 2004; Crofts, 2004b; Osyczka et al., 2004; Cape et al., 2006; Baniulis et al., 2008; Crofts et al., 2008; Lhee et al., 2010).

2. Rate-Limiting Step in Electron Transfer?

Plastoquinol oxidation at the Cyt b_6f Q_p-site has long been recognized as the typical rate-limiting

step in photosynthetic electron transport (Witt, 1971; Joliot and Joliot, 1992). Specific steps have been discussed that may limit the overall, coupled, two electron–two proton oxidation of a quinol (QH_2) molecule in the Cyt b_6f-bc_1 Q_p-pocket. These are: (i) the diffusion of the Rieske ISP cluster domain from its proximal position in the Q_p-pocket to a position near the Cyt *f* heme and back (see Cramer et al., 2006 for a discussion); (ii) movement of the bulky PQH_2 molecule (see Fig. 21.7) through the narrow, ~11 Å × 12 Å, portal into the Q_p-pocket and movement of PQ back out (see Baniulis et al., 2009, and Fig. 21.6), and (iii) rate limitation imposed by the coupled proton-electron transfer from PQH_2 to the Rieske 2Fe-2S cluster (Crofts, 2004b). Diffusion of the Rieske ISP is unlikely as the rate-limiting step because the calculated diffusion rate, unless constrained, is faster than the rate of PQH_2 oxidation (reviewed by Darrouzet et al., 2001). Crofts and colleagues (Crofts, 2004b; Lhee et al., 2010) present compelling evidence favoring deprotonation of QH_2 to QH^- as the activation energy barrier and overall rate-limiting step. Direct electron transfer from the protonated quinol (QH_2) to the ~+300 mV Riekse 2Fe-2S cluster is extremely unlikely because the bound protons stabilize the electrons on the quinol and thus the potential of a QH_2/QH_2^+ couple is much too high for reduction of the Rieske ISP. The estimated potential ($E_{m,7}$) of the QH_2/QH_2^+ couple is +900 mV (Rich, 1984) and that of $QH_2/Q^{\bullet-}$ is ~+400 mV or higher (Hauska et al., 1983; Crofts and Wang, 1989). Accordingly, QH_2 must first be deprotonated to QH^- (as illustrated in Fig. 21.7) before an electron can flow to the Rieske ISP cluster. The QH^-/ QH• couple at ~+200 mV (Brandt and Okun, 1997) is well suited for electron transfer to the Rieske ISP cluster. Note again that TDS in the Q_p-pocket forms a hydrogen bond to an imidazole nitrogen of one of the Riekse ISP cluster-binding His residues (Fig. 21.8). Spectroscopic and mutagenesis studies indicate that the native quinol binds in the same way and suggest that both the first proton and electron transfers from QH_2 occur via a hydrogen bond from a quinol –OH to the Rieske ISP imidazole nitrogen (Crofts, 2004b; Lhee et al., 2010). Given the pKa's of the quinol hydroxyl and His imidazole nitrogen, the quinol –OH provides the hydrogen atom. After loss of the second proton, the generated, reactive semiquinone anion ($Q^{\bullet-}$) has an estimated midpoint potential of ~−300 mV relative to quinone ($Q^{\bullet-}/Q$ couple) (Crofts and Wang, 1989; Muller, 2000) and rapidly injects an electron into the low-potential chain. Thus the first proton and electron transfer step from QH_2 to the Rieske ISP cluster is considered slow and endergonic, and has presumably evolved to minimize the lifetime of the dangerous semiquinone ($Q^{\bullet-}$) radical. Once formed, electron transfer from the semiquinone to heme b_p is exergonic and rapid. Details of these quinol oxidation reaction steps and evidence for movement of quinol and semiquinone species within the Q_p-pocket are discussed in comprehensive reviews and recent articles (Berry et al., 2000; Crofts, 2004b; Cape et al., 2006; Crofts et al., 2006; Darrouzet et al., 2004; Lhee et al., 2010).

3. Low-Potential Chain and Quinone-Reduction at the Q_n Site

As noted, heme c_n in the Q_n-pocket is a unique feature of Cyt b_6f relative to bc_1 complexes. The close structural and electronic coupling of hemes b_n and c_n in Cyt b_6f complexes provides a mechanism for concerted, two-electron reduction of PQ to PQH_2 in the Q_n-pocket and a way to avoid or minimize the danger of semiquinone radical anion ($Q^{\bullet-}$) and consequent superoxide ($O_2^{\bullet-}$) formation at this site (Baymann et al., 2007). Details of the Q_n-site structure and current understanding of the role of heme c_n and the mechanism of PQ reduction were discussed above in Sections VII.A.4 and VII.A.5 (see also Figs. 21.5, 21.6, and 21.9). Readers are referred to several reviews and articles for further discussion (Allen, 2004; Cramer and Zhang, 2006; de Lacroix de Lavalette et al., 2008; Cramer et al., 2011).

4. Oxygen Radicals

As noted, formation of a reactive, semiquinone anion radical ($PQ^{\bullet-}$) with a midpoint potential, $E_{m,7}$ of ~ −300 mV is generally considered a necessary intermediate in the bifurcated quinol oxidation reaction at the Q_p-site. Under conditions where the semiquinone cannot readily transfer an electron to heme b_p, 'bypass' reactions can occur, including the diversion of an electron to O_2 to form superoxide ($O_2^{\bullet-}$) and subsequent, 'downstream' reactive oxygen species (ROS) (Muller

et al., 2002; Cape et al., 2006, 2007; Crofts et al., 2006; Forquer et al., 2006; Zhang et al., 2007). The occurrence of 'bypass' reactions has been well documented in Cyt bc_1 complexes, including mitochondrial complex III, where mutations resulting in 'mitochondrial myopathies' and ROS production are linked to cancers, neurodegenerative diseases, and diseases of aging (Muller, 2000; Demmig-Adams and Adams, 2002; Rego and Oliveira, 2003). Semiquinone formed in the Cyt bc_1 Q_{p-} (Q_o)-pocket has been detected by EPR spectroscopy (Cape et al., 2007; Zhang et al., 2007) and is linked to superoxide formation (Sun and Trumpower, 2003; Forquer et al., 2006). Oxygenic photosynthetic organisms live in a precarious balance where oxygen production and hyperoxic conditions must co-exist with low-potential electron carriers. Indeed, ROS production has also been well documented in photosynthetic cells where superoxide formation is primarily attributed to the low-potential iron-sulfur centers of PS I (Ivanov and Khorobrykh, 2003; Foyer and Noctor, 2009).

The Cyt b_6f complex is generally not considered a major source of ROS in photosynthesis, yet the transiently-formed Q_p-site plastosemiquinone should have a midpoint potential sufficiently low to generate superoxide (Muller, 2000; Ivanov and Khorobrykh, 2003). Indeed, we find that impaired electron flow in the Cyt b_6f low-potential chain of *Synechococcus* sp. PCC 7002 mutant PetB-R214H (Nelson et al., 2005) results in superoxide production, cellular damage and slowed growth (Ouyang et al., 2004) (D. Horn, P. Brantmier and T. Kallas, unpublished). These ROS arise from the Cyt b_6f complex and are linked to the redox level of the PQ pool as demonstrated by the highest rate of superoxide formation in dark incubated cells. Normal growth is partially restored by incubating these cells in an N_2-CO_2 atmosphere. These findings show that the Cyt b_6f complex may become a major site of ROS production under conditions of impaired electron flow in the low-potential chain and suggest that superoxide formation arises from the reactive semiquinone ($PQ^{\bullet -}$) in the Q_p-pocket. Alternatively, the PetB-R214H mutation, in the Q_n-pocket, may lower the midpoint potential of heme c_n as does the Q_n-site inhibitor NQNO (Alric et al., 2005) allowing plasto-semiquinone formation at this site. ROS have been implicated in redox signaling (Karpinski et al., 1999; Laloi et al., 2007; Drose and Brandt, 2008; Pfannschmidt et al., 2008) and it remains to be explored whether ROS may be involved in signal transduction by the Cyt b_6f complex.

C. Alternative Reaction Schemes, Resolved and Unresolved Controversies

One long-standing controversy concerns whether Cyt b_6f complexes necessarily operate by a Q-cycle mechanism or whether other schemes are possible, for example under highly reducing conditions, resulting in a variable stoichiometery of H^+ translocated per electron transferred (see Sacksteder et al., 2000; Cape et al., 2006; Baniulis et al., 2008; Cramer et al., 2011 for discussions). A number of interesting alternative schemes have been proposed. These include 'semiquinone' (Joliot and Joliot, 1994), 'proton slip' (Joliot and Joliot, 1998; Finazzi, 2002), and 'bypass' (Soriano et al., 1999) models involving transfer of semiquinones or protons across the membrane, or the transfer of two electrons from PQH_2 to two Rieske ISP clusters in a Cyt b_6f dimer. Part of the controversy arises from the absence of a highly efficient Q_n-site inhibitor in Cyt b_6f complexes. NQNO acts at this site (see Section VII.A.4 and Fig. 21.9) but only partially blocks turnover of the complex (Joliot and Joliot, 1986; Jones and Whitmarsh, 1988) making it difficult to assess the role of the low-potential chain. Further controversy revolves around whether the b_p and b_n hemes have different midpoint potentials in vivo. Thus direct evidence for b_p to b_n electron transfer has been difficult to obtain (see, however, Kramer and Crofts, 1994). Several studies have reported separate midpoint potentials ($E_{m,7}$) of −120 to −150 mV for heme b_p (b_L) and −30 to −50 mV for heme b_n (b_H) in membranes or isolated Cyt b_6f complexes (Baymann et al., 2001; Kramer and Crofts, 1994; Pierre et al., 1995). Others have detected only one midpoint potential for the *b* hemes between 0 and −50 mV (Girvin and Cramer, 1984; Furbacher et al., 1989). Cramer and colleagues argue that the apparent detection of distinctive midpoint potentials for the *b* hemes in vitro arises from a known decrease in heme potentials resulting from degradation or solvent exposure (Kassner, 1972). The question is interesting because isopotential *b* hemes in vivo more easily

allow possible alternative electron flow schemes including possible 'reverse' flow from the Q_n- to the Q_p-pocket under particular conditions (see e.g., Drose and Brandt, 2008). Note, however, that mutational data demonstrate the need for a functional Q_n-site for typical operation of the Cyt b_6f complex, whether by a Q-cycle or other mechanism. This is shown, for example, by the *Synechococcus* sp. PCC 7002 PetB-R214H Q_n-site mutation that results in impaired turnover of the Cyt b_6f complex and slowed growth of the cyanobacterium (Nelson et al., 2005). Readers are referred to additional sources for further discussion (Sacksteder et al., 2000; Cape et al., 2006; Cramer et al., 2006, 2011).

Another question has concerned possible inter-monomer electron transfer in Cyt bc_1-b_6f complexes (see e.g., Cramer et al., 2006). This has recently been resolved by an elegant gene fusion experiment in *Rhodobacter capsulatus*. Swierczek and colleagues fused two modified genes for Cyt *b*, one which carries a mutation that eliminates heme b_h in one monomer, and the other carries a mutation that eliminates quinol binding at the Q_p-site of the other monomer (Swierczek et al., 2010). This rendered impossible intra-monomer electron flow in the low-potential chain of either monomer. However, transmembrane electron transfer still occurred in a mixed dimer having a functional Q_p-site in one monomer and functional low-potential chain in the other monomer. These findings demonstrate inter-monomer electron transfer across the ~14 Å gap separating the two b_p hemes and suggest that the same phenomenon occurs in Cyt b_6f complexes (see Fig. 21.10 for reference). The authors suggest that this 'H-shaped' electron transfer option has evolved to defuse potentially damaging semiquinone ($Q^{\bullet-}$) radicals formed at the Q_p-site (Cape et al., 2007; Zhang et al., 2007; Drose and Brandt, 2008). If the low-potential chain of one monomer is blocked, electrons can flow via the b_p hemes to the other monomer.

D. Cyclic Electron Flow and the Cytochrome b_6f Complex

Cyclic electron flow around PS I has long been recognized (Arnon et al., 1954) but its demonstrated importance in plants (Joliot et al., 2004; Munekage et al., 2004; DalCorso et al., 2008), algae (Finazzi et al., 2002), and cyanobacteria (Yu et al., 1993; Mi et al., 1994) has emerged more recently. Some aspects of cyclic electron transfer were discussed above in Sections (VI.D and VII.A.4) on supercomplexes and the Q_n-domain of Cyt b_6f complexes. As noted, the Cyt b_6f complex is required for both linear electron flow ($H_2O \rightarrow$ PS II $\rightarrow$ PQ pool $\rightarrow$ Cyt b_6f complex $\rightarrow$ Cyt c_6 or plasocyanin $\rightarrow$ PS I $\rightarrow$ Fd), which generates both ATP and reducing equivalents as NADPH, and cyclic flow (between PS I and Cyt b_6f), which generates ATP only (see Fig. 21.2). CO_2 fixation to CH_2O by the Calvin-Benson cycle requires 3ATP and 2NADPH (Allen, 2002). Linear flow alone cannot generate sufficient ATP for carbon fixation (Allen, 2002; Kramer et al., 2004). Thus one role of cyclic, or perhaps 'pseudo-cyclic electron flow (the flow of electrons through PS I to O_2, thus generating ATP but no NADPH, see Allen, 2003) is to increase ATP concentration relative to NADPH. Another, at least in chloroplasts, is to increase ΔpH and thereby trigger the NPQ mechanism of thermal dissipation of excess PS II light energy (Niyogi, 1999; Holt et al., 2004). Possible pathways of cyclic flow include: (1) The NDH pathway (Fd $\rightarrow$ FNR $\rightarrow$ NAD(P)H $\rightarrow$ NADH dehydrogenase (NDH) $\rightarrow$ PQ pool $\rightarrow$ Cyt $b_6f \rightarrow$ PS I) that seems to predominate in cyanobacteria (Yu et al., 1993; Mi et al., 1994) (and also T. Kallas, unpublished) and has recently been demonstrated in plants (Livingston et al., 2010) and (2) direct flow from PS I to the Cyt b_6f Q_n-site, possibly via bound FNR as postulated from the Cyt b_6f structures (Kurisu et al., 2003; Stroebel et al., 2003), evidence for FNR bound to the *bf* complex (Zhang et al., 2001; Whitelegge et al., 2002; Breyton et al., 2006), and high cyclic rates and low NADH dehydrogenase abundance in chloroplasts (Joliot and Joliot, 2005). This is an attractive idea that has now been confirmed by the isolation of a Cyt b_6f–PS I supercomplex that catalyzes light-driven cyclic electron transport in vitro, as discussed above (Iwai et al., 2010).

The shift between linear and cyclic electron flow is mediated by 'state transitions' involving the redistribution of light-harvesting antenna proteins between the two photosystems (Allen, 1992). Events that lead to reduction of the PQ pool (state 2) trigger (via signaling by the Cyt b_6f complex) a displacement of antenna from PS II to PS I

thus favoring cyclic electron flow. This is well documented for *C. reinhardtii* (Finazzi et al., 2002) where up to 80% of the PS II antenna may be displaced. Cyclic flow may be particularly important in algae that experience anaerobic environments and thus decreased ATP levels (Joliot et al., 2006). Anaerobiosis induces the state 1 to state 2 transition and cyclic electron flow predominates in this condition (Finazzi et al., 1999). Cyclic flow has also been shown to be important in plant leaves illuminated after dark adaptation to provide ATP for activating the Calvin-Benson cycle (Joliot and Joliot, 2002). Fluorescence screening of *A. thaliana* mutants has identified mutations in two proteins, PGR5 (p*roton gradient* r*egulator,* Munekage et al., 2004) and pGRL1 (PGR*5*-li*ke*, DalCorso et al., 2008) that result in impaired cyclic flow and therefore decreased ΔpH generation and NPQ. DalCorso et al. (2008) find that PGRL1 and PGR5 form a complex that interacts with PS I, FNR, and the Cyt b_6f complex in a proposed direct cyclic pathway. Mutants, defective in both the PGR5 and NDH pathways are impaired in overall photosynthetic efficiency (Munekage et al., 2004). PGRL1 homologs have not been found in cyanobacteria. Protein Ssr2016 of *Synechocystis* 6803 shares some similarity with the antimycin-A sensitive PGR5 and has been implicated in an antimcycin-A sensitive, Fd-plastoquinone reductase (FQR) cyclic pathway (Bendall and Manasse, 1995; Yeremenko et al., 2005) but there is currently little supporting evidence for an FQR protein. Readers are directed to recent articles and reviews for further discussion of cyclic electron transport and its role (Cramer and Zhang, 2006; Joliot et al., 2006; Alric et al., 2010; Iwai et al., 2010; Livingston et al., 2010).

IX. Cytochrome b_6f Complex in Redox Sensing and Signal Transduction

A. Redox Signaling of 'State Transitions'

The Cyt b_6f complex plays a central role in redox-mediated signaling of light-harvesting protein distribution between the photosystems which influences electron fluxes and prevents the over-absorption of light energy and over-reduction of electron carriers. These 'state transitions' occur to varying degrees in plants (Allen, 2004), algae (Wollman, 2001; Finazzi et al., 2002; Cardol et al., 2009), and cyanobacteria (McConnell et al., 2002; Joshua and Mullineaux, 2004). When PS II absorbs excess light energy, the PQ pool 'upstream' of the Cyt b_6f complex becomes more reduced (state 2). By mechanisms as yet poorly understood, the Cyt b_6f complex senses this redox change and signals redistribution of LHCII or the PBS antenna such that more light energy is funneled into PS I. Conversely, if PS I absorbs excess energy, the PQ pool becomes oxidized (state 1) and antenna proteins are redirected to PS II. This in turn determines the relative fluxes of linear and cyclic electron flow and the cellular ATP/NADPH ratio which has profound impacts on metabolism including carbon fixation as discussed above.

In chloroplasts, state transitions are mediated by a stromal kinase associated with the Cyt b_6f complex (Allen et al., 1981; Keren and Ohad, 1998). State 2 (PQ pool reduced) induces kinase activation (phosphorylation), LHCII phosphorylation, and migration of phosphorylated LHCII proteins to PS I. In state 1 (PQ pool oxidized), LHCII becomes dephosphorylated and reassociates with PS II. Mutants lacking the b_6f complex lack state transitions (Wollman and Lemaire, 1988). Kinase activation requires plastoquinol (PQH_2) binding to the Q_p pocket (Vener et al., 1995) and a triggering conformational change of the b_6f complex is postulated (Finazzi et al., 2001). A state transition kinase, STN7, has been identified in *C. reinhardtii* (Fleischmann et al., 1999; Depege et al., 2003) and *A. thaliana* (Bellafiore et al., 2005). Mutants lacking the kinase have impaired PQ pool regulation and growth responses. The Rieske ISP transmembrane helix and *n*-side domain have been implicated in kinase activation via movement of the ISP cluster domain, but mutations of the hinge and *n*-terminus had little impact on the relatively slow ($t_{1/2}$ ~1 min) state transitions in *C. reinhardtii* (de Vitry et al., 2004). Recent data show association of the STN7 (Stt7) kinase with LHCII, PS I, and the Cyt b_6f complex, and demonstrate that the STN7 transmembrane domain and a disulfide between two lumenal (*p*-side) Cys residues is critical for state transitions (Lemeille et al., 2009).

Cyanobacteria undergo state transitions rapidly, usually in seconds. It remains controversial whether PBS redistribution occurs via conformational changes ('spillover') (McConnell et al., 2002),

lateral migration (Mullineaux et al., 1997), or both. Fluorescence recovery after photobleaching (FRAP) and other studies provide evidence for migration of at least some of the PBS complexes (Joshua and Mullineaux, 2004; Mullineaux, 2008; Yang et al., 2009). No apparent state-transition kinase exists in cyanobacteria; however, the signaling mechanism involves the Cyt b_6f complex as indicated by mutational studies (Schneider et al., 2001; Volkmer et al., 2007) and inhibitor treatments (P. Brantmier and T. Kallas, unpublished). RpaC (Sll1926), a small membrane protein of *Synechocystis* sp. PCC 6803, is essential for state transitions apparently by stabilizing PS II–PBS interactions (Emlyn-Jones et al., 1999; Joshua and Mullineaux, 2004). Other cyanobacterial state transition mutants are defective in NADH dehydrogenase components (Mi et al., 2000) or in the PS I PsaE subunit involved in cyclic flow (Yu et al., 1993), presumably because these alter the redox state of the PQ pool. How PQ pool redox changes trigger Cyt b_6f-mediated signal-transduction and subsequent steps remains largely unclear. The Cyt b_6f small subunit helix bundle (PetG, L, M, N) and chlorophyll molecule whose phytyl tail extends into the Q_p-pocket have postulated roles in signaling (Kurisu et al., 2003; Stroebel et al., 2003). In *Synechocystis* sp. PCC 6803, PetM inactivation lowers PS I content and the PQ pool becomes more oxidized (Schneider et al., 2001). Deletion of Ssr2998, a protein loosely associated with the Cyt b_6f complex, slows turnover and alters photosystem stochiometry and state transitions (Volkmer et al., 2007). Subunit-IV Val104 to Phe and Gly136 to Phe mutations that alter the properties of the Cyt b_6f chlorophyll in *C. reinhardtii* also impair state transitions (de Lacroix de Lavalette et al., 2008). These findings implicate communication between the Q_p and Q_n domains and the redox status or conformational changes in the Cyt b_6f low-potential chain in signaling state transitions. Readers are referred to recent articles and reviews for further discussion (Eberhard et al., 2008; Cardol et al., 2009; Kondo et al., 2009; Murata, 2009; Lemeille and Rochaix, 2010).

B. Redox Signaling of Gene Expression

In chloroplasts, changes in PQ pool redox status alter PS II and PS I gene transcription to balance electron flow between the photosystems (Pfannschmidt et al., 1999a, 1999b, 2008). Similar observations have been made in cyanobacteria (El Bissati and Kirilovsky, 2001). Sensing of PQ pool redox status and signaling to adjust gene and protein expression levels may occur via: (1) proteins directly associated with the PQ pool; (2) the Cyt b_6f complex low- or high-potential chains; (3) ROS generated by the Cyt b_6f complex or PS I, and/or (4) redox components downstream of PS I. These hypotheses are not mutually exclusive, some evidence supports each of these, and several different signaling pathways may be used depending on environmental signals. A chloroplast stromal sensor kinase (CSK), homologous to the cyanobacterial histidine kinase, Hik2 (Ashby and Houmard, 2006), responds to PQ pool redox status and regulates chloroplast gene expression (Puthiyaveetil et al., 2008). RppA (*regulator of photosynthesis and photopigment-related gene expression)* similarly upregulates PS I and downregulates PS II genes in response to increased PQ pool reduction in *Synechocystis* sp. PCC 6803 (Li and Sherman, 2000). The LdpA ISP (Ivleva et al., 2005) and the CikA, quinone-binding, kinase (Ivleva et al., 2006) of *Synechococcus* sp. PCC 7942 both respond to PQ pool redox changes and regulate circadian proteins. These serve as interesting examples of redox-sensing, signal-tranduction proteins.

Microarray analysis of *Synechocystis* sp. PCC 6803 revealed ~140 genes differentially regulated in response to the PS II inhibitor DCMU, which oxidizes the PQ pool, and the Cyt b_6f Q_p-site inhibitor DBMIB, which reduces the PQ pool (Hihara et al., 2003). Other microarray data show that nuclear gene expression in *A. thaliana* depends on the redox state of components on the acceptor side of PS I (Piippo et al., 2006). A thiol-containing, LuxR-type regulator, PedR (ssl0564), of *Synechocystis* sp. PCC 6803 similarly regulates a set of genes in response to electron flux, apparently downstream of PS I (Nakamura and Hihara, 2006). Redox signaling may also be mediated by ROS such as H_2O_2, which dramatically influences gene expression in cyanobacteria (Li et al., 2004; Singh et al., 2005) and plants (Laloi et al., 2007). Our preliminary microarray data from *Synecococcus* sp. PCC 7002 show that mutations that specifically slow electron flow in the Cyt b_6f low- (Nelson et al., 2005) and high- (Yan and Cramer, 2003) potential chains alter the expression of hundreds of genes (Weir et al., 2009 and G. Weir, K. Short and T. Kallas, unpublished).

Some hundred genes (at $p < 0.01$) were up- or down-regulated in the same direction in both mutants indicating responses at the level of the PQ pool or downstream of PS I. However, a smaller set of genes was regulated uniquely in the high- or low-potential chain mutants. These data suggest that redox or conformational changes occurring specifically in the Cyt b_6f high- or low-potential chains elicit the expression of different sets of genes. The mechanism by which these redox-mediated gene expression events are signaled remain poorly understood and largely unexplored.

X. Assembly of Subunits and Prosthetic Groups

Because of space and time constraints, the important topics of biogenesis of the Cyt b_6f complex and regulation of the assembly process cannot be covered in this review. Readers are referred to excellent reviews and discussions on assembly and targeting of the membrane-bound b_6 hemes (Dreher et al., 2008); *c* hemes (Ferguson et al., 2008); and the unique, high-spin c_n heme (Kuras et al., 2007; Saint-Marcoux et al., 2009). The unique, high-potential Rieske 2Fe-2S cluster remains largely unexplored in terms of its biogenesis although the assembly and repair of iron-sulfur clusters in bacteria and plants have been investigated extensively (Balk and Lobreaux, 2005; Imlay, 2006; Shcolnick et al., 2009). The assembly of the Cyt b_6f complex has been reviewed (Wollman, 1999). An intriguing phenomenon termed, 'control by epistasy of synthesis' governs the synthesis of some of the subunits of thylakoid membrane protein complexes. The rate of Cyt *f* synthesis decreases markedly if neighboring subunits of the Cyt b_6f complex are unavailable to interact with the C-terminus of the Cyt *f* protein (Choquet et al., 1998, 2003; see also de Vitry et al., 2004).

XI. Concluding Remarks

The Cyt b_6f complex lies at the heart of energy transduction in photosynthetic electron transfer chains of cyanobacteria and chloroplasts. In addition, the complex is central to environmental reponses of photosynthetic organisms mediated via changes in redox status of the plastoquinone pool. The Cyt b_6f complex serves in sensing plastoquinone pool redox status and in signaling that triggers rapid adjustments in photosynthesis as well as longer-term gene expression events. Remarkable discoveries have been made in the past decade that have greatly advanced understanding of the structure of Cyt b_6f complexes and their role in energy conversion and regulation of photosynthesis. Many intriguing questions remain. These include ones concerning the assembly of the complex and its cofactors, roles of peripherally associated proteins, reaction mechanisms and structural modifications in response to environmental changes, and the components and mechanisms of PQ pool redox sensing and signaling.

Note: Malnoë et al. (2011) describe a *Chlamydomonas reinhardtii* FtsH1 protease mutant that stabilizes a Cyt b_6f complex lacking heme b_n (b_H) and therefore a Q-cycle. Amazingly this mutant grows photosynthetically although at a very low rate. Plastoquinol (PQH_2) oxidation at the Q_p-site still injects one electron into the high-potential chain and a second electron onto heme b_p (b_L). A second PQH_2 may again inject an electron into the high-potential chain but now the reduced heme b_p is thought to re-reduce the resulting semiquinone at the Q_p-site. This process allows linear electron flow through a crippled Cyt b_6f complex that cannot catalyze a Q-cycle and transmembrane electron transfer. These data demonstrate that while a Q-cycle or equivalent is necessary for efficient energy transduction, it is not obligatory (see sections VIII B and C).

Secondly, the Cyt b_6f plastoquinol-oxidation reaction is not always the rate-limiting step in photosynthetic electron transfer as demonstrated recently by David Kramer, Michigan State University (D. Kramer, personal communication). An *Arabidopsis thaliana* mutant lacks kinetic control of the ATP synthase and more rapidly discharges the ΔpH gradient. This relieves the "back-pressure" that the pH gradient normally imposes on the PQH_2 oxidation (coupled electron-proton) reaction and allows the Cyt b_6f complex to run freely. In this condition, plastoquinol oxidation speeds up and is no longer the rate-limiting step in electron transfer.

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References

Albertsson P (2001) A quantitative model of the domain structure of the photosynthetic membrane. Trends Plant Sci 6: 349–358

Aldridge C, Spence E, Kirkilionis MA, Frigerio L and Robinson, C (2008) Tat-dependent targeting of Rieske iron-sulphur proteins to both the plasma and thylakoid membranes in the cyanobacterium *Synechocystis* PCC6803. Mol Microbiol 70: 140–150

Allen J (2002) Photosynthesis of ATP-electrons, proton pumps, rotors, and poise. Cell 110: 273–276

Allen JF (1992) Protein phosphorylation in regulation of photosynthesis. Biochim Biophys Acta 1098: 275–335

Allen JF (2003) Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. Trends Plant Sci 8: 15–19

Allen JF (2004) Cytochrome b_6f: structure for signalling and vectorial metabolism. Trends Plant Sci 9: 130–137

Allen JF, Bennett J, Steinback KE and Arntzen CJ (1981) Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. Nature 291: 25–29

Alric J, Pierre Y, Picot D, Lavergne J and Rappaport F (2005) Spectral and redox characterization of the heme ci of the cytochrome b_6f complex. Proc Natl Acad Sci U S A 102: 15860–15865

Alric J, Lavergne J and Rappaport F (2010) Redox and ATP control of photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii* (I) aerobic conditions. Biochim Biophys Acta 1797: 44–51

Arieli B, Shahak Y, Taglicht D, Hauska G and Padan E (1994) Purification and characterization of sulfide-quinone reductase, a novel enzyme driving anoxygenic photosynthesis in *Oscillatoria limnetica*. J Biol Chem 269: 5705–5711

Arnon DI, Allen MB and Whatley FR (1954) Photosynthesis by isolated chloroplasts. Nature 174: 394–396

Ashby MK and Houmard J (2006) Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. Microbiol Mol Biol Rev 70: 472–509

Atteia A, Van Lis R, Wetterskog D, Gutierrez-Cirlos EB, Ongay-Larios L, Franzen LG and Gonzalez-Halphen D (2003) Structure, organization and expression of the genes encoding mitochondrial cytochrome *c*(1) and the Rieske iron-sulfur protein in *Chlamydomonas reinhardtii*. Mol Genet Genomics 268: 637–644

Baciou L and Michel H (1995) Interruption of the water chain in the reaction center from *Rhodobacter sphaeroides* reduces the rates of the proton uptake and of the second electron transfer to Q_B. Biochemistry 34: 7967–7972

Bailey S, Melis A, Mackey KR, Cardol P, Finazzi G, Van Dijken G, Berg GM, Arrigo K, Shrager J and Grossman A (2008) Alternative photosynthetic electron flow to oxygen in marine *Synechococcus*. Biochim Biophys Acta 1777: 269–276

Bald D, Kruip J, Boekema EJ, and Rögner M (1992) Structural investigations on Cyt b_6/f complex and PS I-complex from the cyanobacterium *Synechocystis* PCC 6803. In: Murata N (ed) Research in Photosynthesis, 629–632. Kluwer, Dordecht

Balk J and Lobreaux S (2005) Biogenesis of iron-sulfur proteins in plants. Trends Plant Sci 10: 324–331

Ballesteros M, Fredriksson A, Henriksson J and Nystrom T (2001) Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. EMBO J 20: 5280–5289

Baniulis D, Yamashita E, Zhang H, Hasan SS and Cramer WA (2008) Structure-function of the cytochrome b_6f complex. Photochem Photobiol 84: 1349-1358

Baniulis D, Yamashita E, Whitelegge JP, Zatsman AI, Hendrich MP, Hasan SS, Ryan CM and Cramer WA (2009) Structure-function, stability, and chemical modification of the cyanobacterial cytochrome b_6f complex from *Nostoc* sp. PCC 7120. J Biol Chem 284: 9861–9869

Baymann F and Nitschke W (2010) Heliobacterial Rieske/cyt*b* complex. Photosynth Res 104: 177–187

Baymann F, Rappaport F, Joliot P and Kallas T (2001) Rapid electron transfer to photosystem I and unusual spectral features of cytochrome *c*(6) in *Synechococcus* sp. PCC 7002 in vivo. Biochemistry 40: 10570–10577

Baymann F, Lebrun E and Nitschke W (2004) Mitochondrial cytochrome c_1 is a collapsed di-heme cytochrome. Proc Natl Acad Sci USA 101: 17737–17740

Baymann F, Giusti F, Picot D and Nitschke W (2007) The ci/bH moiety in the b_6f complex studied by EPR: a pair of strongly interacting hemes. Proc Natl Acad Sci USA 104: 519–524

Bellafiore S, Barneche F, Peltier G and Rochaix JD (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895

Bendall DS and Manasse RS (1995) Cyclic phosphorylation and electron transport. Biochim Biophys Acta 1229: 23–38

Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. Proc Natl Acad Sci USA 79: 4352–4356

Berry EA, Zhang Z, Huang LS, and Kim SH (1999) Structures of quinone-binding sites in bc complexes: functional implications. Biochem Soc Trans 27: 565–572

Berry EA, Guergova-Kuras M, Huang LS and Crofts AR (2000) Structure and function of cytochrome *bc* complexes. Annu Rev Biochem 69: 1005–1075

Berry S, Schneider D, Vermaas WF and Rögner M (2002) Electron transport routes in whole cells of *Synechocystis* sp. strain PCC 6803: the role of the cytochrome *bd*-type oxidase. Biochemistry 41: 3422–3429

Berthold DA, Schmidt CL and Malkin R (1995) The deletion of *petG* in *Chlamydomonas reinhardtii* disrupts the cytochrome *bf* complex. J Biol Chem 270: 29293–29298

Betterle N, Ballottari M, Zorzan S, de Bianchi S, Cazzaniga S, Dall'osto L, Morosinotto T and Bassi R (2009) Light-induced dissociation of an antenna hetero-oligomer is needed for non-photochemical quenching induction. J Biol Chem 284: 15255–15266

Bialek W, Nelson M, Tamiola K, Kallas T and Szczepaniak A (2008) Deeply branching c_6-like cytochromes of cyanobacteria. Biochemistry 47: 5515–5522

Bibby TS, Nield J and Barber J (2001). Iron deficiency induces the formation of an antenna ring around trimeric photosystem I in cyanobacteria. Nature 412: 743–745

Boekema EJ, Hifney A, Yakushevska AE, Piotrowski M, Keegstra W, Berry S, Michel KP, Pistorius EK and Kruip J (2001) A giant chlorophyll-protein complex induced by iron deficiency in cyanobacteria. Nature 412: 745–748

Boronowsky U, Wenk S, Schneider D, Jager C and Rögner M (2001) Isolation of membrane protein subunits in their native state: evidence for selective binding of chlorophyll and carotenoid to the b_6 subunit of the cytochrome b_6f complex. Biochim Biophys Acta 1506: 55–66

Brandt U and Okun JG (1997) Role of deprotonation events in ubihydroquinone:cytochrome *c* oxidoreductase from bovine heart and yeast mitochondria. Biochemistry 36: 11234–11240

Brasseur G, Saribas AS and Daldal F (1996) A compilation of mutations located in the cytochrome b subunit of the bacterial and mitochondrial bc_1 complex. Biochim Biophys Acta 1275: 61–69

Brasseur G, Bruscella P, Bonnefoy V and Lemesle-Meunier,D (2002) The bc_1 complex of the iron-grown acidophilic chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* functions in the reverse but not in the forward direction. Is there a second bc_1 complex? Biochim Biophys Acta 1555: 37–43

Breyton C (2000) Conformational changes in the cytochrome b_6f complex induced by inhibitor binding. J Biol Chem 275: 13195–13201

Breyton C, Nandha B, Johnson GN, Joliot P and Finazzi G (2006) Redox modulation of cyclic electron flow around photosystem I in C3 plants. Biochemistry 45: 13465–13475

Britt RD, Sauer K, Klein MP, Knaff DB, Kriauciunas A, Yu CA, Yu L and Malkin R (1991) Electron spin echo envelope modulation spectroscopy supports the suggested coordination of two histidine ligands to the Rieske Fe-S centers of the cytochrome b_6f complex of spinach and the cytochrome bc_1 complexes of *Rhodospirillum rubrum*, *Rhodobacter sphaeroides* R-26, and bovine heart mitochondria. Biochemistry 30: 1892–1901

Brugna M, Rodgers S, Schricker A, Montoya G, Kazmeier M, Nitschke W and Sinning I (2000) A spectroscopic method for observing the domain movement of the Rieske iron-sulfur protein. Proc Natl Acad Sci USA 97: 2069–2074

Bruscella P, Appia-Ayme C, Levican G, Ratouchniak J, Jedlicki E, Holmes DS and Bonnefoy V (2007) Differential expression of two bc_1 complexes in the strict acidophilic chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* suggests a model for their respective roles in iron or sulfur oxidation. Microbiology 153: 102–110

Bulte L, Gans P, Rebeille F and Wollman FA (1990) ATP control on state transitions in vivo in *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1020: 72–80

Burrows PA, Sazanov LA, Svab Z, Maliga P and Nixon PJ (1998) Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid *ndh* genes. EMBO J 17: 868–876

Cape JL, Bowman MK and Kramer DM (2006) Understanding the cytochrome *bc* complexes by what they don't do. The Q-cycle at 30. Trends Plant Sci 11: 46–55

Cape JL, Bowman MK and Kramer DM (2007) A semiquinone intermediate generated at the Qo site of the cytochrome bc1 complex: importance for the Q-cycle and superoxide production. Proc Natl Acad Sci USA 104: 7887–7892

Cardol P, Bailleul B, Rappaport F, Derelle E, Beal D, Breyton C, Bailey S, Wollman FA, Grossman A, Moreau H, et al. (2008) An original adaptation of photosynthesis in the marine green alga *Ostreococcus*. Proc Natl Acad Sci USA 105: 7881–7886

Cardol P, Alric J, Girard-Bascou J, Franck F, Wollman FA and Finazzi G (2009) Impaired respiration discloses the physiological significance of state transitions in *Chlamydomonas*. Proc Natl Acad Sci USA 106: 15979–15984

Carrell CJ, Schlarb BG, Bendall DS, Howe CJ, Cramer WA and Smith JL (1999) Structure of the soluble domain of cytochrome *f* from the cyanobacterium *Phormidium laminosum*. Biochemistry 38: 9590–9599

Carrell CJ, Zhang H, Cramer WA and Smith JL (1997) Biological identity and diversity in photosynthesis and respiration: structure of the lumen-side domain of the chloroplast Rieske protein. Structure 5: 1613–1625

Chi YI, Huang LS, Zhang Z, Fernandez-Velasco JG and Berry EA (2000) X-ray structure of a truncated form of cytochrome *f* from *Chlamydomonas reinhardtii*. Biochemistry 39: 7689–7701

Choquet Y, Stern DB, Wostrikoff K, Kuras R, Girard-Bascou J and Wollman FA (1998) Translation of cytochrome *f* is autoregulated through the 5' untranslated region of petA mRNA in *Chlamydomonas* chloroplasts. Proc Natl Acad Sci USA 95: 4380–4385

Choquet Y, Zito F, Wostrikoff K and Wollman FA (2003) Cytochrome *f* translation in *Chlamydomonas* chloroplast is autoregulated by its carboxyl-terminal domain. Plant Cell 15: 1443–1454

Clark RD, Hawkesford MJ, Coughlan SJ, Bennett J and Hind G (1984). Association of ferredoxin-$NADP^+$ oxidoreductase with the chloroplast cytochrome *b-f* complex. FEBS Lett 174: 137–142

Colbert CL, Couture MM, Eltis LD and Bolin JT (2000) A cluster exposed: structure of the Rieske ferredoxin from biphenyl dioxygenase and the redox properties of Rieske Fe-S proteins. Structure 8: 1267–1278

Cooley JW and Vermaas WF (2001) Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of *Synechocystis* sp. strain PCC 6803: capacity comparisons and physiological function. J Bacteriol 183: 4251–4258

Cooley JW, Howitt CA and Vermaas WF (2000) Succinate: quinol oxidoreductases in the cyanobacterium *Synechocystis* sp. strain PCC 6803: presence and function in metabolism and electron transport. J Bacteriol 182: 714–722

Cournac L, Guedeney G, Peltier G and Vignais PM (2004) Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. J Bacteriol 186: 1737–1746

Couture MM, Colbert CL, Babini E, Rosell FI, Mauk AG, Bolin JT and Eltis LD (2001) Characterization of BphF, a Rieske-type ferredoxin with a low reduction potential. Biochemistry 40: 84–92

Cramer WA (2004) *Ironies* in photosynthetic electron transport: a personal perspective. Photosynth Res 80: 293–305

Cramer WA and Zhang H (2006) Consequences of the structure of the cytochrome b_6f complex for its charge transfer pathways. Biochim Biophys Acta 1757: 339–345

Cramer WA, Furbacher PN, Szczepaniak A and Tae G-S (1991) Electron transport between photosystem II and photosystem I. Curr Top Bioenerg 16: 179–222

Cramer WA, Soriano GM, Ponomarev M, Huang D, Zhang H, Martinez SE and Smith JL (1996) Some new structural aspects and old controversies concerning the cytochrome b_6f complex of oxygenic photosynthesis. Ann Rev Plant Physiol Plant Mol Biol 47: 477–508

Cramer WA, Zhang H, Yan J, Kurisu G and Smith JL (2004) Evolution of photosynthesis: time-independent structure of the cytochrome b_6f complex. Biochemistry 43: 5921–5929

Cramer WA, Yan J, Zhang H, Kurisu G and Smith JL (2005) Structure of the cytochrome b_6f complex: new prosthetic groups, Q-space, and the 'hors d'oeuvres hypothesis' for assembly of the complex. Photosynth Res 85: 133–143

Cramer WA Zhang H, Yan J, Kurisu G and Smith JL (2006) Transmembrane traffic in the cytochrome b_6f complex. Annu Rev Biochem 75: 769–790

Cramer WA, Yamashita E, Baniulis D and Hassan SS (2011) The cytochrome b_6f complex of oxygenic photosynthesis. In: Messerschmidt A (ed) Handbook of Metalloproteins, John Wiley, Chichester DOI: 10.1002/0470028637.met287

Crofts AR (2004a) The cytochrome bc_1 complex: function in the context of structure. Annu Rev Physiol 66: 689–733

Crofts AR (2004b) Proton-coupled electron transfer at the Q_o-site of the bc_1 complex controls the rate of ubihydroquinone oxidation. Biochim Biophys Acta 1655: 77–92

Crofts AR (2004c) The Q-cycle – a personal perspective. Photosynth Res 80: 223–243

Crofts AR and Wang Z (1989) How rapid are the internal reactions of the ubiquinol: cytochrome c_2 oxidoreductase? Photosynth Res 22: 69–87

Crofts AR, Meinhardt SW, Jones KR and Snozzi M (1983) The role of the quinone pool in the cyclic electron-transfer chain of *Rhodopseudomonas sphaeroides*. Biochim Biophys Acta 723: 202–218

Crofts AR, Hong S, Ugulava N, Barquera B, Gennis R, Guergova-Kuras M and Berry EA (1999) Pathways for proton release during ubihydroquinone oxidation by the bc(1) complex. Proc Natl Acad Sci USA 96: 10021–10026

Crofts AR, Lhee S, Crofts SB, Cheng J and Rose S (2006) Proton pumping in the bc_1 complex: a new gating mechanism that prevents short circuits. Biochim Biophys Acta 1757: 1019–1034

Crofts AR, Holland JT, Victoria D, Kolling DR, Dikanov SA, Gilbreth R, Lhee S, Kuras R and Kuras MG (2008) The Q-cycle reviewed: How well does a monomeric mechanism of the bc_1 complex account for the function of a dimeric complex? Biochim Biophys Acta 1777: 1001–1019

DalCorso G, Pesaresi P, Masiero S, Aseeva E, Schunemann D, Finazzi G, Joliot P, Barbato R and Leister D (2008) A complex containing PGRL1 and PGR5 is involved in the switch between linear and cyclic electron flow in *Arabidopsis*. Cell 132: 273–285

Darrouzet E, Valkova-Valchanova M and Daldal F (2000a) Probing the role of the Fe-S subunit hinge region during Q_o site catalysis in *Rhodobacter capsulatus* bc_1 complex. Biochemistry 39: 15475–15483

Darrouzet E, Valkova-Valchanova M, Moser CC, Dutton PL and Daldal F (2000b) Uncovering the [2Fe2S] domain movement in cytochrome bc_1 and its implications for energy conversion. Proc Natl Acad Sci USA 97: 4567–4572

Darrouzet E, Moser CC, Dutton PL and Daldal F (2001) Large scale domain movement in cytochrome bc_1: a new device for electron transfer in proteins. Trends Biochem Sci 26: 445–451

Darrouzet E, Cooley JW and Daldal F (2004) The cytochrome bc_1 complex and its homologue the b_6f complex: similarities and differences. Photosynth Res 79: 25–44

Dashdorj N, Zhang H, Kim H, Yan J, Cramer WA and Savikhin S (2005) The single chlorophyll *a* molecule in the cytochrome b_6f complex: unusual optical properties protect the complex against singlet oxygen. Biophys J 88: 4178–4187

Davenport HE and Hill R (1952) The preparation and some properties of cytochrome *f*. Proc Royal Soc B 139: 327–345

Davidson E and Daldal F (1987) *fbc* operon, encoding the Rieske Fe-S protein cytochrome *b*, and cytochrome c_1 apoproteins previously described from *Rhodopseudomonas sphaeroides*, is from *Rhodopseudomonas capsulata*. J Mol Biol 195: 25–29

de Lacroix de Lavalette A, Finazzi G, and Zito F (2008) b_6f-associated chlorophyll: Structural and dynamic contribution to the different cytochrome functions. Biochemistry 47: 5259–5265

de Vitry C, Breyton C, Pierre Y and Popot JL (1996) The 4-kDa nuclear-encoded PetM polypeptide of the chloroplast cytochrome b_6f complex. Nucleic acid and protein sequences, targeting signals, transmembrane topology. J Biol Chem 271: 10667–10671

de Vitry C, Finazzi G, Baymann F and Kallas T (1999) Analysis of the nucleus-encoded and chloroplast-targeted rieske protein by classic and site-directed mutagenesis of *Chlamydomonas*. Plant Cell 11: 2031–2044

de Vitry C, Ouyang Y, Finazzi G, Wollman FA, and Kallas T (2004) The chloroplast Rieske iron-sulfur protein. At the crossroad of electron transport and signal transduction. J Biol Chem 279: 44621–44627

Degli Esposti M, Ballester F, Solaini G and Lenaz G (1987) The circular-dichroic properties of the 'Rieske' iron-sulphur protein in the mitochondrial ubiquinol: cytochrome *c* reductase. Biochem J 241: 285–290

Demmig-Adams B and Adams WW 3rd (2002) Antioxidants in photosynthesis and human nutrition. Science 298: 2149–2153

Denke E, Merbitz-Zahradnik T, Hatzfeld OM, Snyder CH, Link TA and Trumpower BL (1998) Alteration of the midpoint potential and catalytic activity of the rieske iron-sulfur protein by changes of amino acids forming hydrogen bonds to the iron-sulfur cluster. J Biol Chem 273: 9085–9093

Depege N, Bellafiore S and Rochaix JD (2003) Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in *Chlamydomonas*. Science 299: 1572-1575

Desplats C, Mus F, Cuine S, Billon E, Cournac L and Peltier G (2009) Characterization of Nda2, a plastoquinone-reducing type II NAD(P)H dehydrogenase in *Chlamydomonas* chloroplasts. J Biol Chem 284: 4148–4157

Diaz-Quintana A, Navarro JA, Hervas M, Molina-Heredia FP, De la Cerda B and De la Rosa MA (2003) A comparative structural and functional analysis of cyanobacterial plastocyanin and cytochrome c_6 as alternative electron donors to photosystem I. Photosynth Res 75: 97–110

Dong C, Tang A, Zhao J, Mullineaux CW, Shen G and Bryant DA (2009) ApcD is necessary for efficient energy transfer from phycobilisomes to photosystem I and helps to prevent photoinhibition in the cyanobacterium *Synechococcus* sp. PCC 7002. Biochim Biophys Acta 1787: 1122–1128

Dreher C, Prodohl A, Hielscher R, Hellwig P and Schneider D (2008) Multiple step assembly of the transmembrane cytochrome b_6. J Mol Biol 382: 1057–1065

Drose S and Brandt U (2008) The mechanism of mitochondrial superoxide production by the cytochrome bc_1 complex. J Biol Chem 283: 21649–21654

Ducluzeau AL, Chenu E, Capowiez L and Baymann F (2008) The Rieske/cytochrome *b* complex of Heliobacteria. Biochim Biophys Acta 1777: 1140-1146

Dudkina NV, Sunderhaus S, Boekema EJ and Braun HP (2008) The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. J Bioenerg Biomembr 40: 419–424

Dudkina NV, Kouril R, Bultema JB and Boekema EJ (2010) Imaging of organelles by electron microscopy reveals protein-protein interactions in mitochondria and chloroplasts. FEBS Lett 584: 2510–2515

Eberhard S, Finazzi G and Wollman FA (2008) The dynamics of photosynthesis. Annu Rev Genet 42: 463–515

El Bissati K and Kirilovsky D (2001) Regulation of *psbA* and *psaE* expression by light quality in *Synechocystis* species PCC 6803. A redox control mechanism. Plant Physiol 125: 1988–2000

Elsen NL, Moe LA, McMartin LA and Fox BG (2007) Redox and functional analysis of the Rieske ferredoxin component of the toluene 4-monooxygenase. Biochemistry 46: 976–986

Emlyn-Jones D, Ashby MK and Mullineaux CW (1999) A gene required for the regulation of photosynthetic light harvesting in the cyanobacterium *Synechocystis* 6803. Mol Microbiol 33: 1050-1058

Esser L, Elberry M, Zhou F, Yu CA, Yu L and Xia D (2008) Inhibitor-complexed structures of the cytochrome bc_1 from the photosynthetic bacterium *Rhodobacter sphaeroides*. J Biol Chem 283: 2846–2857

Fee JA, Kuila D, Mather MW and Yoshida T (1986) Respiratory proteins from extremely thermohilic, aerobic bacteria. Biochim Biophys Acta 853: 153–185

Ferguson SJ, Stevens JM, Allen JW and Robertson IB (2008) Cytochrome *c* assembly: a tale of ever increasing variation and mystery? Biochim Biophys Acta 1777: 980–984

Fernandez-Velasco JG, Jamshidi A, Gong XS, Zhou J and Ueng RY (2001) Photosynthetic electron transfer through the cytochrome b_6f complex can bypass cytochrome *f*. J Biol Chem 276: 30598–30607

Fiebig A, Stegemann S and Bock R (2004) Rapid evolution of RNA editing sites in a small non-essential plastid gene. Nucleic Acids Res 32: 3615–3622

Finazzi G (2002) Redox-coupled proton pumping activity in cytochrome b_6f, as evidenced by the pH dependence of electron transfer in whole cells of *Chlamydomonas reinhardtii*. Biochemistry 41: 7475–7482

Finazzi G, Furia A, Barbagallo RP and Forti G (1999) State transitions, cyclic and linear electron transport and photophosphorylation in *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1413: 117–129

Finazzi G, Zito F, Barbagallo RP and Wollman FA (2001) Contrasted effects of inhibitors of cytochrome b6f complex on state transitions in *Chlamydomonas reinhardtii*: the role of Q_o site occupancy in LHCII kinase activation. J Biol Chem 276: 9770–9774

Finazzi G, Rappaport F, Furia A, Fleischmann M, Rochaix JD, Zito F and Forti G (2002) Involvement of state transitions in the switch between linear and cyclic electron flow in *Chlamydomonas reinhardtii*. EMBO Rep 3: 280–285

Fleischmann MM, Ravanel S, Delosme R, Olive J, Zito F, Wollman FA and Rochaix JD (1999) Isolation and characterization of photoautotrophic mutants of *Chlamydomonas reinhardtii* deficient in state transition. J Biol Chem 274: 30987–30994

Forquer I, Covian R, Bowman MK, Trumpower BL and Kramer DM (2006) Similar transition states mediate the Q-cycle and superoxide production by the cytochrome bc_1 complex. J Biol Chem 281: 38459–38465

Foyer CH and Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Redox Signal 11: 861–905

Fromme P and Grotjohann I (2008) Structure of Photosystems I and II. Results Probl Cell Differ 45: 33–72

Furbacher PN, Girvin ME and Cramer WA (1989) On the question of interheme electron transfer in the chloroplast cytochrome b_6 in situ. Biochemistry 28: 8990–8998

Gabellini N and Sebald W (1986) Nucleotide sequence and transcription of the *fbc* operon from *Rhodopseudomonas sphaeroides*. Evaluation of the deduced amino acid sequences of the FeS protein, cytochrome *b* and cytochrome c_1. Eur J Biochem 154: 569–579

Gabellini N, Bowyer JR, Hurt E, Melandri BA and Hauska G (1982) A cytochrome b/c_1 complex with ubiquinol–cytochrome c_2 oxidoreductase activity from *Rhodopseudomonas sphaeroides* GA. Eur J Biochem 126: 105–111

Gao X, Wen X, Esser L, Quinn B, Yu L, Yu CA and Xia D (2003) Structural basis for the quinone reduction in the bc_1 complex: a comparative analysis of crystal structures of mitochondrial cytochrome bc_1 with bound substrate and inhibitors at the Q_i site. Biochemistry 42: 9067–9080

Garlick S, Oren A and Padan E (1977) Occurrence of facultative anoxygenic photosynthesis among filamentous and unicellular cyanobacteria. J Bacteriol 129: 623–629

Girvin ME and Cramer WA (1984) A redox study of the electron transport pathway responsible for generation of the slow electrochromic phase in chloroplasts. Biochim Biophys Acta 767: 29–38

Gray JC (1992) Cytochrome *f*: structure, function and biosynthesis. Photosynth Res 34: 359–374

Guergova-Kuras M, Kuras R, Ugulava N, Hadad I and Crofts AR (2000) Specific mutagenesis of the rieske iron-sulfur protein in *Rhodobacter sphaeroides* shows that both the thermodynamic gradient and the pK of the oxidized form determine the rate of quinol oxidation by the bc_1 complex. Biochemistry 39: 7436–7444

Gurbiel RJ, Ohnishi T, Robertson DE, Daldal F and Hoffman BM (1991) Q-band ENDOR spectra of the Rieske protein from *Rhodobactor capsulatus* ubiquinol-cytochrome *c* oxidoreductase show two histidines coordinated to the [2Fe-2S] cluster. Biochemistry 30: 11579–11584

Hager M, Biehler K, Illerhaus J, Ruf S and Bock R (1999) Targeted inactivation of the smallest plastid genome-encoded open reading frame reveals a novel and essential subunit of the cytochrome b_6f complex. EMBO J 18: 5834–5842

Haley J and Bogorad L (1989) A 4-kDa maize chloroplast polypeptide associated with the cytochrome b_6-*f* complex: subunit 5, encoded by the chloroplast *petE* gene. Proc Natl Acad Sci USA 86: 1534–1538

Hamel P, Olive J, Pierre Y, Wollman FA and de Vitry C (2000) A new subunit of cytochrome b_6f complex undergoes reversible phosphorylation upon state transition. J Biol Chem 275: 17072–17079

Hart SE, Schlarb-Ridley BG, Bendall DS and Howe CJ (2005) Terminal oxidases of cyanobacteria. Biochem Soc Trans 33: 832–835

Hauska G (2004) The isolation of a functional cytochrome b_6f complex: from lucky encounter to rewarding experiences. Photosynth Res 80: 277–291

Hauska G, Hurt E, Gabellini N and Lockau W (1983) Comparative aspects of quinol-cytochrome *c*/plastocyanin oxidoreductases. Biochim. Biophys. Acta 726: 97–133

Hauska, G, Nitschke, W, and Herrmann, RG (1988). Amino acid identities in the three redox center-carrying polypeptides of cytochrome bc_1/b_6f complexes. J Bioenerg Biomembr 20: 211–228

Hauska G, Herold E, Huber C, Nitschke W and Sofrova D (1989) Stigmatellin affects both hemes of cytochrome *b* in cytochrome b_6f/bc_1-complexes. Z Naturforsch 44: 462–467

Heimann S, Ponamarev MV and Cramer WA (2000) Movement of the Rieske iron-sulfur protein in the p-side bulk aqueous phase: effect of lumenal viscosity on redox reactions of the cytochrome b_6f complex. Biochemistry 39: 2692–2699

Helman Y, Tchernov D, Reinhold L, Shibata M, Ogawa T, Schwarz R, Ohad I and Kaplan A (2003) Genes encoding A-type flavoproteins are essential for photoreduction of O_2 in cyanobacteria. Curr Biol 13: 230–235

Hihara Y, Sonoike K, Kanehisa M and Ikeuchi M (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 185: 1719–1725

Hill R (1954) The cytochrome *b* component of chloroplasts. Nature 174: 501–503

Hill R and Scarisbrick R (1951) The hematin compounds of leaves. New Phytol 50: 98–111

Hiller A, Henninger T, Schafer G and Schmidt CL (2003) New genes encoding subunits of a cytochrome bc_1-analogous complex in the respiratory chain of the hyperthermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*. J Bioenerg Biomembr 35: 121–131

Ho KK and Krogmann DW (1980) Cytochrome *f* from spinach and cyanobacteria. Purification and characterization. J Biol Chem 255: 3855–3861

Holt NE, Fleming GR and Niyogi KK (2004) Toward an understanding of the mechanism of nonphotochemical quenching in green plants. Biochemistry 43: 8281–8289

Holton B, Wu X, Tsapin AI, Kramer DM, Malkin R and Kallas T (1996) Reconstitution of the 2Fe-2S center and g = 1.89 electron paramagnetic resonance signal into overproduced *Nostoc* sp. PCC 7906 Rieske protein. Biochemistry 35: 15485–15493

Hope AB (1993) The chloroplast cytochrome *bf* complex: a critical focus on function. Biochim Biophys Acta 1143: 1–22

Hope AB (2000) Electron transfers amongst cytochrome *f*, plastocyanin and photosystem I: kinetics and mechanisms. Biochim Biophys Acta 1456: 5–26

Hope AB, Huilgol RR, Panizza M, Thompson M and Matthews DB (1992) The flash-induced turnover of cytochrome *b*-563, cytochrome *f* and plastocyanin in chloroplasts. Models and estimation of kinetic parameters. Biochim Biophys Acta 1100: 15–26

Houot L, Floutier M, Marteyn B, Michaut M, Picciocchi A, Legrain P, Aude JC, Cassier-Chauvat C and Chauvat F (2007) Cadmium triggers an integrated reprogramming of the metabolism of *Synechocystis* PCC6803, under the control of the Slr1738 regulator. BMC Genomics 8: 350

Howitt CA and Vermaas WF (1998) Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry 37: 17944–17951

Huang D, Everly RM, Cheng RH, Heymann JB, Schagger H, Sled V, Ohnishi T, Baker TS and Cramer WA (1994) Characterization of the chloroplast cytochrome b_6f complex as a structural and functional dimer. Biochemistry 33: 4401–4409

Huang LS, Cobessi D, Tung EY and Berry EA (2005) Binding of the respiratory chain inhibitor antimycin to the mitochondrial bc_1 complex: a new crystal structure reveals an altered intramolecular hydrogen-bonding pattern. J Mol Biol 351: 573–597

Hunsicker-Wang LM, Heine A, Chen Y, Luna EP, Todaro T, Zhang YM, Williams PA, McRee DE, Hirst J, Stout CD et al. (2003) High-resolution structure of the soluble, respiratory-type Rieske protein from *Thermus thermophilus*: analysis and comparison. Biochemistry 42: 7303–7317

Hunte C (2001) Insights from the structure of the yeast cytochrome bc_1 complex: crystallization of membrane proteins with antibody fragments. FEBS Lett 504: 126–132

Hunte C and Richers S (2008) Lipids and membrane protein structures. Curr Opin Struct Biol 18: 406–411

Hunte C, Koepke J, Lange C, Rossmanith T and Michel H (2000) Structure at 2.3 Å resolution of the cytochrome bc_1 complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment. Structure 8: 669–684

Hurt E and Hauska G (1981) A cytochrome f/b_6 complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chloroplasts. Eur J Biochem 117: 591–599

Hurt E and Hauska G (1982) Identification of the polypeptides in the cytochrome b_6f complex from spinach chloroplasts with redox center carrying subunits. J Bioenerg Biomembr 14: 405–424

Imlay JA (2006) Iron-sulphur clusters and the problem with oxygen. Mol Microbiol 59: 1073–1082

Ivanov B and Khorobrykh S (2003) Participation of photosynthetic electron transport in production and scavenging of reactive oxygen species. Antioxid Redox Signal 5: 43–53

Ivleva NB, Bramlett MR, Lindahl PA and Golden SS (2005) LdpA: a component of the circadian clock senses redox state of the cell. Embo J 24: 1202–1210

Ivleva NB, Gao T, LiWang AC and Golden SS (2006) Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. Proc Natl Acad Sci USA 103: 17468–17473

Iwai M, Takizawa K, Tokutsu R, Okamuro A, Takahashi Y and Minagawa J (2010). Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. Nature 464: 1210–1213

Iwasaki T, Imai T, Urushiyama A and Oshima T (1996) Redox-linked ionization of sulredoxin, an archaeal Rieske-type [2Fe-2S] protein from *Sulfolobus* sp. strain 7. J Biol Chem 271: 27659–27663

Iwata S, Saynovits M, Link TA and Michel H (1996) Structure of a water soluble fragment of the 'Rieske' iron-sulfur protein of the bovine heart mitochondrial cytochrome bc_1 complex determined by MAD phasing at 1.5 Å resolution. Structure 4: 567–579

Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S and Jap BK (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc_1 complex. Science 281: 64–71

Jans F, Mignolet E, Houyoux PA, Cardol P, Ghysels B, Cuine S, Cournac L, Peltier G, Remacle C and Franck F (2008) A type II NAD(P)H dehydrogenase mediates light-independent plastoquinone reduction in the chloro-

plast of *Chlamydomonas*. Proc Natl Acad Sci USA 105: 20546–20551

Joliot P and Joliot A (1984) Electron transfer between the two photosystems. I. Flash excitation under oxidizing conditions. Biochim Biophys Acta 765: 210–218

Joliot P and Joliot A (1986) Proton pumping and electron transfer in the cytochrome *b*/*f* complex of algae. Biochim Biophys Acta 849: 211–222

Joliot P and Joliot A (1988) The low-potential electron-transfer chain in the cytochrome *bf* complex. Biochim Biophys Acta 933: 319–333

Joliot P and Joliot A (1992) Electron transfer between photosystem II and the cytochrome *b*/*f* complex: mechanistic and structural implications. Biochim Biophys Acta 1102: 53–61

Joliot P and Joliot A (1994) Mechanism of electron transfer in the cytochrome *b*/*f* complex of algae: Evidence for a semiquinone cycle. Proc Natl Acad Sci USA 91: 1034–1038

Joliot P and Joliot A (1998) In vivo analysis of the effect of dicyclohexylcarbodiimide on electron and proton transfers in cytochrome *bf* complex of *Chlorella sorokiniana*. Biochemistry 37: 10404–10410

Joliot P and Joliot A (2002) Cyclic electron transfer in plant leaf. Proc Natl Acad Sci USA 99: 10209–10214

Joliot P and Joliot A (2005) Quantification of cyclic and linear flows in plants. Proc Natl Acad Sci USA 102: 4913–4918

Joliot P, Beal D and Joliot A (2004) Cyclic electron flow under saturating excitation of dark-adapted Arabidopsis leaves. Biochim Biophys Acta 1656: 166–176

Joliot P, Joliot A and Johnson G (2006) Cyclic electron transfer around photosystem I. In: Golbeck JH (ed) Photosystem I: The Light-Driven Plastocyanin:Ferredoxin Oxidoreductase, Advances in Photosynthesis and Respiration, Vol 24, pp 177–192. Springer, Dordrecht

Jones RW and Whitmarsh J (1988) Inhibition of electron transfer and the electrgenic reaction in the cytochrome *b*/*f* complex by 2-n-nonyl-4-hdroxyquinoline N-oxide (NQNO) and 2,5-dibromo-3-methuyl −6-isopropyl-p benzoquinone (DBMIB). Biochim Biophys Acta 933: 258–268

Joshua S and Mullineaux CW (2004) Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiol 135: 2112

Junge W, Sielaff H and Engelbrecht S (2009) Torque generation and elastic power transmission in the rotary F_OF_1-ATPase. Nature 459: 364–370

Kallas T (1994) The cytochrome b_6f complex. In: Bryant DA (ed) The Molecular Biology of the Cyanobacteria, Advances in Photosynthesis, Vol 1, pp 259–317. Kluwer Academic Publishers, Dordrecht

Kallas T, Spiller S and Malkin R (1988a) Characterization of two operons encoding the cytochrome b_6-*f* complex of the cyanobacterium *Nostoc* PCC 7906. Highly conserved sequences but different gene organization than in chloroplasts. J Biol Chem 263: 14334–14342

Kallas T, Spiller S and Malkin R (1988b) Primary structure of cotranscribed genes encoding the Rieske iron-sulfur protein and cytochrome *f* proteins of the cyanobacterium *Nostoc* PCC 7906. Proc Natl Acad Sci USA 85: 5794–5798

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S et al. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3: 109–136

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G and Mullineaux P (1999) Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. Science 284: 654–657

Kassner RJ (1972) Effects of nonpolar environments on the redox potentials of heme complexes. Proc Natl Acad Sci USA 69: 2263–2267

Keren N and Ohad I (1998) State transition and photoinhibition. In: Rochaix J-D, Goldschmidt-Clermont M and Merchant S (eds) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7, pp 569–596. Kluwer Academic Publishers, Dordrecht

Kerfeld CA and Krogmann DW (1998) Photosynthetic cytochromes *c* in cyanobacteria, algae, and plants. Annu Rev Plant Physiol Plant Mol Biol 49: 397–425

Kim H, Xia D, Yu CA, Xia JZ, Kachurin AM, Zhang L, Yu L and Deisenhofer J (1998) Inhibitor binding changes domain mobility in the iron-sulfur protein of the mitochondrial bc_1 complex from bovine heart. Proc Natl Acad Sci USA 95: 8026–8033

Kim H, Dashdorj N, Zhang H, Yan J, Cramer WA and Savikhin S (2005) An anomalous distance dependence of intraprotein chlorophyll-carotenoid triplet energy transfer. Biophys J 89: L28–L30

Kolling DR, Samoilova RI, Holland JT, Berry EA, Dikanov SA and Crofts AR (2003) Exploration of ligands to the Q_i site semiquinone in the bc_1 complex using high-resolution EPR. J Biol Chem 278: 39747–39754

Kondo K, Mullineaux CW and Ikeuchi M (2009) Distinct roles of CpcG1-phycobilisome and CpcG2-phycobilisome in state transitions in a cyanobacterium *Synechocystis* sp. PCC 6803. Photosynth Res 99: 217–225

Kramer DM and Crofts AR (1994) Re-examination of the properties of the *b* cytochromes of the thylakoid cytochrome *bf* complex. Biochim Biophys Acta 1184: 193–201

Kramer DM, Avenson TJ and Edwards GE (2004) Dynamic flexibility in the light reactions of photosynthesis governed

by both electron and proton transfer reactions. Trends Plant Sci 9: 349–357

Kramer DM, Nitschke W and Cooley JW (2008) The cytochrome bc_1 and related *bc* complexes: the Rieske/cytochrome *b* complex as the functional core of a central electron/proton transfer complex. In: Hunter CN, Daldal F, Thurnauer MC and Beatty JT (eds) The Purple Phototrophic Bacteria, Advances in Photosynthesis and Respiration, Vol 28, pp 451–473. Springer, Dordrecht

Kufryk GI and Vermaas WF (2006) Sll1717 affects the redox state of the plastoquinone pool by modulating quinol oxidase activity in thylakoids. J Bacteriol 188: 1286–1294

Kuila D, Schoonover JR, Dyer RB, Batie CJ, Ballou DP, Fee JA and Woodruff WH (1992) Resonance Raman studies of Rieske-type proteins. Biochim Biophys Acta 1140: 175–183

Kuras R and Wollman FA (1994) The assembly of cytochrome b_6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. EMBO J 13: 1019–1027

Kuras R, de Vitry C, Choquet Y, Girard-Bascou J, Culler D, Buschlen S, Merchant S and Wollman FA (1997) Molecular genetic identification of a pathway for heme binding to cytochrome b_6. J Biol Chem 272: 32427–32435

Kuras R, Saint-Marcoux D, Wollman FA and de Vitry C (2007) A specific *c*-type cytochrome maturation system is required for oxygenic photosynthesis. Proc Natl Acad Sci USA 104: 9906–9910

Kurisu G, Zhang H, Smith JL and Cramer WA (2003) Structure of the cytochrome b_6f complex of oxygenic photosynthesis: tuning the cavity. Science 302: 1009–1014

Laisk A (1993) Mathematical modelling of free-pool and channelled electron transport in photosynthesis: evidence for a functional supercomplex around photosystem 1. Proc Biol Sci 251: 243–251

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I and Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104: 672–677

Lange C and Hunte C (2002) Crystal structure of the yeast cytochrome bc_1 complex with its bound substrate cytochrome *c*. Proc Natl Acad Sci USA 99: 2800–2805

Lange C, Nett JH, Trumpower BL and Hunte C (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome bc_1 complex structure. EMBO J 20: 6591–6600

Lavergne J (1983) Membrane potential-dependent reduction of cytochrome b_6 in an algal mutant lacking photosystem I centers. Biochim Biophys Acta 725: 25–33

Lavergne J and Joliot P (1991) Restricted diffusion in photosynthetic membranes. Trends Biochem Sci 16: 129–134

Lebrun E, Brugna M, Baymann F, Muller D, Lievremont D, Lett MC and Nitschke W (2003) Arsenite oxidase, an ancient bioenergetic enzyme. Mol Biol Evol 20: 686–693

Lebrun E, Santini JM, Brugna M, Ducluzeau AL, Ouchane S, Schoepp-Cothenet B, Baymann F and Nitschke W (2006) The Rieske protein: a case study on the pitfalls of multiple sequence alignments and phylogenetic reconstruction. Mol Biol Evol 23: 1180–1191

Lee WJ and Whitmarsh J (1989) Photosynthetic apparatus of pea thylakoid membranes: Response to growth light intensity. Plant Physiol 89: 932–940

Lee T, Metzger SU, Cho YS, Whitmarsh J and Kallas T (2001) Modification of inhibitor binding sites in the cytochrome *bf* complex by directed mutagenesis of cytochrome b_6 in *Synechococcus* sp. PCC 7002. Biochim Biophys Acta 1504: 235–247

Lemeille S and Rochaix JD (2010) State transitions at the crossroad of thylakoid signalling pathways. Photosynth Res 106: 33–46

Lemeille S, Willig A, Depege-Fargeix N, Delessert C, Bassi R and Rochaix JD (2009) Analysis of the chloroplast protein kinase Stt7 during state transitions. PLoS Biol 7: e45

Lhee S, Kolling DR, Nair SK, Dikanov SA and Crofts AR (2010) Modifications of protein environment of the [2Fe-2S] cluster of the bc_1 complex: Effects on the biophysical properties of the rieske iron-sulfur protein and on the kinetics of the complex. J Biol Chem 285: 9233–9248

Li H and Sherman LA (2000) A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 182: 4268–4277

Li H, Singh AK, McIntyre LM and Sherman LA (2004) Differential gene expression in response to hydrogen peroxide and the putative PerR regulon of *Synechocystis* sp. strain PCC 6803. J Bacteriol 186: 3331–3345

Li Z, Wakao S, Fischer BB and Niyogi KK (2009). Sensing and responding to excess light. Annu Rev Plant Biol 60: 239–260

Lieutaud C, Alric J, Bauzan M, Nitschke W and Schoepp-Cothenet B (2005) Study of the high-potential iron sulfur protein in *Halorhodospira halophila* confirms that it is distinct from cytochrome *c* as electron carrier. Proc Natl Acad Sci USA 102: 3260–3265

Link TA, Hagen WR, Pierik AJ, Assmann C and von Jagow G (1992) Determination of the redox properties of the Rieske [2Fe-2S] cluster of bovine heart bc_1 complex by direct electrochemistry of a water-soluble fragment. Eur J Biochem 208: 685–691

Link TA, Hatzfeld OM, Unalkat P, Shergill JK, Cammack R and Mason JR (1996) Comparison of the "Rieske" [2Fe-2S] center in the bc_1 complex and in bacterial dioxygenases by circular dichroism spectroscopy and cyclic voltammetry. Biochemistry 35: 7546–7552

Livingston AK, Cruz JA, Kohzuma K, Dhingra A and Kramer DM (2010) An *Arabidopsis* mutant with high

cyclic electron flow around photosystem I (*hcef*) involving the NADPH dehydrogenase complex. Plant Cell 22: 221–233

Louro RO, Bento I, Matias PM, Catarino T, Baptista AM, Soares CM, Carrondo MA, Turner DL and Xavier AV (2001) Conformational component in the coupled transfer of multiple electrons and protons in a monomeric tetraheme cytochrome. J Biol Chem 276: 44044–44051

Mackey KRM, Payton A, Grossman A and Bailey S (2008) A photosynthetic strategy for coping in a high-light, low-nutrient environment. Limnol Oceanogr 53: 900–913

Madueno F, Napier JA, Cejudo FJ and Gray JC (1992). Import and processing of the precursor of the Rieske FeS protein of tobacco chloroplasts. Plant Mol Biol 20: 289–299

Maiwald D, Dietzmann A, Jahns P, Pesaresi P, Joliot P, Joliot A, Levin JZ, Salamini F and Leister D (2003) Knock-out of the genes coding for the Rieske protein and the ATP-synthase δ-subunit of Arabidopsis. Effects on photosynthesis, thylakoid protein composition, and nuclear chloroplast gene expression. Plant Physiol 133: 191–202

Majeran W, Wollman FA and Vallon O (2000) Evidence for a role of ClpP in the degradation of the chloroplast cytochrome b_6f complex. Plant Cell 12: 137–150

Malkin R (1981) Redox properties of the DBMIB-Rieske iron-sulfur complex in spinach chloroplast membranes. FEBS Lett 131: 169–172

Malkin R (1982) Interaction of photosynthetic electron transport inhibitors and the Rieske Iron-Sulfur center in chloroplasts and the cytochrome b_6-f complex. Biochem 21: 2945–2950

Malkin R (1992) Cytochrome-bc_1 and cytochrome-b_6f complexes of photosynthetic membranes. Photosynth Res 33: 121–136

Malkin R and Aparicio PJ (1975) Identification of a g = 1.90 high-potential iron-sulfur protein in chloroplasts. Biochem Biophys Res Commun 63: 1157–1160

Malkin R, Adam Z, Chain R, Kallas T, Spiller S and Willms I (1988) Biochemical and molecular biological approaches to the study of the thylakoid cytochrome b_6-f complex. In: Stevens SEJ and Bryant DA (eds) Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models, pp 274–289. American Society of Plant Physiologists, Rockville

Malnoë A, Wollman F-A, de Vitry C and Rappaport F (2011) Photosynthetic growth despite a broken Q-cycle. Nat Commun 2: 301. doi: 10. 1038/ncomms1299

Martinez SE, Huang D, Szczepaniak A, Cramer WA and Smith JL (1994) Crystal structure of chloroplast cytochrome *f* reveals a novel cytochrome fold and unexpected heme ligation. Structure 2: 95–105

Martinez SE, Huang D, Ponomarev M, Cramer WA, and Smith JL (1996) The heme redox center of chloroplast cytochrome *f* is linked to a buried five-water chain. Protein Sci 5: 1081–1092

McConnell MD, Koop R, Vasil'ev S and Bruce D (2002) Regulation of the distribution of chlorophyll and phycobilin-absorbed excitation energy in cyanobacteria. A structure-based model for the light state transition. Plant Physiol 130: 1201–1212

Mehler AH (1951) Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch Biochem 33: 65–77

Merbitz-Zahradnik T, Zwicker K, Nett JH, Link TA and Trumpower BL (2003) Elimination of the disulfide bridge in the Rieske iron-sulfur protein allows assembly of the [2Fe-2S] cluster into the Rieske protein but damages the ubiquinol oxidation site in the cytochrome bc_1 complex. Biochemistry 42: 13637–13645

Merchant S and Dreyfuss BW (1998) Posttranslational assembly of photosynthetic metalloproteins. Annu Rev Plant Physiol Plant Mol Biol 49: 25–51

Meyer TE and Kamen MD (1982). New perspectives on *c*-type cytochromes. Adv Protein Chem 35: 105–212

Mi HL, Endo T, Schreiber U, Ogawa T and Asada K (1994) NAD(P)H dehydrogenase-dependent cyclic electron flow around photosystem I in the cyanobacterium *Synechocystis* PCC 6803. Plant Cell Physiol 35: 163–173

Mi H, Klughammer C and Schreiber U (2000) Light-induced dynamic changes of NADPH fluorescence in *Synechocystis* PCC 6803 and its *ndhB*-defective mutant M55. Plant Cell Physiol 41: 1129–1135

Mitchell P (1975) The protonmotive Q cycle: a general formulation. FEBS Lett 59: 137–139

Mitchell P (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. J Theor Biol 62: 327–367

Moser CC, Keske JM, Warncke K, Farid RS and Dutton PL (1992) Nature of biological electron transfer. Nature 355: 796–802

Moser CC, Page CC, Farid R and Dutton PL (1995) Biological electron transfer. J Bioenerg Biomembr 27: 263–274

Moss DA and Bendall DS (1984) Cyclic electron transport in chloroplasts. The Q-cycle and the site of action of antimycin. Biochim Biophys Acta 767: 389–395

Muller F (2000) The nature and mechanism of superoxide production by the electron transport chain: Its relevance to aging. J Amer Aging Assoc 23: 227–253

Muller F, Crofts AR and Kramer DM (2002) Multiple Q-cycle bypass reactions at the Q_o site of the cytochrome bc_1 complex. Biochemistry 41: 7866-7874

Mullineaux CW (2008) Phycobilisome-reaction centre interaction in cyanobacteria. Photosynth Res 95: 175–182

Mullineaux CW, Tobin MJ and Jones MR (1997) Mobility of photosynthetic complexes in thylakoid membranes. Nature 390: 421–424

Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M and Shikanai T (2002) *PGR5* is involved in cyclic electron flow around photosystem I and is essential for photoprotection in *Arabidopsis*. Cell 110: 361–371

Munekage Y, Hashimoto M, Miyake C, Tomizawa K, Endo T, Tasaka M and Shikanai T (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature 429: 579–582

Murata N (2009) The discovery of state transitions in photosynthesis 40 years ago. Photosynth Res 99: 155–160

Nakamura K and Hihara Y (2006) Photon flux density-dependent gene expression in *Synechocystis* sp. PCC 6803 is regulated by a small, redox-responsive, LuxR-type regulator. J Biol Chem 281: 36758–36766

Nandha B, Finazzi G, Joliot P, Hald S and Johnson GN (2007) The role of *PGR5* in the redox poising of photosynthetic electron transport. Biochim Biophys Acta 1767: 1252–1259

Navarro JA, Duran RV, De la Rosa MA and Hervas M (2005) Respiratory cytochrome *c* oxidase can be efficiently reduced by the photosynthetic redox proteins cytochrome c_6 and plastocyanin in cyanobacteria. FEBS Lett 579: 3565–3568

Nelson ME, Finazzi G, Wang QJ, Middleton-Zarka KA, Whitmarsh J and Kallas T (2005) Cytochrome b_6 arginine 214 of *Synechococcus* sp. PCC 7002, a key residue for quinone-reductase site function and turnover of the cytochrome *bf* complex. J Biol Chem 280: 10395–10402

Nelson N and Neumann J (1972) Isolation of a cytochrome b_6-*f* particle from chloroplasts. J Biol Chem 247: 1817–1824

Nett JH, Hunte C and Trumpower BL (2000) Changes to the length of the flexible linker region of the Rieske protein impair the interaction of ubiquinol with the cytochrome bc_1 complex. Eur J Biochem 267: 5777–5782

Nitschke W and Hauska G (1987) On the nature of the g = 6 EPR signal in isolated cytochrome b_6f complex from spinach chloroplasts. Biochim Biophys Acta 892: 314–219

Nitschke W, Hauska G and Crofts AR (1988) Fast electron transfer from low- to high-potential cytochrome b_6 in isolated cytochrome b_6f complex. FEBS Lett 232: 204–208

Nitschke W, Joliot P, Liebl U, Rutherford AW, Hauska G, Muller A and Riedel A (1992) The pH dependence of the redox midpoint potential of the 2Fe2S cluster from cytochrome b_6f complex (the 'Rieske center'). Biochim Biophys Acta 1102: 266–268

Nitschke W, Van Lis R, Schoepp-Cothenet B and Baymann F (2010) The "green" phylogenetic clade of Rieske/cyt*b* complexes. Photosynth Res 104: 347–355

Nixon PJ (2000) Chlororespiration. Philos Trans R Soc Lond B Biol Sci 355: 1541–1547

Niyogi KK (1999) Photoprotection revisited: Genetic and molecular approaches. Annu Rev Plant Physiol Plant Mol Biol 50: 333–359

Nomura CT, Persson S, Shen G, Inoue-Sakamoto K and Bryant DA (2006a) Characterization of two cytochrome oxidase operons in the marine cyanobacterium *Synechococcus* sp. PCC 7002: inactivation of *ctaDI* affects the PS I:PS II ratio. Photosynth Res 87: 215–228

Nomura CT, Sakamoto T and Bryant DA (2006b) Roles for heme-copper oxidases in extreme high-light and oxidative stress response in the cyanobacterium *Synechococcus* sp. PCC 7002. Arch Microbiol 185: 471–479

Ochman H, Lawrence JG and Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. Nature 405: 299–304

Ohnishi T, Brandt U and von Jagow G (1988) Studies on the effect of stigmatellin derivatives on cytochrome *b* and the Rieske iron-sulfur cluster of cytochrome *c* reductase from bovine heart mitochondria. Eur J Biochem 176: 385–389

Osiewacz HD (1994) Construction of insertion mutants of *Synechocystis* sp. PCC 6803: evidence for an essential function of subunit IV of the cytochrome b_6/f complex. Arch Microbiol 157: 336–342

Osyczka A, Moser CC, Daldal F and Dutton PL (2004) Reversible redox energy coupling in electron transfer chains. Nature 427: 607–612

Ouchane S, Nitschke W, Bianco P, Vermeglio A and Astier C (2005) Multiple Rieske genes in prokaryotes: exchangeable Rieske subunits in the cytochrome *bc*-complex of *Rubrivivax gelatinosus*. Mol Microbiol 57: 261–275

Ouyang Y, Horn D, Grebe R, Guo LW, Nelson M, Wang Q, Whitmarsh J, Finazzi G and Kallas T (2004) Mutational analysis of the cytochrome *bf* Rieske iron-sulfur protein and quinone-reductase (Q_i) site. In: Van der Est A and Bruce D (eds) Photosynthesis: Fundamental Aspects to Global Perspectives, pp 434–426. Allen Press, Lawrence, Kansas

Page CC, Moser CC, Chen X and Dutton PL (1999) Natural engineering principles of electron tunnelling in biological oxidation-reduction. Nature 402: 47–52

Peterman EJ, Wenk SO, Pullerits T, Palsson LO Van Grondelle R, Dekker JP, Rögner M and Van Amerongen H (1998) Fluorescence and absorption spectroscopy of the weakly fluorescent chlorophyll *a* in cytochrome b_6f of *Synechocystis* PCC6803. Biophys J 75: 389–398

Pfannschmidt T, Nilsson A and Allen JF (1999a) Photosynthetic control of chloroplast gene expression. Nature 397: 625–628

Pfannschmidt T, Nilsson A, Tullberg A, Link G and Allen JF (1999b) Direct transcriptional control of the chloroplast genes *psbA* and *psaAB* adjusts photosynthesis to light energy distribution in plants. IUBMB Life 48: 271–276

Pfannschmidt T, Brautigam K, Wagner R, Dietzel L, Schroter Y, Steiner S and Nykytenko A (2008) Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. Ann Bot 103: 599–607

Pierre Y, Breyton C, Kramer D and Popot J-L (1995) Purification and characterization of the cytochrome b_6f complex from *Chlamydomonas reinhardtii*. J Biol Chem 270: 29342–29349

Pierre Y, Breyton C, Lemoine Y, Robert B, Vernotte C and Popot JL (1997) On the presence and role of a molecule of chlorophyll *a* in the cytochrome b_6f complex. J Biol Chem 272: 21901–21908

Piippo M, Allahverdiyeva Y, Paakkarinen V, Suoranta UM, Battchikova N and Aro EM (2006) Chloroplast-mediated regulation of nuclear genes in *Arabidopsis thaliana* in the absence of light stress. Physiol Genomics 25: 142–152

Pils D, Wilken C, Valladares A, Flores E and Schmetterer G (2004) Respiratory terminal oxidases in the facultative chemoheterotrophic and dinitrogen fixing cyanobacterium *Anabaena variabilis* strain ATCC 29413: characterization of the *cox2* locus. Biochim Biophys Acta 1659: 32–45

Ponamarev MV and Cramer WA (1998) Perturbation of the internal water chain in cytochrome *f* of oxygenic photosynthesis: loss of the concerted reduction of cytochromes *f* and b_6. Biochemistry 37: 17199–17208

Ponamarev MV, Schlarb BG, Howe CJ, Carrell CJ, Smith JL, Bendall DS and Cramer WA (2000) Tryptophan-heme pi-electrostatic interactions in cytochrome *f* of oxygenic photosynthesis. Biochemistry 39: 5971–5976

Price GD, Yu J-W, von Caemmerer S, Evans JR, Chow WS, Anderson JM, Hurry V and Badger MR (1995) Chloroplast cytochrome b_6/f and ATP synthase complexes in tobacco: transformation with antisense RNA against nuclear-encoded transcripts for the Rieske FeS and ATPδ polypeptides. Aus J Plant Physiol 22: 285–297

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, Van der Giezen M, Rogers MB and Allen JF (2008) The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proc Natl Acad Sci USA 105: 10061–10066

Rego AC and Oliveira CR (2003) Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: Implications for the pathogenesis of neurodegenerative diseases. Neurochem Res 28: 1563–1574

Rich PR (1984) Electron and proton transfers through quinones and cytochrome *bc* complexes. Biochim Biophys Acta 768: 53–79

Rieske JS, Zaugg WS and Hansen RE (1964) Studies on the electron transfer system. LIX. Distribution of iron and of the component giving an electron paramagnetic resonance signal at g = 1.90 in subfractions of complex 3. J Biol Chem 239: 3023–3030

Roberts AG, Bowman MK and Kramer DM (2002) Certain metal ions are inhibitors of cytochrome b_6f complex 'Rieske' iron-sulfur protein domain movements. Biochemistry 41: 4070–4079

Roberts AG, Bowman MK and Kramer DM (2004) The inhibitor DBMIB provides insight into the functional architecture of the Q_o site in the cytochrome b_6f complex. Biochemistry 43: 7707–7716

Ryan CM, Souda P, Bassilian S, Ujwal R, Zhang J, Abramson J, Ping P, Durazo A, Bowie JU, Hasan SS et al. (2010) Post-translational modifications of integral membrane proteins resolved by top-down Fourier transform mass spectrometry with collisionally activated dissociation. Mol Cell Proteomics 9: 791–803

Sacksteder CA, Kanazawa A, Jacoby ME and Kramer DM (2000) The proton to electron stoichiometry of steady-state photosynthesis in living plants: A proton-pumping Q cycle is continuously engaged. Proc Natl Acad Sci USA 97: 14283–14288

Saint-Marcoux D, Wollman FA and de Vitry C (2009) Biogenesis of cytochrome b_6 in photosynthetic membranes. J Cell Biol 185: 1195–1207

Sainz G, Carrell CJ, Ponamarev MV, Soriano GM, Cramer WA and Smith JL (2000) Interruption of the internal water chain of cytochrome *f* impairs photosynthetic function. Biochemistry 39: 9164–9173

Schagger H and Pfeiffer K (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 19: 1777–1783

Scherer S (1990) Do photosynthetic and respiratory electron transport chains share common redox proteins? Trends Biol Sci 15: 458–462

Schettle S and Kallas T (2007) Isoforms of the major PetC1 Rieske iron-sulfur protein of photosynthesis in a unicellular cyanobacterium. Oshkosh Scholar 2: 77–85

Schmetterer G (1994) Cyanobacterial respiration. In: Bryant DA (ed) The Molecular Biology of Cyanobacteria, Advances in Photosynthesis, Vol 1, pp 409–435. Kluwer Academic Publishers, Dordrecht

Schmidt CL and Malkin R (1993) Low molecular weight subunits associated with the cytochrome b_6f complexes from spinach and *Chlamydomonas reinhardtii*. Photosynth Res 38: 73–81

Schmidt CL and Shaw L (2001) A comprehensive phylogenetic analysis of Rieske and Rieske-type iron-sulfur proteins. J Bioenerg Biomembr 33: 9–26

Schneider D and Schmidt CL (2005) Multiple Rieske proteins in prokaryotes: where and why? Biochim Biophys Acta 1710: 1–12

Schneider D, Berry S, Rich P, Seidler A and Rögner, M (2001). A regulatory role of the PetM subunit in a cyanobacterial cytochrome b_6f complex. J Biol Chem 276: 16780–16785

Schneider D, Skrzypczak S, Anemuller S, Schmidt CL, Seidler A and Rögner, M (2002). Heterogeneous Rieske proteins in the cytochrome b_6f complex of *Synechocystis* PCC6803? J Biol Chem 277: 10949–10954

Schneider D, Berry S, Volkmer T, Seidler A and Rögner M (2004) PetC1 is the major Rieske iron-sulfur protein in the cytochrome b_6f complex of *Synechocystis* sp. PCC 6803. J Biol Chem 279: 39383–39388

Schneider D, Volkmer T and Rögner, M (2007) PetG and PetN, but not PetL, are essential subunits of the cytochrome b_6f complex from *Synechocystis* PCC 6803. Res Microbiol 158: 45–50

Schoepp B, Parot P, Menin L, Gaillard J, Richaud P and Vermeglio A (1995) In vivo participation of a high potential iron-sulfur protein as electron donor to the

photochemical reaction center of *Rubrivivax gelatinosus*. Biochemistry 34: 11736–11742

Schoepp B, Brugna M, Riedel A, Nitschke W and Kramer DM (1999) The Q_o-site inhibitor DBMIB favours the proximal position of the chloroplast Rieske protein and induces a pK-shift of the redox-linked proton. FEBS Lett 450: 245–250

Schoepp-Cothenet B, Lieutaud C, Baymann F, Vermeglio A, Friedrich T, Kramer DM and Nitschke W (2009) Menaquinone as pool quinone in a purple bacterium. Proc Natl Acad Sci USA 106: 8549–8554

Schottler MA, Flugel C, Thiele W and Bock R (2007) Knock-out of the plastid-encoded PetL subunit results in reduced stability and accelerated leaf age-dependent loss of the cytochrome b_6f complex. J Biol Chem 282: 976–985

Schultze M, Forberich B, Rexroth S, Dyczmons NG, Rögner M and Appel J (2009) Localization of cytochrome b_6f complexes implies an incomplete respiratory chain in cytoplasmic membranes of the cyanobacterium *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 1787: 1479–1485

Schütz M, Zirngibl S, Coutre J, Büttner M, Xie D-L, Nelson N, Deutzmann R and Hauska G (1994). A transcription unit for the Rieske FeS-protein and cytochrome *b* in *Chlorobium limicola*. Photosynth Res 39: 163–174

Schütz M, Brugna M, Lebrun E, Baymann F, Huber R, Stetter KO, Hauska G, Toci R, Lemesle-Meunier D, Tron P et al. (2000) Early evolution of cytochrome *bc* complexes. J Mol Biol 300: 663–675

Schütz M, Schoepp-Cothenet B, Lojou E, Woodstra M, Lexa D, Tron P, Dolla A, Durand MC, Stetter KO and Baymann F (2003). The naphthoquinol oxidizing cytochrome bc_1 complex of the hyperthermophilic knallgasbacterium *Aquifex aeolicus*: properties and phylogenetic relationships. Biochemistry 42: 10800–10808

Schwenkert S, Legen J, Takami T, Shikanai T, Herrmann RG and Meurer J (2007) Role of the low-molecular-weight subunits PetL, PetG, and PetN in assembly, stability, and dimerization of the cytochrome b_6f complex in tobacco. Plant Physiol 144: 1924–1935

Selak MA and Whitmarsh J (1982) Kinetics of the electrogenic step and cytochrome b_6 and *f* redox changes in chloroplasts. FEBS Lett 150: 286–292

Shcolnick S, Summerfield TC, Reytman L, Sherman LA and Keren N (2009) The mechanism of iron homeostasis in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and its relationship to oxidative stress. Plant Physiol 150: 2045–2056

Singh AK and Sherman LA (2007) Reflections on the function of IsiA, a cyanobacterial stress-inducible, Chl-binding protein. Photosynth Res 93: 17–25

Singh AK, Li H, Bono L and Sherman LA (2005) Novel adaptive responses revealed by transcription profiling of a *Synechocystis* sp. PCC 6803 delta-*isiA* mutant in the presence and absence of hydrogen peroxide. Photosynth Res 84: 65–70

Smith DR and Lee RW (2009) Nucleotide diversity of the *Chlamydomonas reinhardtii* plastid genome: addressing the mutational-hazard hypothesis. BMC Evol Biol 9: 120

Smith JL, Zhang H, Yan J, Kurisu G and Cramer WA (2004) Cytochrome *bc* complexes: a common core of structure and function surrounded by diversity in the outlying provinces. Curr Opin Struct Biol 14: 432–439

Snyder CH, Merbitz-Zahradnik T, Link TA and Trumpower BL (1999) Role of the Rieske iron-sulfur protein midpoint potential in the protonmotive Q-cycle mechanism of the cytochrome bc_1 complex. J Bioenerg Biomembr 31: 235–242

Soriano GM, Ponamarev MV, Piskorowski RA, and Cramer WA (1998) Identification of the basic residues of cytochrome *f* responsible for electrostatic docking interactions with plastocyanin in vitro: Relevance to the electron transfer reaction in vivo. Biochemistry 37: 15120–15128

Soriano GM, Ponamarev MV, Carrell CJ, Xia D, Smith JL and Cramer WA (1999) Comparison of the cytochrome bc_1 complex with the anticipated structure of the cytochrome b_6f complex: Le plus ca change le plus c'est la meme chose. J Bioenerg Biomembr 31: 201–213

Soriano GM, Smith JL and Cramer WA (2001) Cytochrome *f*. In: Messerschmidt A, Huber R, Wieghardt K and Poulos T (eds) Handbook of Metalloproteins, pp 172–181. Wiley, London

Soriano GM, Guo LW, de Vitry C, Kallas T and Cramer WA (2002) Electron transfer from the Rieske iron-sulfur protein (ISP) to cytochrome *f* in vitro. Is a guided trajectory of the ISP necessary for competent docking? J Biol Chem 277: 41865–41871

Stirewalt VL and Bryant DA (1989) Molecular cloning and nucleotide sequence of the *petG* gene of the cyanelle genome of *Cyanophora paradoxa*. Nucleic Acids Res 17: 10095

Streb P, Josse EM, Gallouet E, Baptist F, Kuntz M and Cornic G (2005) Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*. Plant Cell Environ 28: 1123–1135

Stroebel D, Choquet Y, Popot JL and Picot D (2003) An atypical haem in the cytochrome b_6f complex. Nature 426: 413–418

Stuart RA (2008) Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. J Bioenerg Biomembr 40: 411–417

Summerfield TC, Toepel J and Sherman LA (2008) Low-oxygen induction of normally cryptic *psbA* genes in cyanobacteria. Biochemistry 47: 12939–12941

Sun J and Trumpower BL (2003) Superoxide anion generation by the cytochrome bc_1 complex. Arch Biochem Biophys 419: 198–206

Swierczek M, Cieluch E, Sarewicz M, Borek A, Moser CC, Dutton PL and Osyczka A (2010) An electronic bus bar lies in the core of cytochrome bc_1. Science 329: 451–454

Takahashi Y, Rahire M, Breyton C, Popot JL, Joliot P and Rochaix JD (1996) The chloroplast *ycf7* (*petL*) open reading frame of *Chlamydomonas reinhardtii* encodes a small functionally important subunit of the cytochrome b_6f complex. EMBO J 15: 3498–3506

Tanaka T, Inoue M, Sakamoto J and Sone N (1996) Intra- and inter-complex cross-linking of subunits in the quinol oxidase super-complex from thermophilic *Bacillus* PS3. J Biochem 119: 482–486

Thierbach G, Kunze B, Reichenbach H, and Hôfle G (1984) The mode of action of stigmatellin, a new inhibitor of the cytochrome b-c_1 segment of the respiratory chain. Biochim Biophys Acta 765: 227–235

Thony-Meyer L, James P and Hennecke H (1991) From one gene to two proteins: the biogenesis of cytochromes b and c_1 in *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 88: 5001–5005

Tian H, Yu L, Mather MW and Yu CA (1998) Flexibility of the neck region of the Rieske iron-sulfur protein is functionally important in the cytochrome bc_1 complex. J Biol Chem 273: 27953–27959

Tian H, White S, Yu L and Yu CA (1999) Evidence for the head domain movement of the Rieske iron-sulfur protein in electron transfer reaction of the cytochrome bc_1 complex. J Biol Chem 274: 7146–7152

Trebst A (2007) Inhibitors in the functional dissection of the photosynthetic electron transport system. Photosynth Res 92: 217–224

Trebst A, Harth E and Draber W (1970) On a new inhibitor of photosynthetic electron-transport in isolated chloroplasts. Z Naturforsch 25b: 1157–1159

Trebst A, Wietoska H, Draber W and Knops HJ (1978) The inhibition of photosynthetic electron flow in chloroplasts by the dinitrophenylether of bromo or iodonitrothynol. Z Naturforsch 33c: 919–927

Tsukihara T, Fukuyama K and Katsube Y (1986) Structure-function relationship of [2Fe-2S] ferredoxins. In: Matsubara H (ed) Iron-Sulfur Protein Research, pp 59–68. Japan Sci Soc Press/Springer Verlag, Tokyo

Tsunoyama Y, Bernat G, Dyczmons NG, Schneider D and Rögner M (2009) Multiple Rieske proteins enable short- and long-term light adaptation of *Synechocystis* sp. PCC 6803. J Biol Chem 284: 27875–27883

Twigg AI, Baniulis D, Cramer WA and Hendrich MP (2009) EPR detection of an O_2 surrogate bound to heme c_n of the cytochrome b_6f complex. J Am Chem Soc 131: 12536–12537

Ugulava NB and Crofts AR (1998) CD-monitored redox titration of the Rieske Fe-S protein of *Rhodobacter sphaeroides*: pH dependence of the midpoint potential in isolated bc_1 complex and in membranes. FEBS Lett 440: 409–413

Vallon O, Bulte L, Dainese P, Olive J, Bassi R and Wollman FA (1991) Lateral redistribution of cytochrome b_6/f complexes along thylakoid membranes upon state transitions. Proc Natl Acad Sci USA 88: 8262–8266

Van Baalen C (1962) Studies on marine blue-green algae. Bot Marina 4: 129–139

Vener AV, Van Kan PJ, Gal A, Andersson B and Ohad I (1995) Activation/deactivation cycle of redox-controlled thylakoid protein phosphorylation. Role of plastoquinol bound to the reduced cytochrome *bf* complex. J Biol Chem 270: 25225–25232

Vener AV, Van Kan PJ, Rich PR, Ohad I and Andersson B (1997) Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: thylakoid protein kinase deactivation by a single turnover flash. Proc Natl Acad Sci USA 94: 1585–1590

Volkmer T, Schneider D, Bernat G, Kirchhoff H, Wenk SO and Rögner, M (2007) Ssr2998 of *Synechocystis* sp. PCC 6803 is involved in regulation of cyanobacterial electron transport and associated with the cytochrome b_6f complex. J Biol Chem 282: 3730–3737

Wastl J, Bendall DS and Howe CJ (2002) Higher plants contain a modified cytochrome c_6. Trends Plant Sci 7: 244–245

Weir GLIV, Short K and Kallas T (2009) Global gene expression in cyanobacterial electron transfer mutants. Oshkosh Scholar 4: 17–27

Wenk SO, Schneider D, Boronowsky U, Jager C, Klughammer C, de Weerd FL, Van Roon H, Vermaas WF, Dekker JP and Rögner, M (2005) Functional implications of pigments bound to a cyanobacterial cytochrome b_6f complex. FEBS J 272: 582–592

Wenz T, Hielscher R, Hellwig P, Schagger H, Richers S and Hunte C (2009) Role of phospholipids in respiratory cytochrome bc_1 complex catalysis and supercomplex formation. Biochim Biophys Acta 1787: 609–616

White CC, Chain RK and Malkin R (1978) Duroquinol as an electron donor for chloroplast electron transfer reactions. Biochim Biophys Acta 502: 127–137

Whitelegge JP, Zhang H, Aguilera R, Taylor RM and Cramer WA (2002) Full subunit coverage liquid chromatography electrospray ionization mass spectrometry (LCMS+) of an oligomeric membrane protein: cytochrome b_6f complex from spinach and the cyanobacterium *Mastigocladus laminosus*. Mol Cell Proteomics 1: 816–827

Widger WR, Cramer WA, Herrmann RG and Trebst A (1984) Sequence homology and structural similarity between cytochrome b of mitochondrial complex III and the chloroplast *bf* complex: position of the cytochrome b hemes in the membrane. Proc Natl Acad Sci USA 81: 674–678

Willms I, Malkin R and Chain RK (1988) Quinone interactions with the chloroplast cytochrome b_6-f complex. Arch Biochem Biophys 263: 36–44

Witt HT (1971) Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. Results by pulse spectroscopic methods. Q Rev Biophys 4: 365–477

Wollman FA (1999) The structure, function and biogenesis of cytochrome b_6f complexes. In: Rochaix JD, Goldschmidt-Clermont M and Merchant S (eds) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7, pp 459–476. Kluwer Academic Publishers, Dordrecht

Wollman FA (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J 20: 3623–3630

Wollman F-A and Lemaire C (1988) Studies on kinase-controlled state transitions in photosystem II and b_6f mutants from *Chlamydomonas reinhardtii* which lack quinone-binding proteins. Biochim Biophys Acta 85: 85–94

Wood PM and Bendall DS (1976) The reduction of plastocyanin by plastoquinol-1 in the presence of chloroplasts: a dark electron transfer reaction involving components between the two photosystems. Eur. J. Biochem. 61: 337–344

Worrall JA, Luisi BF, Schlarb-Ridley BG, Bendall DS and Howe CJ (2008) Cytochrome c_{6A}: discovery, structure and properties responsible for its low haem redox potential. Biochem Soc Trans 36: 1175–1179

Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, Yu L and Deisenhofer J (1997) Crystal structure of the cytochrome bc_1 complex from bovine heart mitochondria. Science 277: 60–66

Xiao K, Yu L and Yu CA (2000) Confirmation of the involvement of protein domain movement during the catalytic cycle of the cytochrome bc_1 complex by the formation of an intersubunit disulfide bond between cytochrome *b* and the iron-sulfur protein. J Biol Chem 275: 38597–38604

Yamashita E, Zhang H and Cramer WA (2007) Structure of the cytochrome b_6f complex: quinone analogue inhibitors as ligands of heme c_n. J Mol Biol 370: 39–52

Yan J and Cramer WA (2003) Functional insensitivity of the cytochrome b_6f complex to structure changes in the hinge region of the Rieske iron-sulfur protein. J Biol Chem 278: 20925–20933

Yan J and Cramer WA (2004) Molecular control of a bimodal distribution of quinone-analogue inhibitor binding sites in the cytochrome b_6f complex. J Mol Biol 344: 481–493

Yan J, Kurisu G and Cramer WA (2006) Intraprotein transfer of the quinone analogue inhibitor 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone in the cytochrome b_6f complex. Proc Natl Acad Sci USA 103: 69–74

Yan J, Dashdorj N, Baniulis D, Yamashita E, Savikhin S and Cramer WA (2008) On the structural role of the aromatic residue environment of the chlorophyll *a* in the cytochrome b_6f complex. Biochemistry 47: 3654–3661

Yang S, Zhang R, Hu C, Xie J and Zhao J (2009) The dynamic behavior of phycobilisome movement during light state transitions in cyanobacterium *Synechocystis* PCC6803. Photosynth Res 99: 99–106

Yeremenko N, Jeanjean R, Prommeenate P, Krasikov V, Nixon PJ, Vermaas WF, Havaux M and Matthijs HC (2005) Open reading frame ssr2016 is required for antimycin A-sensitive photosystem I-driven cyclic electron flow in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol 46: 1433–1436

Yu J, Hederstedt L and Piggot PJ (1995) The cytochrome *bc* complex (menaquinone:cytochrome *c* reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J Bacteriol 177: 6751–6760

Yu L, Zhao J, Muhlenhoff U, Bryant DA and Golbeck JH (1993) PsaE is required for in vivo cyclic electron flow around photosystem I in the cyanobacterium *Synechococcus* sp. PCC 7002. Plant Physiol 103: 171–180

Yu S-G, Romanowska E, Xue Z-T and Albertsson P-A (1994) Evidence for two different Rieske iron-sulfur proteins in the cytochrome *bf* complex of spinach chloroplast. Biochim Biophys Acta 1185: 239–242

Zara V, Conte L and Trumpower BL (2009) Biogenesis of the yeast cytochrome bc_1 complex. Biochim Biophys Acta 1793: 89-96

Zatsman AI, Zhang H, Gunderson WA, Cramer WA and Hendrich MP (2006) Heme-heme interactions in the cytochrome b_6f complex: EPR spectroscopy and correlation with structure. J Am Chem Soc 128: 14246-14247

Zhang Z, Huang L, Shulmeister VM, Chi YI, Kim KK, Hung LW, Crofts AR, Berry EA, and Kim SH (1998) Electron transfer by domain movement in cytochrome bc_1. Nature 392: 677–684

Zhang H, Huang D and Cramer WA (1999) Stoichiometrically bound beta-carotene in the cytochrome b_6f complex of oxygenic photosynthesis protects against oxygen damage. J Biol Chem 274: 1581–1587

Zhang H, Whitelegge JP and Cramer WA (2001) Ferredoxin:$NADP^+$ oxidoreductase is a subunit of the chloroplast cytochrome b_6f complex. J Biol Chem 276: 38159–38165

Zhang H, Kurisu G, Smith JL and Cramer WA (2003) A defined protein-detergent-lipid complex for crystallization of integral membrane proteins: The cytochrome b_6f complex of oxygenic photosynthesis. Proc Natl Acad Sci USA 100: 5160–5163

Zhang H, Primak A, Cape J, Bowman MK, Kramer DM and Cramer WA (2004a) Characterization of the high-spin heme *x* in the cytochrome b_6f complex of oxygenic photosynthesis. Biochemistry 43: 16329–16336

Zhang P, Battchikova N, Jansen T, Appel J, Ogawa T and Aro EM (2004b). Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition

complex NdhD3/NdhF3/CupA/Sll1735 in *Synechocystis* sp. PCC 6803. Plant Cell 16: 3326–3340

Zhang H, Osyczka A, Dutton PL and Moser CC (2007) Exposing the complex III Q_o semiquinone radical. Biochim Biophys Acta 1767: 883–887

Zhou J, Fernandez-Velasco JG and Malkin R (1996) N-terminal mutants of chloroplast cytochrome *f*. Effect on redox reactions and growth in *Chlamydomonas reinhardtii*. J Biol Chem 271: 6225–6232

Zito F, Finazzi G, Delosme R, Nitschke W, Picot D and Wollman FA (1999) The Q_o site of cytochrome b_6f complexes controls the activation of the LHCII kinase. Embo J 18: 2961–2969

Zito F, Finazzi G, Joliot P and Wollman FA (1998) Glu78, from the conserved PEWY sequence of subunit IV, has a key function in cytochrome b_6f turnover. Biochemistry 37: 10395–10403

Zito F, Vinh J, Popot JL and Finazzi G (2002) Chimeric fusions of subunit IV and PetL in the b_6f complex of *Chlamydomonas reinhardtii*: structural implications and consequences on state transitions. J Biol Chem 277: 12446–12455

Chapter 22

Energy Transduction by the Two Molecular Motors of the F_1F_o ATP Synthase

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Summary

The F_1F_o ATP synthase has nearly universal importance as the major source of ATP among all life forms. These molecular motors couple the energy provided by a transmembrane proton gradient to the production of ATP from ADP and phosphate. The intrinsic membrane complex of ab_2c_{10-15} subunits, known as F_o, functions as a proton channel via a Brownian ratchet mechanism and the F_1 peripheral membrane complex of $\alpha_3\beta_3\gamma\delta\varepsilon$ subunits contains one site for ATP synthesis/hydrolysis per $\alpha\beta$ heterodimer. When F_1 is purified from F_o and the membrane, it retains the ability to hydrolyze ATP. The ring of three $\alpha\beta$ heterodimers form the stator around the γ-subunit rotor that rotates in response to ATP hydrolysis activity producing a torque of 63 pN nm. Rotation occurs via the alternating site mechanism in which ATP binds to one site, while product release occurs at another site. It uses the non-equilibrium transmembrane electrochemical proton gradient derived from the oxidation of metabolites or light during photosynthesis

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to drive the reaction ADP + Pi ↔ ATP + H_2O away from equilibrium, and thereby maintains high cellular concentrations of ATP. Under some conditions, the enzyme can catalyze ATPase-driven proton pumping in the reverse direction across the membrane. However, the enzymes from mitochondria and chloroplasts employ mechanisms to minimize this reverse reaction.

I. Introduction

The F_1F_o ATP synthase couples the energy provided by a transmembrane proton gradient to the production of ATP from ADP and phosphate. The intrinsic membrane complex of $ab_2c_{10–15}$ subunits, known as F_o, functions as a proton channel and the F_1 peripheral membrane complex of $\alpha_3\beta_3\gamma\delta\varepsilon$ subunits contains one site for ATP synthesis/hydrolysis per αβ heterodimer. The photosynthetic ATP synthase is found in the stroma lamellae of the chloroplast where the F_1 complex faces the stroma. In mitochondria, the ATP synthase resides in the inner mitochondrial membrane with the F_1 complex facing the matrix, while the enzymes of bacterial origin are in the inner membrane where the F_1 faces the cytoplasm.

The F_1F_o ATP synthase uses the transmembrane electrochemical proton gradient derived from the oxidation of metabolites or light during photosynthesis to drive the reaction ADP + Pi ↔ ATP + H_2O away from equilibrium, and thereby maintains high cellular concentrations of ATP (Nakamoto et al., 1999; Frasch, 2000; Stock et al., 2000; Yoshida et al., 2001; Fillingame et al., 2002; Senior et al., 2002). A variety of essential cellular functions depend on the energy gained by ATP hydrolysis to return the [ATP]/[ADP][Pi] chemical gradient toward equilibrium. The F_1F_o ATP synthase has nearly universal importance as the major source of ATP among all life forms. Under some conditions, the enzyme can catalyze ATPase-driven proton pumping in the reverse direction across the membrane. However, the enzymes from mitochondria and chloroplasts employ mechanisms to minimize this reverse reaction.

The F_1 and F_o complexes are both rotary molecular motors. During ATP synthesis, a proton gradient provides the energy for the F_o motor to drive the rotation of the rotor composed of the cγε subunits. This has been observed to occur in 120° rotational steps by single molecule fluorescence resonance energy transfer (FRET) (Börsch et al., 2002). Rotation forces conformational changes of the three sites that catalyze ATP synthesis on F_1, and results in changes in nucleotide affinities that promote the release of ATP despite an unfavorable [ATP]/[ADP][Pi] chemical gradient. The sequential changes in conformation at each catalytic site are staggered from the others such that binding of substrate at one site results in the release of product from another site. Conversely, ATP hydrolysis can drive conformational changes in F_1 that drives the rotation of the cγε in the opposite direction relative to the stator, resulting in proton pumping. When F_1 is solubilized away from F_o and the membrane, ATPase-driven γ subunit rotation can be observed by single molecule assays (Noji et al., 1997). Due to its regulatory mechanisms the ATPase activity of purified chloroplast F_1 is latent, but can be activated by any of several treatments.

A number of independent experiments have demonstrated that the hydrolysis of ATP drives the rotation of the γ subunit. The most compelling evidence was obtained by adding histidines to the β subunit N-terminus to form histidine "tags" that allow F_1 to bind tightly to a nickel nitrilotriacetate (Ni-NTA) coated coverslip such that the γ subunit is normal to the surface. This positioned the γ subunit foot region distal from the surface, to which an actin filament was attached via an introduced cysteine residue that had been biotinylated. Counterclockwise rotation of the actin filament was observed upon addition of ATP.

Abbreviations: DCCD – Dicyclohexylcarbodiimide; EPR – Electron paramagnetic resonance; ESEEM – Electron spin-echo envelop modulation; FRET – Fluorescent resonance energy transfer; k_B – Boltzman constant; LHCC – Left-handed coiled-coil; Ni-NTA – Nickel nitrilotriacetate resin; NMR Nuclear magnetic resonance; OSCP – Oligomycin sensitivity conferring protein; QM/MM – Quantum mechanical molecular modeling; RHCC – Right-handed coiled-coil; S_N2 – Nucleophilic aliphatic substitution with second order kinetics; WHB – Walker homology B sequence

At saturating concentrations of ATP, the γ subunit rotates in 120° steps consistent with the consumption of three ATP by the three catalytic sites per 360° of rotation. The dwell time between rotational events is 2 ms and 8 ms for F_1 from the thermophilic bacterium *Bacillus PS3* (Yasuda et al., 2001) and *Escherichia coli* (Spetzler et al., 2006), respectively, consistent with the rate-limiting step in the reaction for these enzymes. At rate-limiting concentrations of ATP, the 120° rotational steps are resolved into 80° and 40° sub-steps (Sakaki et al., 2005) (though some data support 90° and 30° sub-steps). The duration of the dwell prior to the 80° sub-step is inversely proportional to the substrate concentration indicating that the binding of ATP to an empty catalytic site initiates the 80° rotation. The dwell prior to the 40° rotational step does not change with ATP concentration, is comparable to the rate-limiting step in the reaction, and is thus likely the same dwell observed at saturating ATP. Evidence suggests that product release initiates the final 40º of rotation to complete the catalytic cycle (Sielaff et al., 2008a). This rotational scheme couples the energy from the chemiosmotic process to the rotation of the F_1F_o complex.

A. Chemiosmotic Energy Coupling

In the chemiosmotic coupling process, the magnitude of the energy gradient, designated the proton-motive force (pmf or $\Delta\mu_{H^+}$), is related to the trans-membrane concentration gradient (ΔpH) and membrane potential (ΔΨ) differences in mV at 30°C by Eq. 22.1. Even though F_oF_1 transports only protons across the membrane, nonequilibrium concentration gradients of other ions like K^+ can contribute to the energy of the proton gradient by changing the ΔΨ if the ion is permeable to the membrane. Most F_1F_o ATP synthases are driven by proton translocation, though some thermoalkaliphilic bacteria are capable of using a transmembrane Na^+ gradient to drive ATP synthesis (Ferguson et al., 2006). The protonmotive force translates into more conventional energy terms because it is the sum of the free energy derived from the trans-membrane concentration difference (Eq. 22.2), and the electrical potential gradient generated by the trans-membrane concentration gradient of charged species (Eq. 22.3) where n is the charge on the ion (+1 for protons). This relationship simplifies to Eq. 22.4.

$$\Delta\mu_{H^+} = \Delta\psi - 60\Delta pH, \tag{22.1}$$

$$\Delta G = 2.3RT\log\left[H^+_{lumen}\right]/\left[H^+_{stroma}\right] = 2.3RT\Delta pH, \tag{22.2}$$

$$\Delta G = -n\mathcal{F}\Delta E = -n\mathcal{F}\Delta\psi, \tag{22.3}$$

$$\Delta G = -F\Delta\mu_{H^+}. \tag{22.4}$$

The free energy of the proton gradient generated in thylakoids must exceed a minimum threshold before ATP can be synthesized. This threshold provides information concerning the number of protons that must be transported across the membrane per ATP synthesized. The measurements have been complicated by the mechanisms that regulate the catalytic activity of the enzyme. The most accurate measurements of 4–4.25 H^+ per ATP synthesized were made under conditions in which the $\Delta\mu_{H^+}$ required to activate catalytic activity was accounted for, and the dithiol unique to the chloroplast γ subunit had been reduced (Samra et al., 2006).

Now that it is known that ATP synthesis occurs via a rotary mechanism in which each 360° rotation results in the transport of 14 H^+ protons for the chloroplast enzyme (one per c subunit) and the synthesis of 3 ATP, the consumption of protons per ATP will be 4 or 5 depending on the alignment of the γ subunit relative to the c-ring. The threshold values of $\Delta\mu_{H^+}$ used to measure the proton/ATP ratio were measured in the presence of known concentrations of ADP, Pi, and ATP to allow the chemical gradient to be determined. The threshold value of energy needed to drive ATP synthesis increased with the chemical gradient as might be expected. When measured in the presence of different mixtures of ADP, Pi, and ATP, the energy of the proton gradient had to exceed that of the chemical gradient by 43–66 pN·nm per ATP synthesized. For example, in the presence of 0.5 mM ADP containing a 4% contamination of ATP, and 3 mM Pi, the chemical gradient was 208 pN·nm/3 ATP molecules while the threshold for ATP synthesis was 407 pN·nm/14 H^+ such that the energy of the proton gradient had to exceed the chemical gradient by 66 pN·nm/ATP for the chloroplast F_1F_o to catalyze ATP synthesis.

It is noteworthy that the maximum torque on the γ subunit measured to date by the *E. coli* F_1-ATPase was 63 pN·nm (Spetzler et al., 2006; Hornung et al., 2008a, b). This suggests that the energy of the proton gradient is needed to force the rotation of the γ subunit against the ATPase-driven F_1 motor as well as overcoming the chemical gradient to make ATP.

Rotation of the c-ring during ATP synthesis is believed to be driven by the attractive forces between aR210 on subunit a-transmembrane helix 4 and the cD61 carboxyl during the H^+-transport cycle to lower its pK transiently (Fillingame et al., 2002). Stepwise rotary movement of the c-ring has been postulated to result from a rocking motion of subunit a-transmembrane helix 4 that alternately exposes the aR210-cD61 salt bridge that forms to the thylakoid lumen and stroma proton access channels and results in a motion similar to the intermeshing of gears between the helices of subunits a and c (Fillingame et al., 2003). Since cD61 is constantly supplied with protons from the thylakoid lumen via aQ214, the motor has been postulated to behave as a molecular ratchet (Oster et al., 2000).

II. Structure of F_1F_o

The first high-resolution structure of F_1 was determined for the enzyme from bovine mitochondria. That structure resolved most of the $(\alpha\beta)_3$ ring and part of the γ subunit, but did not include the smaller subunits. The α and β subunits were resolved nearly completely, while subunit γ was resolved only partially (Abrahams et al., 1994). Since that time structures of F_1 complexes from several sources have been successfully crystallized under various conditions (Hausrath et al., 1999; Gibbons et al., 2000; Menz et al., 2001; Kabaleeswaran et al., 2006; Bowler et al. 2007). The most complete F_1 complex structure, including all subunits in the central stalk, was obtained in 2000 (Braig et al., 2000).

F_1 complex has a spherical shape, 8–10 nm in diameter (Fig. 22.1). Three α and three β subunits are arranged in an alternating way with six-fold pseudo symmetry as well as α-β dimers with three-fold pseudo symmetry, making a central cavity where N- and C-terminal α-helices of subunit γ are located. Subunit ε and the central part of subunit γ protrude toward the F_o part making a central stalk of the enzyme, which interacts with the F_o part. Three ATP binding catalytic sites are located on the interface of subunits α-β, mostly β. Three non-catalytic sites, usually filled with very tightly bound ATP, are located also on the interface of subunits α-β, but mostly α.

The structures of subunits α and β are similar in both their primary sequence and tertiary structure. They are composed of three distinct domains. The N-terminal segment (up to residues 95 in α, 82 in β) folds into a six-stranded β – barrel at the top of F_1, away from the membrane. These barrels appear to stabilize the αβ – hexamer by making a continuous β-sheet crown clearly seen on the top of the F_1 complex. The first 20 N-terminal residues of subunit α are thought to be involved in binding of subunit δ.

The central segment (α – residues 96–379, β – residues 83–363) contains a Rossmann fold with nine-stranded β-sheet and nine α-helices. This domain binds nucleotides and includes the P-loop, or Walker-A motif – β156GXXXXGKT163, that is a signature motif for many nucleotide-binding proteins. Residues α287-394 and β274-381 form hydrophobic regions that closely interact with the γ C-terminal hydrophobic end. This structure is called a "sleeve" and appears to ensure friction-free rotation of γ. The C-terminal segment (α – residues 380–510, β – residues 364–474) folds into a bundle of seven (α subunit) or six (β subunit) α-helices. The motif DELSEED (β394-400) is responsible for interaction with subunit ε and appears to have a regulatory role (Bi et al., 2008; Volkov et al., 2009).

The conformation of all six α and β subunits is similar, but in general it is dependent upon the binding of ligands at the catalytic sites. Therefore, the overall structure of F_1 complex generally appears asymmetrical, which also results from the eccentric position of subunit γ relatively to each αβ–dimer. This structural asymmetry is closely related to a functional mechanism of F_1 operation described in terms of the binding-change mechanism.

The C-terminal part of subunit γ (residues 209–272) forms a very long (90 Å) α-helix entering into the central cavity almost to the top of the enzyme. The lower part of that helix forms an anti-parallel left-handed coiled-coil structure with the N-terminal (residues 1–45) part of the subunit

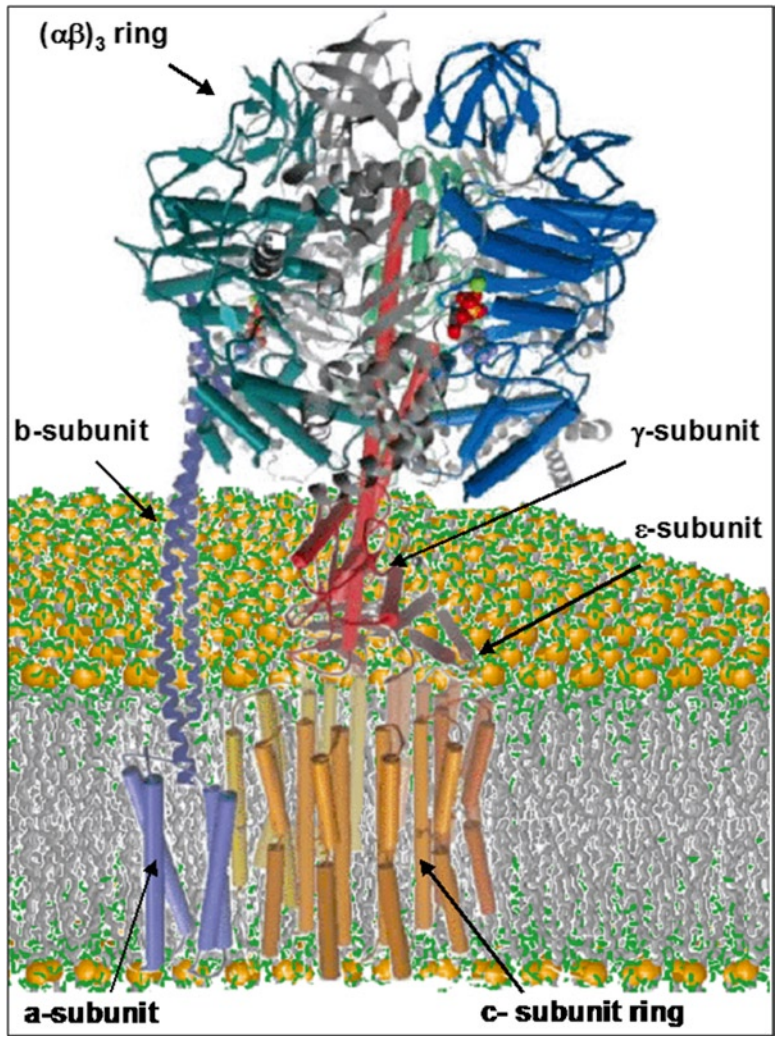

Fig. 22.1. Composite structure of the F_1F_o ATP synthase.

(Wilkens, 2005). The "foot" of the subunit has a globular structure and is supposed to interact with both c and ε subunits to form the rotor.

Subunit δ is composed of a N-terminal six-helix bundle domain (residues 2–106) and a C-terminal domain (residues 107–177) that appeared largely unstructured in a nuclear magnetic resonance (NMR) study (Dmitriev et al., 1999). The N-terminal domain was shown to interact with the N-terminal region of subunit α (Greie et al., 2000). The C-terminal region is thought to interact with the very end of the C-terminus of the subunit b dimer (Caviston et al., 1998). In mitochondria the δ subunit is referred to as the oligomycin sensitivity conferring protein (OSCP).

The ε subunit is attached to the foot of γ and binds to the c-ring of F_o. It contains an N-terminal β-barrel domain and a helix-turn-helix domain at the C-terminus. In mitochondria this subunit is referred to as δ, while a different small peptide is called ε. To minimize confusion, this mitochondrial peptide will be referred to as ε'. It is composed of a helix-turn-helix followed by a single β strand that is part of the β sheet of the γ subunit.

Structural information concerning the assembled F_o complex is currently lacking. However, with the exception of subunit a, structures of the individual components of this complex have recently been determined, and in several cases, differ

between organisms. For example, known c-ring structures include the bovine c_8-ring-F_1 complex (Watt et al., 2010), the yeast c_{10}-ring-F_1 complex (Stock et al., 1999; Dautant et al., 2010), the c_{11}-ring from the Na^+-F-ATPase of bacterium *Ilyobacter tartaricus* (Meier et al., 2005, 2009; Zaida et al., 2008), the c_{13}-ring from *B. pseudofirmus* (Preiss et al., 2010), the c_{14}-ring from spinach chloroplasts (Vollmar et al., 2009), the c_{15}-ring from Spirulina (Pogoryleov et al., 2009), and the c-ring from Na^+-dependent V-ATPase of bacterium *Enterococcus hirae* (Revington et al., 1999). NMR structures of isolated c subunit monomers and the transmembrane segment of subunit's b dimer are also available (Takeyama et al., 1988; McLachlin et al., 1998), and the peripheral stalk of the mitochondrial enzyme, which differs significantly in subunit composition from that of the other ATP synthases, has also recently been determined (Dickson et al., 2006). All F_o subunits have been studied extensively using mutational analysis and protein biochemical techniques, including site-specific labeling and cross-linking formation, which have contributed to the current conception of its structure and function.

Subunit c folds like a hairpin with both termini in the periplasm and the polar loop in the cytoplasm. It assembles into a ring structure made of 10 monomeric subunits in the case of *E. coli* (Jiang et al., 2001), thermophilic bacterium *Bacillus PS3* (Mitome et al., 2004) and yeast (Stock et al., 1999). Bacterium *I. tartaricus* has 11 monomers (Meier et al., 2005, 2009), V-ATPase from *Thermus thermophilus* has 12 subunits (Toei et al., 2007), *Synechococcus elongatus* SAG 89,79 has 13 subunits (Pogoryelov et al., 2007), spinach chloroplast has 14 (Seelert et al., 2003), and cyanobacterium *Spirulina platensis* contains 15 (Pogoryelov et al., 2005). In the *E. coli* enzyme the N-terminal helices (residues 2–40) (Girvin et al., 1998) assemble the internal ring of the rotor, while an external ring is thought to be made of C-terminal helices (47–79). The cytoplasmic loop is formed by seven residues containing R-Q/N-P that is involved in binding the γ and ε subunit (Zhang and Fillingame, 1995; Watts et al., 1996; Watts and Capaldi, 1997; Pogoryelov et al., 2008).

The structure of the c-ring from sodium-pumping *I. tartaricus* was determined with bound Na^+ (Meier et al., 2005). The binding side is assembled of residues of an inner N-terminal helix and two external C-terminal helices of the c-ring. The sodium ion is bound by side-chain oxygens of Q32 and E65 of one c subunit and the hydroxyl of S66 and backbone carbonyl oxygen of V66 of the neighboring c subunit. In addition, the binding site conformation is stabilized by hydrogen bonding of E65 with V66 that tightly locks the sodium ion in a closed conformation. Although this high affinity binding conformation is thermodynamically favored to carry a charged ion through a hydrophobic environment it also suggested that the conformation of the binding site needs to be unlocked to release Na^+ while in the active site.

The overall structure of the rotor has an hourglass shape (Meier et al., 2005), as seen in the c oligomer from *I. tartaricus*, because all helices are bent about 20° in the middle of the membrane at aP28 and aE65, causing the narrow part of the shape. The C-terminal outer ring is thought to be laterally-shifted relative to the N-terminal inner ring in a counter-clockwise direction, as seen from the cytoplasm. This bend tilts the C-terminal helices 10° counter-clockwise out of the axis of radial symmetry, making a right-handed twisted packing. The key residue of the c subunit in ion translocation, aD61 and aE65 in *E. coli* and *I. tartaricus*, respectively, is located in the middle of the membrane in the narrowest part of the rotor, and is surrounded by hydrophobic residues (Aksimentiev et al., 2004; Meier et al., 2005).

Subunit a is located on the outside of the c-ring (Birkenhager et al., 1995; Singh et al., 1996; Takeyasu et al., 1996). Though detailed structural information about subunit a is not available, it is well established that it couples vectorial H^+ (Na^+ in some halophilic bacteria) translocation across the membrane with rotation of the rotor, establishing a dynamic interface with the c-ring. It also interacts with the subunit b dimer, fixing the stator relative to the mobile rotor (Vik et al., 2000). The N-terminus of subunit a is in the periplasm, while the C-terminus is in the cytoplasm. The subunit folds into five transmembrane helices, with two cytoplasmic loops and two periplasmic loops (Vik et al., 2000). A model of an overall arrangement of transmembrane helices of subunit a in the membrane is given in (Vik et al., 2000; Aksimentiev et al., 2004; Schwem and Fillingame, 2006). All five helices are arranged consecutively either clockwise or counter-clockwise

(Zhang and Vik, 2003a). Helices 1 and 2 and the first periplasmic loop are believed to be in contact with subunits b based on second site suppression mutants and cross-linking formation (Caviston et al., 1998; Vik et al., 2000; DeLeon-Rangel et al., 2003). This section of subunit a also has multiple contacts with the c-ring (Long et al., 2002; Zhang and Vik, 2003b). Though mutation of some residues in the helices 1–3 affect proton translocation, none is essential for function (Vik et al., 1988; Patterson et al., 1999; Long et al., 2002; DeLeon-Rangel et al., 2003).

The fourth and the fifth transmembrane helices are directly involved in dynamic interactions with the c-ring rotor and contain residues required for ion translocation including aR210 and aR227 in helix 4 from *E. coli* and *Propionigenium modestum*, respectively, that is an essential residue (Vik et al., 2000; Aksimentiev et al., 2004). Other conserved residues in these helices include aN214, aE219, aH245 and aQ252 (in *E. coli*). Although not essential for function, these groups are involved in proton translocation. It is noteworthy that in the majority of organisms, aE219 on helix 4 and aH245 on helix 5 are essentially switched with the histidine and an aspartate on helices 4 and 5, respectively (Feniouk and Junge, 2009). Function of F_o is retained when these residues are swapped by mutagenesis. The topography of the periplasmic and cytoplasmic half-channels through subunit a are best characterized for the *E. coli* F_o that is specific for proton pumping (Angevine and Fillingame, 2003; Angevine et al., 2003; Angevine et al., 2007). The channels are likely a stretch of polar/charged residues inside of the fourth and fifth transmembrane helices, leading a proton toward the active site through a hydrophobic environment.

The b subunits are the main component of the second stalk that connects F_1 with F_o to form the stator of the motor (Ogilvie et al., 1997; Rodgers et al., 1997; Rodgers and Capaldi, 1998; Wilkens and Capalldi, 1998a, b). Most eubacteria have ATP synthases with homodimeric second stalks. In other organisms such as cyanobacteria and plant chloroplasts, the b_2 subunit is present as a b and b′ heterodimer (Hennig and Herrmann, 1986; Walker et al., 1987; McCarn et al., 1988). The peripheral stalk of mitochondrial ATP synthase has one b subunit that has little sequence similarity to b subunits from other ATP synthases (Senior et al., 2002; Duncan, 2004; Wilkens, 2005; Nakanishi-Matsui and Futai, 2006) and several other unique subunits (Walker and Dickson, 2006). In general a comparison of the sequences of b subunits from various organisms reveals a very low number of identifiable conserved amino acids (Hornung et al. 2008a, b). One of roles of the second stalk is to hold the $(\alpha\beta)_3$ ring against the rotational torque of the rotor. Otherwise the $(\alpha\beta)_3$ ring would rotate with $\gamma\varepsilon c_{10}$ instead of undergoing the conformational changes that lead to the production of ATP.

The N-terminus of subunit b forms a transmembrane α-helix (Dmitriev et al., 1999), while the amino acids 25–156 are located in the cytoplasm and form mostly an α-helix (Greie et al., 2000). The b_2 helices can be cross-linked to each other indicating close spatial proximity in the membrane (Dmitriev et al., 1999). The cytoplasmic part of b_2 contains the tether domain (Caviston et al., 1998; McLachlin et al., 2000; Zaida et al., 2008), the dimerization domain (Sorgen et al., 1998a; Revington et al., 1999; Zaida et al., 2008) and the δ-binding domain (Takeyama et al., 1990; McLachlin et al., 1998; Bhatt et al., 2005). It is not resolved whether the tether domain extends from amino acid 25 to amino acid 53 (Caviston et al., 1998; McLachlin et al., 2000) or 65 (Zaida et al., 2008), and the dimerization domain from 53 to 122 (Sorgen et al., 1998a; Revington et al., 1999) or from 65 to 139 (Zaida et al., 2008). The C-terminus of subunit b contains the δ-binding domain that binds the peripheral stalk to F_1 (Takeyama et al., 1990; McLachlin et al., 1998). However, other interactions also exist between b_2 and the $(\alpha\beta)_3$ ring (McLachlin et al., 2000; Motz et al., 2004; Weber et al., 2004). High affinity binding of the b dimer to F_1 is dependent upon Mg^{2+} (Weber et al., 2004).

The tether region is known to interact with subunit a and to play a role in coupling (Caviston et al., 1998; McLachlin et al., 2000). Up to 14 amino acids can be inserted and as many as 11 can be deleted in the area between amino acids 50 and 65 with little effect on the activity of the protein (Sorgen et al., 1998b; Sorgen et al., 1999). These results indicate that this region of subunit-b is flexible. From electron paramagnetic resonance (EPR) spectroscopy experiments it is known that both b monomers are far apart from each other between the amino acid positions 31

and 65 (Steigmiller et al., 2005; Zaida et al., 2008). It has been suggested that the b subunit forms a "bubble" that might be involved in the storage of elastic energy produced during the rotation of the enzyme (Zaida et al., 2008). However, direct measurements of the spring constant of the stator from *E. coli* indicate that the peripheral stalk is at least ten-fold stiffer than the rotor (Wachter et al., 2011).

Dimerization of the b subunits has been found to be essential for the binding to F_1 and ATP synthase activity (Sorgen et al., 1998a; Cipriano et al., 2006). Unlike the results obtained for the tether domain, deletions of any single amino acid between 100 and 105 led to an enzyme that assembled but was not able to support oxidative phosphorylation in vivo (Cipriano et al., 2006). A crystal structure of subunit b from amino acid 62 to 122 as a monomer shows that this segment forms a single α-helix with a slight right-handed twist. Based on these results and other studies, the dimerization domain of subunit b has been postulated to exist as a right-handed coiled-coil (RHCC) in which the helices are offset rather than in register (Del Rizzo et al., 2002). Results from low-resolution X-ray and NMR studies on b_2 and $b_2\delta$, spin label EPR, and molecular modeling support the presence of a left-handed coiled-coil (LHCC) that is commonly found in several proteins (Priya et al., 2008). However, examinations of the amino acid sequences of subunit b support either a RHCC or a LHCC. The recently derived structure of the peripheral stalk of the A-type ATPase/synthase from *T. thermophilus* contains a heterodimeric RHCC (Lee et al., 2010), which provides some support for the postulate that the comparable stalk in the F-type enzyme is right handed.

III. Observation of Single Molecule Rotation

The first direct observation of counterclockwise rotation of single F_1 molecules was accomplished by attaching a fluorescent actin filament to γ while fixing the $(\alpha\beta)_3$ cylinder from a thermophilic bacterium F_1 to a glass surface (Noji et al., 1997). Subsequent experiments have confirmed rotation in F_1 and *E. coli* (Noji et al., 1999; Omote et al., 1999) and plant chloroplasts (Hisabori et al., 1999). Reporter groups other than actin have successfully been used to observe rotation (Adachi et al., 2003), including a single fluorophore (Adachi et al., 2000), spherical bead or bead duplex (Hirono-Hara et al., 2001; Yasuda et al., 2001), metal bar (Soong et al., 2000), and a single donor-acceptor pair for FRET.

Rotation of the γ subunit relative to subunit b in F_1F_o via a proton gradient in liposomes was observed to occur in discrete 120° steps using single molecule FRET (Börsch et al., 2002) in the opposite direction as that observed during ATP hydrolysis. Single molecule ATP hydrolysis-driven rotation of purified F_1F_o has also been reported (Sambongi et al., 1999; Pänke et al., 2000; Tsunoda et al., 2000, 2001a; Tanabe et al., 2001; Nishio et al., 2002; Ueno et al., 2005), though it is not clear if the subunit a-c interface was always intact due to the lack of dicyclohexylcarbodiimide (DCCD) inhibition (Tsunoda et al., 2000). In all of the single molecule studies, rotation followed the 120° step pattern of F_1 rotation without any additional friction imposed by interactions between the c-ring and subunit a (Ueno et al., 2005). This indicates that F_1 is more powerful than any interactions between the c-ring and subunit a.

The kinetics of the time-averaged stepping rate for TF_1 ATPase-driven rotation follows the trend predicted by Michaelis-Menten in an [ATP] dependent manner, with a V_{max} of 390 steps s^{-1} and a K_m of 15 μM, where TF_1 is F_1 from the thermophilic bacterium *PS3* (Yasuda et al., 2001). For *E.coli* F_1, k_{cat} has been measured in a bulk assay to be 120 s^{-1} with a K_m of 10 μM (Greene and Frasch, 2003). Statistical analysis of intervals between steps at various [ATP] indicates that each step is associated with the hydrolysis of one ATP molecule (Yasuda et al., 1998, 2001; Adachi et al., 2000). However, the rate is likely underestimated since the hydrolysis rate in solution is reduced due to inhibition by MgADP (Jault et al., 1996; Boyer, 1997; Matsui et al., 1997; Kinosita et al., 2000; Hirono-Hara et al., 2001), as compared to the observation of single active molecules.

A high speed imaging system was used to measure the rotation of an F_1-ATPase labeled with a gold nanosphere on the 'foot' region of the γ subunit. The data showed that there are two sub-steps in each 120° rotational event of the γ subunit. At limiting ATP concentrations (20 nM),

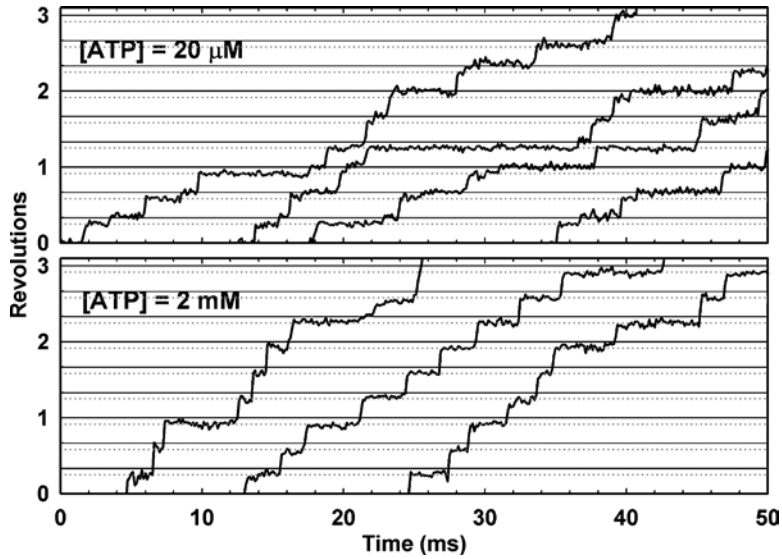

Fig. 22.2. Sub-steps in F_1 rotation at 20 mM ATP (*upper panel*) recorded with a temporal resolution of 0.125 ms (Yasuda et al., 2001). With this resolution, sub-steps are not discerned at 2 mM ATP (*lower panel*), implying that the ATP-waiting dwell is <0.125 ms and the next 80° rotation is complete within 0.125 ms of ATP binding; it also implies that F_1 is completely reset and ready to bind a next ATP by the end of a 40° sub-step. *Horizontal solid lines* are separated by 120°, and *dotted lines* are drawn 40° below (Taken from Kinosita et al., 2004).

the 120° steps were resolved into ~80° and ~40° sub-steps (Fig. 22.2). However, at 20 nM, the ATP concentration is too low for the third site to fill. Thus patterns observed under these conditions may not conform to typical rotation. The dwell before each 80° sub-step is much longer at lower [ATP].

Since the average dwell is inversely proportional to [ATP], the implication is that the 80° sub-step is triggered by the binding of ATP. Later experiments suggest that this pause may occur between 80° and 40° rotation events and that the completion of a catalytic cycle at any one site involves the rotation of the γ subunit by 240° (Shimabukuro et al., 2003). These data support a tri-site mechanism of rotation.

A. Energy for Rotation

Gao and co-workers (Gao et al., 2003) predicted that the free energy profiles of each of the three sites follow the trend shown in Fig. 22.3 for a single 120° rotation event. The figure indicates which species is dominant at each site. The results of Yasuda and co-workers (Yasuda et al., 2001) were used to build the diagram, which is based upon the fact that each 120° rotation event is composed of two parts: a 90° rotation followed by a 30° rotation. The model is based upon assumptions that the pause at 90° is due to product release, and the 30° pause is due to the waiting time for ATP binding to the empty (E) site since this occurs only at low concentrations of ATP.

The free energy from ATP binding to the E site was calculated to be 19.4 pN nm/molecule using K_D=25 μM and a cellular concentration of ATP equal to 3 mM (Gao et al., 2003). Binding of ATP causes the conformation of the E site to change to that of the ATP binding (TP) site. This is due to stabilization of the enzyme, which corresponds to the decrease of K_D from 25 μM to 0.2 nM. Once the ATP has bound to the empty site, the α and β subunits close around the nucleotide which drives γ-subunit rotation. The free energy from binding is the initial and primary source of energy driving the rotary binding change mechanism. There is almost no difference in the free energy between ATP and ADP+Pi at the TP site. Thus, ATP hydrolysis may begin as soon as the TP site is formed. However, before product release occurs, there is a conformational change in the TP that changes it to the ADP-bound (DP) state. Gao and co-workers postulate that this is the

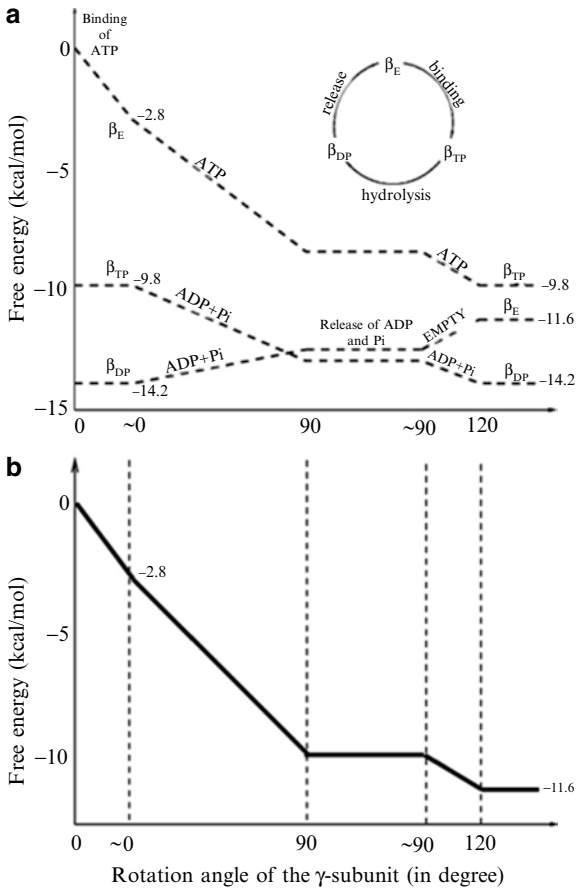

Fig. 22.3. Free energy (kcal/mol) profile for ATP hydrolysis in the tri-site mechanism. (**a**) The individual free energy changes as a function of the conformational transitions. (**b**) The change of the total free energy, which is the sum of the free energy changes of the three sites (Taken from Gao et al., 2003).

mechanism of free energy generation since the hydrolysis rate is faster for multisite than in unisite catalysis (Gao et al., 2003). As γ-subunit rotation occurs due to the change of one subunit from the E to TP conformation, the former TP site is changed to a DP site. They assumed that the ATP hydrolysis reaction is completed during this process, since the binding energy in the DP site is 68 pN nm/molecule. The DP site has a higher binding affinity for ADP+Pi than does the TP site, thus the change of TP to DP is a potential source for a second driving force on the γ-subunit. The free energy associated with this change is 30.5 pN nm/molecule, which corresponds to a free energy change at the TP site of 4.4 pN nm/molecule. Additionally, the K_D changes for ADP from 29 to 0.05 M, while the K_D for Pi remains constant. The rotation of the γ-subunit causes the DP site to open and release ADP+Pi. This process requires 18 pN nm/molecule of energy, which is the free energy from binding of ADP+Pi at the DP site. The net free energy change in a single 120° rotation event is calculated by taking the sum of the contributions of the individual sites.

According to Gao et al. (2003) the net free energy is 80.6 pN nm/molecule, which is the free energy released by hydrolyzing one molecule of ATP. The release of ADP and Pi is thought to be induced by the "opening" of the DP subunit which requires energy. This feature is a result of the cooperativity of the different catalytic sites which are involved in the Binding Change Mechanism of F_1-ATPase (Boyer, 1993). The free energy diagram in Fig. 22.3 differs from the Hongyun Wang and George Oster (Wang and Oster, 1998) model in the hydrolysis cycle. The Wang and Oster model is a transcription of the unisite catalysis measurements (Senior et al., 2002), while the Gao model takes into account the different properties of the other sites, which are essential to the complete hydrolysis cycle. One advantage of the Gao hypothesis is that it allows for cooperativity between the catalytic sites, leading to increased rates of rotation.

B. Torque Determination

The first torque measurements used a micrometer-sized actin filament attached to γ which showed that the F_1 rotated more slowly due to the hydrodynamic drag of the viscous medium against the moving actin filament. The frictional drag coefficient, ξ, is given (Hunt et al., 1994; Adachi et al., 2003) by:

$$\xi = \frac{\frac{4\pi}{3}\eta L^3}{\ln(\frac{L}{r}) - 0.447}, \qquad (22.5)$$

for an actin filament of length L and radius r (~5 nm) rotating around one end, where η is the viscosity of the medium and is $\sim 10^{-3}$ nm^{-2} s at room temperature. If, instead of actin, a spherical bead of radius *a* is attached off-axis such that the distance between the bead center and rotation axis is x, ξ is given by:

$$\xi = 8\pi\eta a^3 + 6\pi\eta a x^2. \qquad (22.6)$$

The torque T needed to rotate the filament against the friction is given by:

$$T = \xi v, \tag{22.7}$$

where v is the velocity of the reporter group (in radians s^{-1}).

In early measurements it was not possible to measure the rotation speed directly, since the temporal resolution of the single molecule measurements was insufficient to resolve the velocity between each 120° step. However, attempts were made to estimate the torque F_1 produces against the friction of the viscous medium. These estimates range from 44 to 55 pN nm (Yasuda et al., 1998; Pänke et al., 2001). As predicted by Stokes equations, the torque appeared independent of the rotary angle since the velocity was constant during each 120° step (Wang and Oster, 2002). The torque estimated from the rotation speed averaged over many revolutions was about 40 pN nm under saturating amounts of [ATP], during which F_1 bearing an actin filament rotated smoothly without apparent steps (Yasuda et al., 1998). However, this time-averaged rate includes the time that the γ-subunit does not move (i.e., dwells between rotational events due to the rate-limiting step of the reaction). Thus, these values are lower than the actual torque generated by the enzyme. From these estimates, it has been calculated that the work F_1 can do in a 120° step is about 80–90 pN nm or ~20 k_BT, calculated as 40–44 pN nm times (2/3)π radians, which should be considered to be the minimum amount of torque that the enzyme can produce. This is close to the physiological |ΔG| for ATP hydrolysis and implies that efficiency of the enzyme to convert chemical energy into mechanical energy is nearly 100%.

Since the actual torque is higher than the estimate from Eqs. 22.5 and 22.6 (Hunt et al., 1994), other methods of determining the torque have been used. A higher value of 50 ± 6 pN nm was calculated by measuring the drag based on the curvature of an actin filament attached to γ (Pänke et al., 2001). This determination was superior to previous attempts since the torque of F_1 is balanced by the elastic recoil of a bent actin which is a conservative force. This method provides an estimate that should be considered as the minimum amount of torque that can be generated because it was not possible to account for the relaxation in the actin that occurs when γ is not moving.

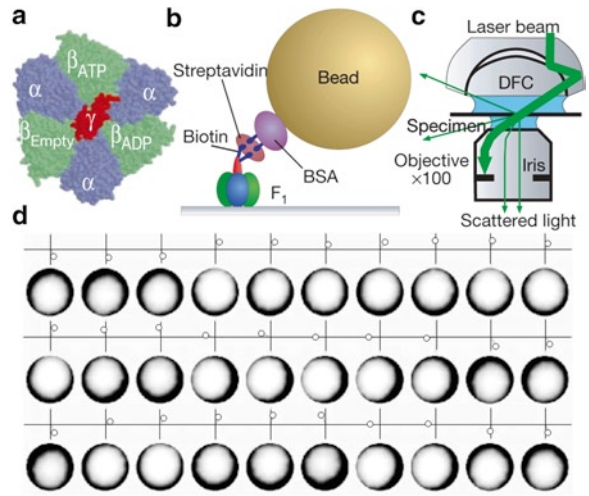

Fig. 22.4. Observation of F_1 rotation. (**a**) Atomic structure of F_1-ATPase viewed from the F_o side (*top* in (**b**)). (**b**) Side view of the observation system. The 40 nm bead gave a large enough optical signal that warranted a sub millisecond resolution; but the bead was small enough not to impede the rotation via viscous drag. (**c**) Laser dark-field microscopy for observation of gold beads. Only light scattered by the beads exited the objective and was detected. *DFC* dark-field condenser. (**d**) Sequential images of a rotating bead at 2 mM ATP. Images are trimmed in circles (diameter 370 nm) to aid identification of the bead position; centroid positions are shown above the images at 100× magnification. The interval between images is 0.5 ms (Taken from Yasuda et al., 2001).

Subsequent studies have used a 40 nm gold bead attached to γ (Fig. 22.4) which was shown not to impede the average stepping rate even though the bead is four times larger than the F_1. In an attempt to resolve the actual velocity of γ as it stepped from one dwell state to the next, time courses of the bead rotation were recorded at 8,000 frames s^{-1} (fps) (Yasuda et al., 2001). However, even 8,000 fps was not sufficient to measure the rate of transition (Fig. 22.5).

More recently, the rotational events of the γ subunit were measured with a time resolution of 0.25 μsec using gold nanorods as probes (Fig. 22.6) such that the speed of rotation could be distinguished from the dwell time (Spetzler et al., 2006). Based on these measurements, values of torque as high as 63 ± 8 pN nm were calculated by using measured values of the drag (Spetzler et al., 2006; Hornung et al., 2008a, b). A torque value of 74 ± 20 pN nm was found for *E. coli* using optical clamps (Pilizota et al., 2007), which is not statistically different from the earlier

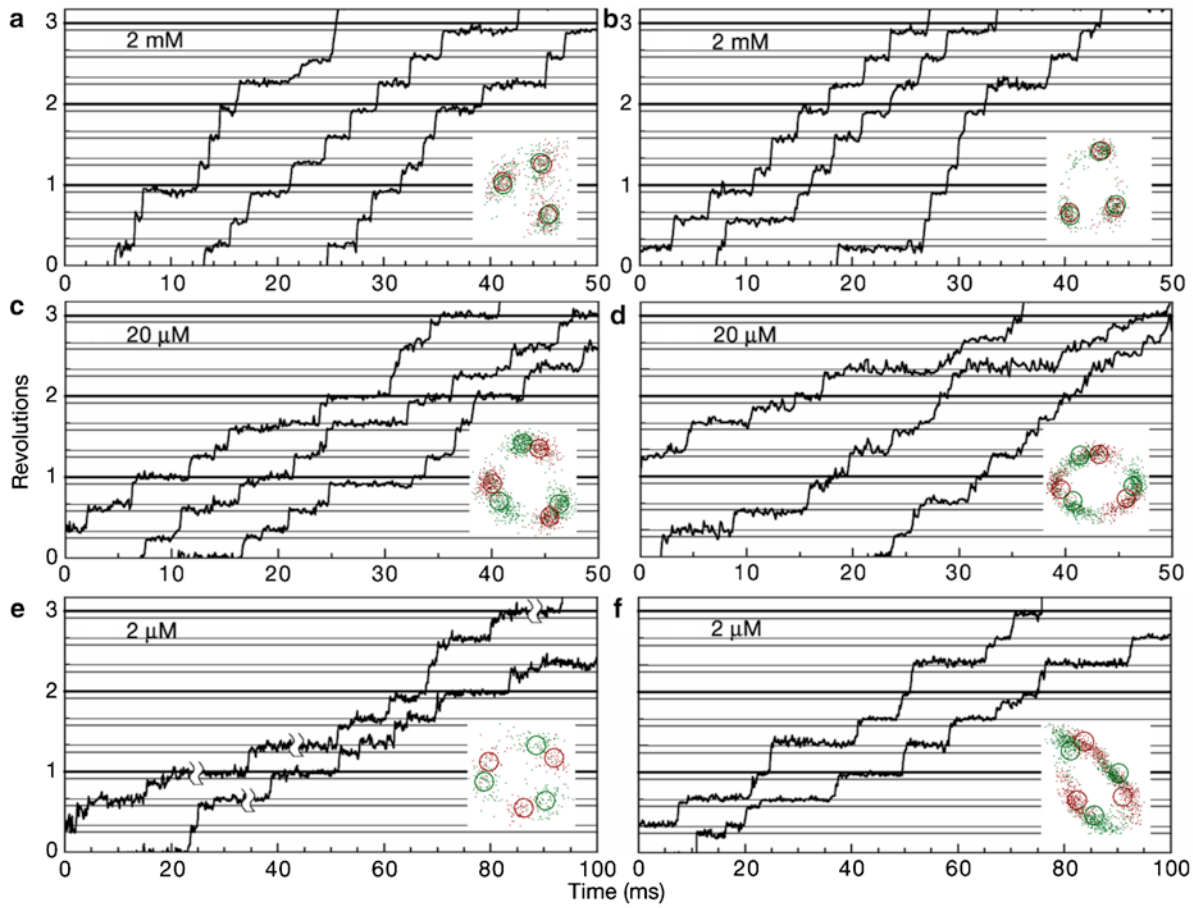

Fig. 22.5. Unfiltered time courses of stepping rotation of 40 nm beads at varying [ATP]. (**a**, **b**) 2 mM; (**c**, **d**) 20 μM; (**e**, **f**) 2 μM [ATP]. All curves in a panel are continuous; later curves are shifted, to save space. *Grey horizontal lines* are placed 30° *below black lines*. In (**e**) some of the long dwells are cut short. Insets, positions of a bead within 0.25 ± 0.5 ms before (*red*) and after (*green*) the main (90° or 120°) steps; runs lasting 0.5 s (2 mM) or 2 s (2 μM and 20 μM) were analyzed. *Circles* indicate projection of 0° and 90° dwell points on an obliquely situated circular trajectory that best fit the data. Angles in the time courses are those on the oblique circle (Taken from Yasuda et al., 2001).

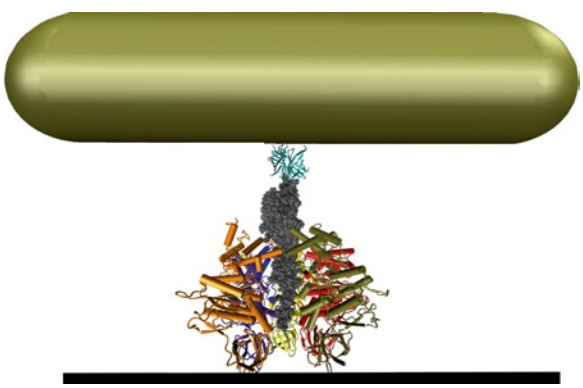

Fig. 22.6. Schematic of F1-ATPase and a gold nanorod used to determine the torque generated by the enzyme.

63 ± 8 pN nm measurement given the error in both measurements.

IV. Mechanism of the F_1F_o ATP Synthase

A. Alternating Site Mechanism of F_oF_1

The "Binding-Change" mechanism proposed by Paul Boyer, postulates that conformation changes of F_1 are linked to the available energy, which is indirectly driven by proton translocation, substrate binding, and product release (Boyer, 1975). Boyer hypothesized that the three asymmetrical catalytic sites of F_1 operate in unison with staggered

conformations, where one site is empty (open) and does not contain substrate or product, one binds product tightly, and the third binds substrate or product loosely. The three catalytic sites are coupled such that the binding of substrate to one site induces conformational changes that result in product release at an adjacent site. The interplay between the three catalytic sites is shown in Fig. 22.7. The sequence of events governing ATP hydrolysis was hypothesized to occur as follows. First, ATP binds to the open catalytic site of F_1-ATPase which causes a conformational change of that site to the loose state. Next, additional binding of ATP causes the loose site to convert to the tight binding conformation. ADP and ATP have about the same affinity in the tight binding conformation. Finally, ADP is released from the tight site when ATP binds to the open site. This is referred to as the bi-site catalysis, as only two catalytic sites are occupied at any one time.

Isotopic exchange experiments demonstrated that when only one catalytic site is filled with ADP and Pi, substrates bind with high affinity, the equilibrium constant of bound substrates and products approaches unity, and product release is slow. Release of ATP as product was greatly accelerated upon the binding of substrates to an additional catalytic site. Based on these results, the F_1F_o ATP synthase was hypothesized to operate via an alternating site mechanism in which binding of ADP and Pi to an empty catalytic site induces conformational changes of all the catalytic sites, and promotes the release of ATP from a second catalytic site. This was proposed to occur because the conformations of the 3 catalytic sites are staggered, and all 3 sites change conformation in response to proton gradient-driven rotation of the $(\alpha\beta)_3$ ring relative to the γ subunit. In this two-site mechanism, one site remains empty through each substrate binding/product release event.

A tenet of the alternating site mechanism is that, at any one time, the three catalytic sites have high, medium, and low affinity for nucleotide binding. This anisotropy of catalytic sites has been demonstrated by measured differences in the dissociation constants for nucleotides, as well as conformational differences of the three $\alpha\beta$ heterodimers in crystal structures. In the first F_1 crystal structure solved ((AMPPNP)(ADP)F_1), one site is filled with the nonhydrolyzable ATP analog AMPPNP (β_{TP}), the second contains ADP (β_{DP}), and the third is empty (β_E). The conformations of the $\alpha\beta$ heterodimers that define each catalytic site also differ. The most dramatic difference is that the β subunit helical domain (DELSEED region) of βE is in an open configuration that differs from the other catalytic sites by about 15 Å. An additional tenet of the mechanism is that the energy from the proton gradient is not required to catalyze the synthesis of ATP from ADP and Pi, but is needed to release ATP against an unfavorable chemical gradient. In several crystal structures, a water molecule is positioned

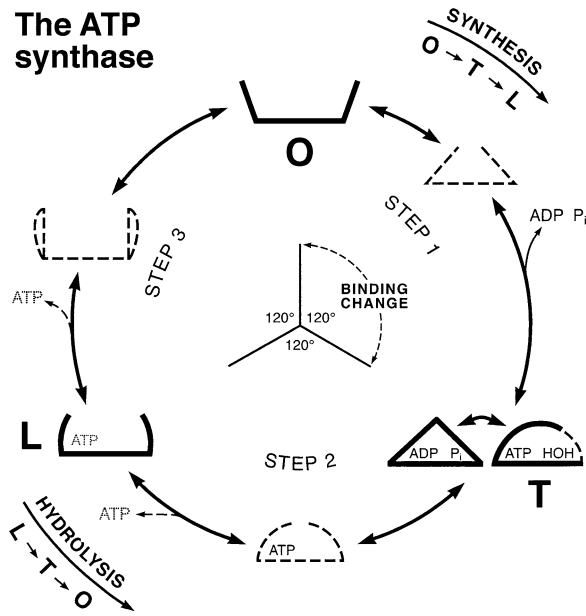

Fig. 22.7. The binding change mechanism. Conformations assumed by one catalytic site of the ATP synthase during synthesis or hydrolysis of ATP during one complete rotation of the γ subunit in 120° steps. The sites are designated as *O* (open), *T* (tight) or *L* (loose), and are related to forms designated as β_E, β_D, or β_T in the Walker structure. Tightness of ATP binding is indicated by the degree of openness of the depicted sites. Intermediate forms during a 120° binding change event are indicated with lighter dashed lines. Forms with some *curved lines* preferentially bind ATP and those with all straight lines preferentially bind ADP and P_i. The sequence of conformational events during ATP synthesis or hydrolysis is shown. Appropriate *arrows* indicate reversible substrate binding or release for rapid bi-site catalysis. *Dashed arrows* and *dotted* ATP indicate that whether ATP is released primarily from the *L* or the *O* form during rapid synthesis is not known. A *dashed* portion of the T site indicates that H_2O formed when ATP is made interchanges readily with the medium water (Boyer, 2000).

between the γ-phosphate of AMPPNP at the β_{TP} site and the aspartate (βD188) that serves as the catalytic base. This would facilitate the nucleophilic aliphatic substitution with second order kinetics (S_N2) reaction for rapid interconversion of ADP and Pi with ATP.

Consistent with the alternating site mechanism, the affinities for nucleotide of the three catalytic sites differ by several orders of magnitude. In spinach chloroplast F_1, the high and medium affinity sites are so tight that they can only be depleted effectively from the site by addition of alkaline phosphatase and by ε subunit depletion, respectively. The low affinity catalytic site has a $K_D \approx 1$ μM which is sufficient to be occupied at stromal ADP and Pi concentrations.

The *E. coli* F_1 catalytic sites are more easily depleted of nucleotides due to proportionally lower affinity than the chloroplast enzyme sites. Using the F_1 βY331W mutant of *E. coli*, Weber and co-workers (1993) monitored the catalytic site occupancy by the extent of tryptophan fluorescence quenching that occurs when nucleotide binds. Since βY345 forms a π-π bond to the nucleotide adenine ring bound at a catalytic site, binding-dependent fluorescence quenching of the substituted tryptophan provides a sensitive and direct measure of the dissociation constants of the three catalytic sites (Shimabukuro et al., 2003). The dissociation constants of the three catalytic sites for Mg^{2+}ATP and Mg^{2+}ADP are 0.001 μM, 1.0 μM, 30 μM and 0.04 μM, 1.8 μM, 35 μM, respectively, while the affinity of Mg^{2+}ITP for these sites is 0.33 μM, 41 μM and 1,400 μM. When the fractional filling of the three catalytic sites was calculated over a range of nucleotide concentrations using these dissociation constants and compared to the rate of hydrolysis, the hydrolysis rate was proportional to the fractional filling of the third catalytic site with the lowest affinity. This supports a three-site mechanism in which all three sites must become occupied with nucleotide before rotation can occur and product can be released. The catalytic sites in *E. coli* F_1-ATPase were designated site 1, site 2, and site 3 in order of decreasing affinity for Mg^{2+}ATP (Weber et al., 1996). Another tryptophan mutation was introduced at position β14. This provides a fluorescent probe able to distinguish bound Mg^{2+}ATP from Mg^{2+}ADP at each of the catalytic sites (Weber and Senior, 1996). At physiological concentrations of Mg^{2+}ATP all three catalytic sites were occupied on average, one with Mg^{2+}ATP, and the other with Mg^{2+}ADP.

Single molecule assays show that ATPase-driven γ subunit rotation occurs in a counter-clockwise direction when viewed from the γ subunit foot domain. In the context of F_1 crystal structures, the sequential conformational changes of any one catalytic site proceeds in the order $\beta_E \rightarrow \beta_{TP} \rightarrow \beta_{DP} \rightarrow \beta_E$ during ATP hydrolysis. Additional structures crystallized under a wider variety of conditions have shown that the nucleotide occupancy of the catalytic sites can be varied. Consequently, a more useful way to distinguish the catalytic site conformations independent of the bound nucleotides is by the designations BF (β_{TP}), CD (β_{DP}), and AE (β_E) where A, B, C, and D, E, F refer to the conformations of α and β subunits, respectively, of each αβ heterodimer that define a catalytic site.

The complex Mg^{2+}ADP-AlF_n has been found to be a potent inhibitor of ATPases because it adopts a stable structure that mimics the transition state. The AlF_n serves as a phosphate analog in which the fluoride ligands of the aluminum adopt a planar configuration, and Jahn-Teller distortion results in elongated O-Al-O bonds as expected when the a catalytic site is in a *conformation* that rapidly inter-converts ADP and Pi with ATP. The structure of (Mg^{2+}ADP-AlF_3^-)F_1 differs little from the first F_1 structure solved except that Mg^{2+}ADP-$AlF_3^-$is now bound at the CD site in lieu of Mg^{2+}ADP (Abrahams et al., 1994; Braig et al., 2000). If the enzyme operated by a two-site mechanism, it is anticipated that Mg^{2+}ADP-AlF_3^- would instead be bound to the BF site.

The (Mg^{2+}ADP-AlF_4)$_2F_1$ structure contains Mg^{2+}ADP-AlF_4^-at both BF and CD catalytic sites, and Mg^{2+}ADP and sulfate at the AE site. As a result, the conformation of AE has become half closed. In addition, the γ subunit foot region is rotated >20º in this structure relative to structures where the AE site is empty. Since the 40º rotational sub-step during ATP hydrolysis is dependent upon product release, this structure may be representative of the rate-limiting step of the reaction, and also suggests that the enzyme operates via a three-site mechanism. Alternatively, the 20º difference of the γ subunit foot region may merely be the result of crystal packing forces.

When the nucleotide occupancy was measured directly during rotation, it was found that each nucleotide remained bound for 240º of rotation, equivalent to two catalytic cycles, supporting a three-site mechanism. However, a few consecutive rotation events have been observed in the presence of nanomolar concentrations of Mg^{2+}ATP, which is insufficient to fill the third catalytic site. Thus, although the majority of evidence strongly favors a three-site mechanism, the ability of the enzyme to work via a two-site mechanism has not been excluded.

Alan Senior proposed a modified alternating site model for the F_1-ATPase mechanism based on the amount of bound Mg^{2+}ATP and Mg^{2+}ADP (Weber and Senior, 1997) (Fig. 22.8). In this model catalytic sites are named according to their relative affinity for Mg^{2+}ATP of high (H), medium, (M) or low (L). In the diagram, the starting point occurs when Mg^{2+}ATP binds to the low affinity site, which was transiently empty.

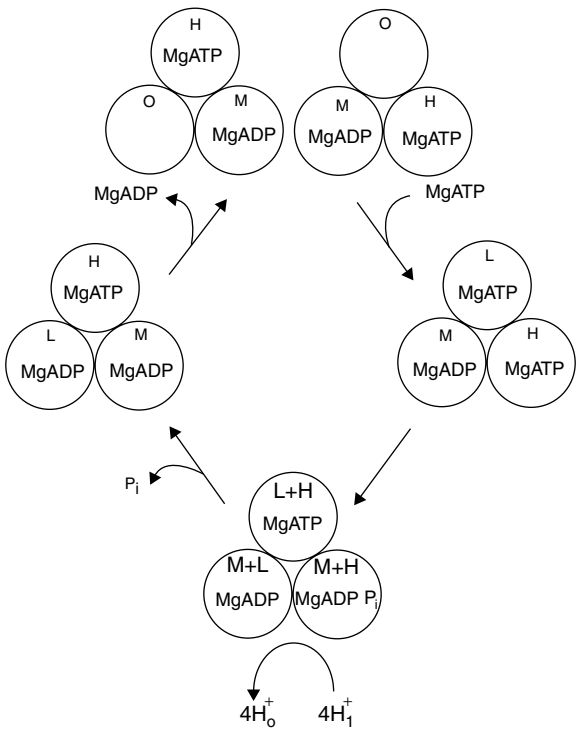

Fig. 22.8. Tri-site catalysis model. Conformations of each of the b catalytic subunits that occur during one catalytic cycle of the enzyme with all the catalytic sites in a 'closed' conformation with nucleotide bound and where the time that the open conformation exists is negligible (Taken from Weber and Senior, 1997).

Nucleotide binding rapidly promotes a conformational change of the enzyme which alters the affinity of each catalytic site for binding a nucleotide ($M \rightarrow L$, $L \rightarrow H$, $H \rightarrow M$). The Mg^{2+}ATP at the high affinity site is then hydrolyzed which forms Mg^{2+}ADP and P_i. Subsequent release of phosphate causes the enzyme to adopt a conformation which promotes the release of Mg^{2+}ADP. Product release from this state returns the enzyme to its initial state and is thought to be the rate-limiting step of the catalytic reaction. The ratio of measured Mg^{2+}ATP to Mg^{2+}ADP is about 1.8, which suggests that the empty site is rapidly filled with Mg^{2+}ATP. Since all three sites are occupied by nucleotides at one time, this mechanism is referred to as the three-site mechanism.

Subsequent investigation of the βL148W mutation by William Allison's group suggested that the mutation is not sufficient to distinguish between bound Mg^{2+}ADP and bound Mg^{2+}ATP (Dong et al., 2002). The ambiguity of the data caused Allison to propose another tri-site model for ATP hydrolysis, shown in (Fig. 22.9). This model suggests that as Mg^{2+}ATP binds to the open catalytic site a signal is propagated through the α_E site to the β_D site, where it promotes hydrolysis of ATP and conversion of β_D from the closed to open conformation, resulting in release of products. The curved arrow indicates the signal which is produced when β_E closes upon the binding Mg^{2+}ATP. In this model, hydrolysis of ATP drives the counterclockwise rotation of the γ subunit. The position of the coiled-coil of γ within the central cavity of the $(\alpha\beta)_3$ hexamer dictates which two sites are in the closed conformation. In addition, the pair of β subunits existing in closed conformations are predicted to simultaneously switch from β_D and β_T to β_T and β_E (Ren and Alison, 2000).

B. The Binding Zipper Model

George Oster and Hongyun Wang (Oster et al., 2000) postulated a model for the mechanism of torque generation called the Binding Zipper model. They surmised that after an ATP binds to the catalytic site, torque is generated by the transition from a weak to a tight binding site. This is consistent with the observed power stroke of GroEL concurrent with ATP binding. The binding zipper model illustrated by Oster is

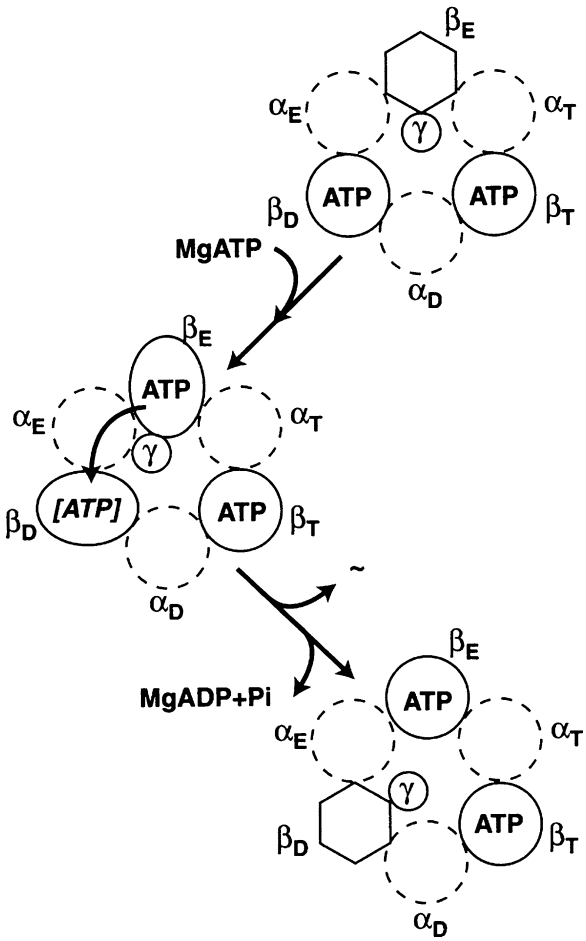

Fig. 22.9. A model for ATP hydrolysis coupled to rotation of the β subunit when three catalytic sites are saturated. *Closed circles* denote β subunits liganded with MgATP in closed conformations. *Hexagons* represent β subunits in open conformations and *ellipses* represent liganded β subunits in open/closed transition. *Stippled circles* represent a subunits liganded with MgATP in closed conformations. The transition state for ATP hydrolysis is represented by [ATP] (Taken from Ren and Allison, 2000).

shown in Fig. 22.10. However this model does not explain the role of hydrolysis. Oster proposed that both ATP binding at one site and hydrolysis at a second are required to complete one power stroke of 120°. This would imply that the power stroke is composed of two steps: a 90° step associated with ATP binding, and a 30° step associated with hydrolysis or product release. The hydrolysis step may contribute to torque generation, or contribute to the completion of the binding transition of ATP at another site. The binding zipper model postulates the electrostatic repulsion of the hydrolysis products does not contribute to torque generation. Instead, this repulsion is used to loosen the tight binding of ATP to the catalytic site for subsequent release, thereby resetting the motor. There may be elastic recoil that contributes to the power stroke as the β subunit relaxes back to its loose conformation.

The binding zipper hypothesis (Oster et al., 2000) proceeds clockwise for ATP hydrolysis. The model begins with ATP in solution, which diffuses at a rate dependent upon the concentration to the open catalytic site and initially becomes weakly bound (ATP docking). The weakly bound ATP may dissociate from the catalytic site, and only occasionally proceeds from weak binding to tight binding (the binding transition). The bonds between the ATP and the catalytic site form during the binding transition, and are accompanied by a gradual increase in the binding affinity ("the binding zipper"). Thus, the conformational change of the catalytic site is inherently linked to the binding affinity. The free energy from binding is used to generate a constant force which rotates the γ subunit during the multi-step ATP binding transition. Thus, ATP concentration only affects how often ATP binds to the catalytic site and not binding transition. Upon completion of the binding transition, the ATP and ADP and Pi are in chemical equilibrium. As the transition continues, the equilibrium is shifted to favor ADP and Pi so that hydrolysis products can be released and the cycle repeated. Product release may be coupled to the nucleotide binding at an adjacent catalytic site. Rotation, concurrent with ADP and Pi release, of the γ subunit is powered by ATP binding transitions at the other two catalytic sites. According to the binding zipper model, γ-subunit rotation should be continuous since the ATP binding affinity would be continuously coupled to γ rotation. Rotation is postulated to be driven by steric hindrance via largely hydrophobic interactions between the γ subunit and the $(\alpha\beta)_3$ ring as the result of the closing of the β subunit and the eccentricity of γ.

Synthesis follows a counterclockwise rotation around Fig. 22.10. The model assumes one of the catalytic sites has ADP and phosphate bound to it. While in the catalytic site, the molecules are in chemical equilibrium continuously changing between being the reactants and product. Thus, ATP is formed from ADP and Pi with only the energy cost of altering the conformation of the catalytic

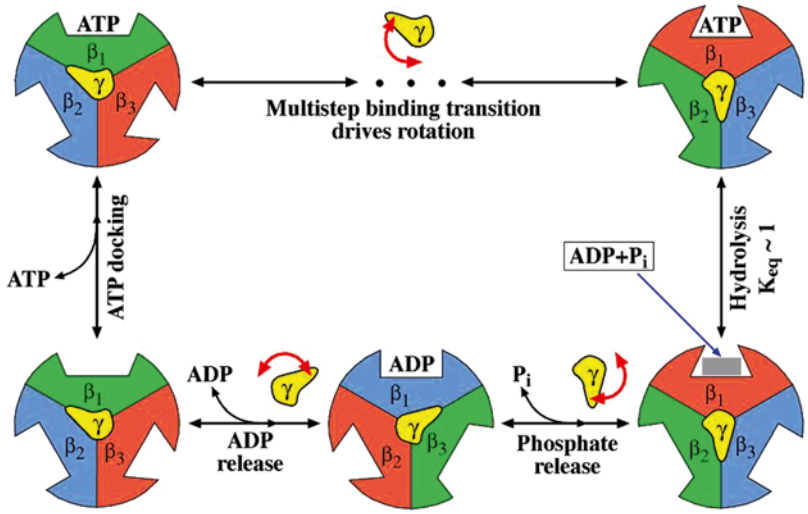

Fig. 22.10. The binding zipper model (Taken from Oster and Wang, 2000).

site. When F_1 and F_o are coupled, the mechanical torque from F_o on γ causes a reduction of the affinity between the bound ATP and the catalytic site. It is during this step that the mechanical torque is generated from the binding free energy of ATP. Because the efficiency of the reaction is high the majority of the 83.4 pN nm/molecule required for synthesis is accounted for by this step. After synthesis the binding affinity is reduced so that the ATP may be released from the catalytic site.

C. The Gamma Dictator Model

The Gamma Dictator model of F_1 rotation proposes that the γ subunit is a rigid body, and that the angular position of the foot domain relative to the $(\alpha\beta)_3$ ring coordinates substrate binding, hydrolysis, and product release. The evidence that most supports this model comes from experiments where a magnetic particle attached to the γ subunit foot domain was used to control rotation by an external magnet in an effort to measure the angular position of the γ subunit that triggers product release during the catalytic cycle (Adachi et al., 2007). Adachi and co-workers concluded that phosphate release initiates the power stroke, and that ADP release occurs about 30–40° into the power stroke. Under these conditions, the magnetic force used to drive rotation acted as a load that limited the speed of the power stroke resulting in encumbered rotation. Since the magnetic force was constant, the rate-limiting step was altered because the γ subunit was forced to rotate through the dwell position. As noted by Senior (Senior, 2007), these results concerning product release (Adachi et al., 2007) are difficult to reconcile with the observed catalytic site occupancy based on tryptophan fluorescence quenching measurements (Nadanaciva et al., 2000; Weber and Senior, 2000a, b, 2004), but would be explained if slow, forced magnetic rotation induced artificial interactions between the foot and coiled-coil domains. Rotational studies of F_1 have been based on attachment of the visible probe to the foot domain (Sabbert et al., 1996; Noji et al., 1997; Omote et al., 1999; Spetzler et al., 2006, 2009), and thus have primarily provided information about the movement of this domain. Since artificially restricting the rotation of the γ subunit affects the dwell time, it is likely to also affect the timing of substrate binding and product release, as is the case for linear motors (Spudich, 2006).

D. The Elastic Coupling Hypothesis

Any rotational mechanism must address the observations that the torque generated by F_1 during hydrolysis involves the coiled-coil of the γ subunit (Furuike et al., 2008), and that the F_1F_o drive shaft is flexible and can push or pull during either ATP hydrolysis or synthesis (Cherepanov et al., 1999; Cherepanov and Junge, 2001; Junge et al., 2001; Sielaff et al., 2008b).

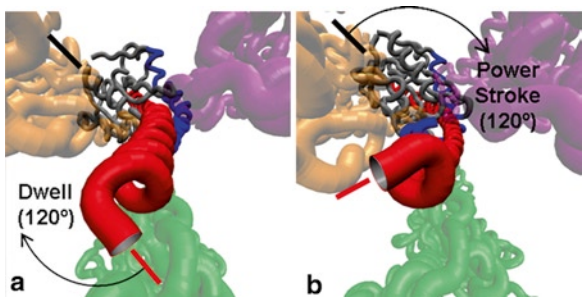

Fig. 22.11. Depiction of how dwells (**a**) and power strokes (**b**) are related in the elastic coupling hypothesis.

These observations may be explained if rotation is driven by elastic energy stored between the two domains of the γ subunit (Fig. 22.11). Differential movement of the coiled-coil and foot domain can store elastic energy in a spring-like manner (Hausrath et al., 1999; Nath, 2008; Sielaff et al., 2008b), and fulfils the requirement that the F_1F_o ATP synthase has a flexible drive shaft (Cherepanov et al., 1999; Cherepanov and Junge, 2001; Junge et al., 2001; Sielaff et al., 2008b), consistent with the mechanism proposed by Cherepanov et al., (1999). The proton-motive force necessary by F_o in the ATP synthase to stall F_1-ATPase activity (Fischer et al., 2000) is comparable to the 63 pN nm of torque generated by ATP hydrolysis (Spetzler et al., 2006; Hornung et al., 2008a, b). Measurements of the compliance of the γ subunit show that the elasticity between the coiled-coil and the foot domain has a spring constant of 68 pN nm (Sielaff et al., 2008b), sufficient to generate the observed torque values.

V. Computer Simulations of F_1 Rotation

By adding an external force on the lower part of γ-ε shaft, a 7 ns simulation of ATP synthesis revealed that the rotation in synthesis direction lowers the ATP affinity of the β_{TP} binding site and triggers a fast and spontaneous closure of the empty β_E subunit (Bockmann and Grubmuller, 2002). A further simulation on isolated β subunits (Bockmann and Grubmüller, 2003) implied that the internal tension of the β_E subunit dominates possible forces from interactions with α or γ subunits. Using targeted molecular dynamics and biased molecular dynamics, a simulation of ATP hydrolysis in F_1-ATPase during a 120° γ rotation showed that both Van der Waals and electrostatic interactions contribute to explain how γ rotation induces conformational changes in the catalytic sites of β subunits (Ma et al., 2002).

The cooperativity among the three β subunits was investigated by using a quantum mechanical molecular modeling (QM/MM) method (Dittrich et al., 2004) as well as free energy simulation (Gao et al., 2003). Although F_1-ATPase by itself catalyzes ATP hydrolysis, the QM/MM study suggested that the ATP hydrolysis in the β_{TP} site is endothermic and the conformation of β_{TP} facilitates ATP synthesis rather than hydrolysis. The reactions in β_{DP} were found almost equienergetic. The free energy profile based on the binding free energy of ATP and ADP + Pi, showed that during one 120° rotation, two β subunits release free energy when their conformations change from β_E to β_{TP} and from β_{TP} to β_{DP}. These two sources were postulated to drive γ rotation: and the energy-consuming conformational change in the third site ($\beta_{DP} \rightarrow \beta_E$) was consequently driven by γ rotation.

A comprehensive Normal Mode Analysis of the inherent plasticity of F_1 ATPase has been made to study the inherent flexibility of individual subunits as well the entire ATPase complex (Cui et al., 2004). It was found that the intrinsic flexibility of the γ subunit contributes to the energy cost of γ rotation relative to $\alpha_3\beta_3$ subunits. It was also found in the slowest modes that the motions of the nucleotide-binding domain and the helical (DELSEED) domain of β subunit were coupled. With regard to the interactions between this helical domain and γ subunit, this phenomenon suggests a communication between β and γ subunits. The low frequency modes of individual β subunits suggest that the structure of the β_E subunit is highly related to the $\beta_E \rightarrow \beta_{TP}$ transition. This further confirmed the previous dynamic simulation on an isolated β subunit, (Bockmann and Grubmüller, 2003) in which the open β_E subunit spontaneously changed its conformation toward the closed β subunit conformation.

Regarding the size of the F_1-ATP synthase, coarse-grained modeling is a compromise of computational resources. Dynamic domain analysis on β and γ subunits and their interactions revealed that the bi-site mechanism proposed by Paul

Boyer may not be sufficient to describe the unidirectional rotation of F_1-ATPase (Liu et al., 2006). A Go-model, which represents each residue as a single point, was built based on the first crystal structure (Abrahams et al., 1994). Starting from the (DP, E, TP) conformation, when the potential was switched to that of (E, DP, TP) conformation after 30,000 time steps, γ rotation of about 120º in 90º and 30º sub-steps was observed. However, if the potential was switched to (DP, TP, TP), a fatal collision occurred. This experiment suggested that F_1-ATPase is driven by a bi-site mechanism (Koga and Takada, 2006). A Targeted Molecular Dynamics simulation applied on a coarse-grained plastic network model supported the tri-site mechanism. Assuming that conformational changes in $\alpha_3\beta_3$ ring cause γ rotation, this two-step simulation, which yields an overall 120º rotation in the hydrolysis direction, suggested that ATP binding and product release drives an 85º rotation and a 35º rotation sequentially (Pu and Karplus, 2008).

Using mathematical models, Oster and co-workers (Sun et al., 2004) made efforts to quantitatively describe the γ rotation sub-steps and dwells. They proposed that ATP-binding triggers the bending of β subunits, which stores elastic energy that drives γ rotation. Consequently, interactions between γ and β subunits open the gate for ADP release and ATP binding (Sun et al., 2004).

VI. F_o Mechanism

A. Brownian Ratchet Energy Transduction

The F_o motor is not only able to generate enough torque to release ATP from F_1 against an unfavorable concentration gradient, but is also capable of pumping ions when driven backwards by ATP hydrolysis in F_1. These features constrain the possible mechanisms, and suggest a possible model for *P. modestum* of how a transmembrane electrochemical difference generates a rotary torque sufficient to account for ATP synthesis. Since the c-ring is rotationally symmetric, and because ATP synthesis requires the rotor to turn, it is likely that there is a structural asymmetry in subunit a of the stator which is involved in the ion channel. It has been suggested that there is a periplasmic half-channel that connects to the cytoplasm via a hydrophilic strip. Ions are prevented from leaking between the acidic periplasmic space and the basic cytoplasm along this path by aR227 (equivalent to *E. coli* aR210). Thus, for ions to pass through the membrane, they must utilize the rotor site, cE65 (equivalent to *E. coli* cD61), and follow the pathway through the hydrophobic region of the stator into the cytoplasm. This is feasible since the ion neutralizes the negatively charged rotor site and does not encounter the large free-energy barrier that inhibits the passage through the aqueous channel into the low dielectric environment of the rotor-stator interface.

Torque generated by the electrostatic interactions between the rotor and stator bias the rotational diffusion of the rotor Fig. 22.12a. Forces that act on the rotor to drive rotation include: (1) the Brownian force; (2) the resistance of the γ shaft connecting the F_1; (3) the electrostatic interactions which consist of the stator charge (aR227) attraction to the nearby empty rotor site (cE65); (4) the membrane potential drop across the horizontal segment of the channel that induces a torque (Fig. 22.12a); and (5) the rotor-stator interface which is hydrophobic so as to be leak-proof to ions. Thus there is a dielectric barrier between the hydrophilic regions of the a-subunit and the c-ring that forms the ion channel. This channel repels the unoccupied rotor sites but allows occupied (neutralized) sites to pass. This barrier creates a bias in the diffusion of the rotor (Fig. 22.12a). In this model, an ion binding to the rotor from the periplasmic channel can pass through the rotor-stator interface, but when the ion is released to the cytoplasm it cannot re-enter the cytoplasmic channel. A typical sequence of events for the stochastic progress of the rotor is shown schematically in Fig. 22.12b, which is consistent with a Brownian Ratchet mechanism (Junge et al., 1997; Elston et al., 1998; Dimroth et al., 1999; Oster and Wang, 1999, 2003; Oster et al., 2000; Oster, 2002; Xing et al., 2004).

A Brownian ratchet mechanism must meet two requirements to function: first, that there are two noncolinear proton access half-channels from each side of the membrane leading to the cD61 carboxyl; and second, that rotational diffusion of the c-ring relative to subunit-a is periodically restricted in some manner. In single molecule FRET measurements, rotation of the

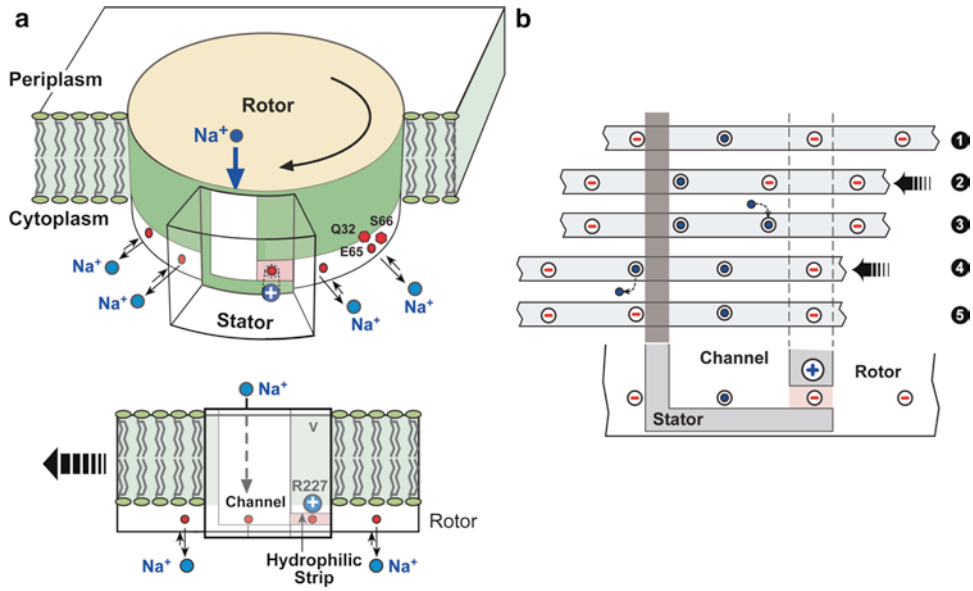

Fig. 22.12. Diagrams illustrating the proposed mechanism of action of F_o. (**a**) Schematic of the rotor-stator assembly in *P. modestum*. During ATP synthesis, the rotor turns to the left (clockwise as viewed from the periplasm). The rotor section below the level of the membrane contains the 12 ion-binding sites. Each binding site consists of the triad Gln32- Glu65-Ser66, which coordinates a sodium ion. The stator contains an aqueous channel that conducts ions from the periplasmic (positive) reservoir to the level of the horizontal hydrophilic strip, below the membrane. The positive stator charge, R227, blocks leakage of ions along this strip to the cytoplasm. The *bottom panel* shows a face-on view. (**b**) A typical sequence of events that advance the rotor by one step of 2p/12. Consider the initial position of the rotor shown at (1). The third site from the left is held by the stator charge. In step (1)→(2) the rotor fluctuates so that the third (empty) site jumps out of the potential well of the stator charge. This jump is biased by the transmembrane potential and is helped by the dielectric barrier preventing the first rotor site (empty) from entering the low dielectric medium of the stator. In step (2)→(3), once the third rotor site moves out of the potential well of the stator charge and moves into the aqueous channel, it quickly binds a sodium ion from the periplasmic (acidic) reservoir. In step (3)→(4) the positive stator charge pulls the empty fourth rotor site into its potential well. As the second rotor site is neutralized, it can pass through the dielectric barrier. Once the second rotor site passes out of the stator its sodium ion quickly hops off into the basic cytoplasmic reservoir (4)→(5). Once empty, this site cannot go back into the low dielectric rotor-stator interface. In the final stage (5) the rotor is in exactly the same state as (1), but shifted to the left by one rotor step (Taken from Oster and Wang, 1999).

c_{10}-ring with step sizes of 36 degrees, consistent with an interaction between subunit-a and adjacent c-subunits, were observed 48% of the time using *E. coli* F_oF_1 proteoliposomes during ATP synthesis in response to a membrane potential of >200 mV (Düser et al., 2009). Larger steps of 72, 108, and 144 degrees were also observed, each separated by 9 ms dwells. The larger step sizes may be the result of the millisecond time resolution limit of the FRET measurements.

Using single molecule rotation measurements with a 10 microsecond time resolution, ATPase-dependent rotation of *E. coli* F_oF_1 embedded in lipid bilayer nanodiscs for stabilization were observed rotate in steps limited to 36 degrees when subjected to a viscosity-induced load (Ishmukhametov et al., 2010). The 100 microsecond interactions between subunit-a and each subunit in the c-ring responsible for this stepping did not depend on the aR210-cD61, but on another previously unknown interaction between these subunits. A striking feature of this interaction is that it forms a leash that limits c-ring rotation to 36 degress in a manner that can satisfy the restricted motion requirement of the F_o Brownian ratchet mechanism. As these 36 degree transient dwells do not form when c-ring rotation and proton transport occur at high rates, the possible use of the leash was anticipated to occur at steady state conditions in which the cellular ATP concentration is high relative to ADP and Pi. Under these conditions, when the free energy of the

proton gradient approaches equilibrium with the chemical potential of ATP, the F_o motor was postulated to use the leash as part of a Brownian ratchet to bias rotation for ATP synthesis against an F_1 motor-imposed load.

B. Protein-Roller Bearing Mechanism

The Protein-roller Bearing Mechanism is a model describing molecular events in the F_o active site of the proton pumping enzyme from *E. coli* based on the results of molecular dynamics simulations (Aksimentiev et al., 2004) and protein cross-linking (Schwem and Fillingame, 2006; Angevine et al., 2007). In a resting state, when no torque is imposed on F_o by $\Delta\mu_{H+}$ or ΔG_{ATP}, aR210 makes a salt bridge with two deprotonated cD61 of two neighboring c subunits. The bridge can be easily transferred from one residue to the other, e.g., the probability of establishing the connection between these aspartates is equal. When a load is imposed from $\Delta\mu_{H+}$, the correct cD61 becomes protonated from aN214. Protonation of that aspartate is followed by concerted rearrangement of transmembrane helices. In turn the C-terminal helix of subunit c with a protonated aspartate rotates clockwise removing the protonated cD61 from the active site. At the same time, the fourth transmembrane helix of subunit a rotates counterclockwise toward the third c subunit, and causes protonation of aS206 by that cD61 (Dmitriev et al., 2008; Moore and Fillingame, 2008; Moore et al., 2008; Steed and Fillingame, 2008, 2009). This deprotonated aspartate makes a salt bridge with the arginine at position aR210. That bridging is accompanied by relaxation of conformation of the triple complex $cD61_1$-aR210-$cD61_2$, returning to the initial resting conformation.

When a force in the opposite direction is applied by F_1, the sequence of these molecular events is reversed such that aS206 is a primary proton donor, and the rotor rotates in a clockwise direction. Thus, the function of the aR210 is proposed to redirect protons from one half-channel to the other by inducing rotations in the rotor as the result of forming salt bridges with two cD61 carboxyls on the rotor. However, the structure of the c-ring and subunit-a is considered by some groups to be too rigid enough to allow such big changes (Meier et al., 2005; Ishmukhametov et al., 2008).

In the case of the sodium-pumping enzyme from *I. tartaricus* (Dimroth et al., 2000, 2006; Xing et al., 2004) aR227 opens and closes the gate between subunit a and the Na^+ binding site on subunit c. In the direction of synthesis, the positively charged arginine repels the Na^+ from the approaching binding site and simultaneously exerts a pull on the negatively charged side chain of cE65, keeping it in its original conformation. Therefore, the Na^+ is thought to escape vertically from the binding site into the cytoplasm. On passing the aR227 residue, the electrostatic attraction to cE65 is retained, thus opening the gate between subunit a and the binding site to allow reloading of the site through the periplasmic entrance channel in subunit a.

VII. Regulation of ATP Synthase

Under some conditions, F_1F_o can catalyze ATPase-dependent proton pumping. However, regulatory mechanisms decrease the efficiency of this activity. An inhibitory protein specific to the mitochondrial enzyme can intercalate into the complex under conditions that favor ATP hydrolysis activity (Lebowitz and Pedersen, 1996). The ATPase activity of chloroplast F_1F_o ATP synthase (CF_1F_o) is instead regulated by the redox state of a disulfide bond between γC199 and γC205. These two cysteine residues are part of an insertion of residues 190–240 found in ATP synthases from chloroplasts and photosynthetic cyanobacteria.

When the disulfide is oxidized, CF_1 has very low ATPase activity. However, reduced thioredoxin produced as the result of the photosynthetic light reactions activates the ATP synthase by reducing the disulfide. Consequently, the enzyme becomes activated when a proton gradient is present to drive ATP synthesis. Other chloroplast enzymes, e.g., NADP-dependent malate dehydrogenase and chloroplast fructose bisphosphatase are activated by disulfide reduction in a similar manner (Carr et al., 1999; Chiadmi et al., 1999; Johansson et al., 1999). Like the chloroplast enzyme, activation of F_1F_o from cyanobacteria requires the formation of a membrane potential even though the cysteines are absent in the analogous peptide γ subunit insertion, which suggests that the insertion is also important to this type of regulation.

After chloroplasts have reached steady state in the light, the onset of darkness leads to a condition in which the proton gradient rapidly dissipates, while the ATP/ADP + Pi ratio remains high. Under these conditions, F_1F_o initially catalyzes ATPase-dependent proton pumping back to the lumen, the reverse of synthase activity. This wasteful consumption of the energy captured in light is rapidly halted by formation of a disulfide bond in the γ subunit that stops rotation and ATP hydrolysis (Nalin and McCarty, 1984; Konno et al., 2000, 2004; Ueoka-Nakanishi et al., 2004; Konno et al., 2006). The dark decay of ATPase activity in thylakoids is accelerated by the addition of ADP that becomes tightly bound in the latent state. Inhibition of ATPase activity and an increase in affinity of the site for ADP only occur upon the addition of Mg^{2+}. Under these conditions, the bound ADP forms a complex with Mg^{2+} that prevents further catalysis. This entrapment of Mg^{2+}-ADP serves a regulatory function in F_1-ATPases from other organisms as well.

Inhibition of ATPase activity by the entrapment of MgADP at a catalytic site is common to many ATP synthases, including those of the chloroplast (Richter et al., 1984) and *E. coli* (Klionsky et al., 1984; Jault and Allison, 1993). Analysis of the VO^{2+}-ADP complex bound to the low affinity site in latent chloroplast F_1 by continuous wave EPR and electron spin-echo envelop modulation (ESEEM) spectroscopies in combination with site-directed mutagenesis, revealed that in the latent state, metal coordination of the metal-nucleotide complex at the catalytic site containing ATP include an amino group from the P-loop lysine, βK162, as well as the Walker homology B sequence (WHB) aspartate, βD256, but does not include the P-loop threonine, βT163. This combination of ligands is likely to contribute significantly to the inability of chloroplast F_1 to catalyze ATP hydrolysis in the latent state. The P-loop lysine also becomes a metal ligand under conditions when F_1 is inhibited by free metal. This suggests that the ability of the P-loop lysine to form a metal ligand in these non-functional conformations of the low affinity catalytic site may contribute significantly to the regulation of ATPase activity in this enzyme. The conformational changes that occur upon activation induced by reduction of the disulfide in the γ subunit cause the insertion of the P-loop threonine and the catch loop tyrosine, βY311, into the coordination sphere, displacing the WHB aspartate and the P-loop lysine.

In the chloroplast and bacterial enzymes, this inhibition has been linked to conformational changes in the C-terminal helix-turn-helix domain of the ε subunit (Hara et al., 2001; Tsunoda et al., 2001b). A high resolution structure of *E. coli* F_1 in this autoinhibited conformation elucidates how the C-terminal helix-turn-helix in the up position disrupts the functional interface between the rotor and stator of the motor (Cingolani and Duncan, 2011). Cross-linking studies indicate that ATP hydrolysis cannot be catalyzed when ε is cross-linked in the up-state, but ATP synthesis can occur (Tsunoda et al., 2001b). When movement of the C-terminal domain was restricted to the folded conformation via the introduction of a specific crosslink between the N- and C- terminal domains of the ε subunit, this subunit was unable to inhibit ATPase activity in F_1 from the thermophilic bacterium *PS3* (i.e., TF_1) (Kato-Yamada et al., 2000). Additional cross-linking studies (Suzuki et al., 2003) indicate that ε is in the up-state when the nucleotide is absent. This suggests that movement of the ε subunit helix-turn-helix domain impacts the activation of F_1-ATPase. Cross-linking experiments also suggested that conditions that promote and retard MgADP entrapment also promote and retard extension of the helix-turn-helix domain (Konno et al., 2006; Feniouk et al., 2007). Although evidence correlates the structure of the extended ε subunit helix-turn-helix with regulatory conformations in the bacterial enzyme (Kato-Yamada et al., 2000) and a similar model has been proposed for light-dependent activation of CF_1F_o (Hisabori et al., 2002; Suzuki et al., 2003), the stabilization of this motif provided by the small ε' subunit decreases the likelihood that such a large conformational change occurs in F_1 from mitochondria.

Regulation by the ε subunit and the γ disulfide are correlated, and the extent of activation by these two subunits is additive in the chloroplast enzyme. Reduction enhances the dissociation of the ε subunit from CF_1, while the attachment of the ε subunit promotes re-oxidization of the enzyme (Soteropoulos et al., 1992). Depletion of the ε subunit from CF_1 or reduction of the disulfide also exposes trypsin-sensitive sites (Hightower and McCarty, 1996; McCarty, 2005).

These observations suggest that the regulatory motifs of these two subunits are in close proximity. The same trypsin-sensitive sites become exposed in response to activation by a membrane potential. When subsequently isolated from the thylakoid membranes, trypsin-cleaved F_1 is partially depleted of the ε subunit suggesting that the membrane potential decreased its affinity. The reversible depletion of the ε subunit partially activates ATPase activity, which makes it easier to reduce the disulfide. Conversely, reducing the disulfide causes εK109 to become solvent exposed (Komatsu-Takaki, 1992), and decreases the affinity for the ε subunit (Soteropoulos et al., 1994), suggesting that the regulatory motif of the ε subunit partially masks the disulfide bridge.

By substituting the regulatory segment of CF_1 γ subunit into TF_1-ATPase, the regulatory activity was observed in the chimeric complex (Bald et al., 2000). Single molecule rotation measurements showed that the oxidized enzyme had longer and more frequent pauses between rotational events than when the disulfide was reduced (Bald et al., 2001). When the disulfide is reduced the ε subunit from CF_1, but not from TF_1, can inhibit ATP hydrolysis in the F_1 containing the chimeric γ subunit (Bald et al., 2000). This indicates that regulation results from specific interactions between the CF_1 γ subunit regulatory region and the CF_1 ε subunit. Deletion of three negatively charged residues (CF_1-$\gamma E_{210}D_{211}E_{212}$) close to the disulfide bridge eliminates the regulation of ATPase activity suggesting that these groups are also involved in the regulatory function (Konno et al., 2000; Ueoka-Nakanishi et al., 2004; Samra et al., 2006).

The absence of a high-resolution structure of CF_1 ε and γ subunits hinders a complete understanding of the regulatory mechanism. A structure for the CF_1 γ subunit was homology-modeled based on the crystal structures of mitochondrial and *E. coli* proteins (Richter et al., 2005; Samra et al., 2006). In this model the γ regulatory region forms a loop that contains the dithiol sulfhydryls. Disulfide formation is hypothesized to induce a movement of this region such that it forms a close association with the open α/β domain of the γ subunit. The model also shows a second loop composed of residues 65–78 to be larger in the chloroplast that is positioned near the regulatory loop. Molecular dynamics simulations suggest that the second loop associates with the dithiol loop in the closed conformation in a manner that might inhibit rotation of the γ subunit.

Sequence comparisons show that the γ subunit disulfide bond responsible for activation of the chloroplast ATP synthase is part of an insertion to the loop that connects the foot and coiled-coil domains. In mitochondrial F_1 structures, an additional small polypeptide (mitochondrion ε subunit) is composed of a helix that makes contact with the helix-turn-helix domain of what is known as the chloroplast ε subunit, and then forms a β-strand that is part of the β-sheet of the γ subunit foot domain. Since this small peptide is close in size to the chloroplast insert that contains the disulfide, it is proposed here that the chloroplast insert takes the place of this polypeptide in chloroplast F_1.

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References

Abrahams JP, Leslie AGW, Lutter R and Walker JE (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature 370: 621–628

Adachi K, Yasuda R, Noji H, Itoh H, Harada Y, Yoshida M and Kinosita K (2000) Stepping rotation of F_1-ATPase visualized through angle-resolved single-fluorophore imaging. Proc Natl Acad Sci USA 97: 7243–7247

Adachi K, Noji H and Kinosita K (2003) Single-molecule imaging of rotation of F_1-ATPase. Methods Enzymol 361: 211–227

Adachi K, Oiwa K, Nishizaka T, Furuike S, Noji H, Itoh H, Yoshida M and Kinosita K (2007) Coupling of rotation and catalysis in F_1-ATPase revealed by single-molecule imaging and manipulation. Cell 130: 309–321

Aksimentiev A, Balabin IA, Fillingame RH and Schulten K (2004) Insights into the molecular mechanism of rotation in the F_o sector of ATP synthase. Biophys J 86: 1332–1344

Angevine CM and Fillingame RH (2003) Aqueous access channels in subunit *a* of rotary ATP synthase. J Biol Chem 278: 6066–6074

Angevine CM, Herold KAG and Fillingame RH (2003) Aqueous access pathways in subunit *a* of rotary ATP synthase extend to both sides of the membrane. Proc Natl Acad Sci USA 100: 13179–13183

Angevine CM, Herold KAG, Vincent OD and Fillingame RH (2007) Aqueous access pathways in ATP synthase subunit *a*. Reactivity of cysteine substituted into transmembrane helices 1, 3, and 5. J Biol Chem 282: 9001–9007

Bald D, Noji H, Stumpp MT, Yoshida M and Hisabori T (2000) ATPase activity of a highly stable $\alpha_3\beta_3\gamma$ subcomplex of thermophilic F_1 can be regulated by the introduced regulatory region of γ subunit of chloroplast F_1. J Biol Chem 275: 12757–12762

Bald D, Noji H, Yoshida M, Hirono-Hara Y and Hisabori T (2001) Redox regulation of the rotation of F_1-ATP synthase. J Biol Chem 276: 39505–39507

Bhatt D, Cole SP, Grabar TB, Claggett SB and Cain BD (2005) Manipulating the length of the *b* subunit F_1 binding domain in F_1F_o ATP synthase from *Escherichia coli*. J Bioenerg Biomembr 37: 67–74

Bi Y, Watts JC, Bamford PK, Briere LK and Dunn SD (2008) Probing the functional tolerance of the *b* subunit of *Escherichia coli* ATP synthase for sequence manipulation through a chimera approach. Biochim Biophys Acta 1777: 583–591

Birkenhager R, Hoppert M, Deckers-Hebestreit G, Mayer F and Altendorf K (1995) The F_o complex of the *Escherichia coli* ATP synthase. Investigation by electron spectroscopic imaging and immunoelectron microscopy. Eur J Biochem 230: 58–67

Bockmann RA and Grubmuller H (2002) Nanoseconds molecular dynamics simulation of primary mechanical energy transfer steps in F_1-ATP synthase. Nat Struct Biol 9: 198–202

Bockmann RA and Grubmüller H (2003) Conformational dynamics of the F_1-ATPase β-subunit: A molecular dynamics study. Biophys J 85: 1482–1491

Börsch M, Diez M, Zimmermann B, Reuter R and Gräber P (2002) Stepwise rotation of the γ-subunit of EF_oF_1-ATP synthase observed by intramolecular single-molecule fluorescence resonance energy transfer. FEBS Lett 527: 147–152

Bowler MW, Montgomery MG, Leslie AGW and Walker JE (2007) Ground state structure of F_1-ATPase from bovine heart mitochondria at 1.9 Å resolution. J Biol Chem 282: 14238–14242

Boyer PD (1975) A model for conformational coupling of membrane potential and proton translocation to ATP synthesis and to active transport. FEBS Lett 58: 1–6

Boyer PD (1993) The binding change mechanism for ATP synthase--some probabilities and possibilities. Biochim Biophys Acta 1140: 215–250

Boyer PD (1997) The ATP synthase - A splendid molecular machine. Annu Rev Biochem 66: 717–749

Boyer PD (2000) Catalytic site forms and controls in ATP synthase catalysis. Biochim Biophys Acta 1458: 252–262

Braig K, Menz RI, Montgomery MG, Leslie AGW and Walker JE (2000) Structure of bovine mitochondrial F_1-ATPase inhibited by Mg^{2+}ADP and aluminium fluoride. Structure 8: 567–573

Carr PD, Verger D, Ashton AR and Ollis DL (1999) Chloroplast NADP-malate dehydrogenase: structural basis of light-dependent regulation of activity by thiol oxidation and reduction. Structure 7: 461–475

Caviston TL, Ketchum CJ, Sorgen PL, Nakamoto RK and Cain BD (1998) Identification of an uncoupling mutation affecting the *b* subunit of F_1F_o ATP synthase in *Escherichia coli*. FEBS Lett 429: 201–206

Cherepanov DA and Junge W (2001) Viscoelastic dynamics of actin filaments coupled to rotary F-ATPase: Curvature as an indicator of the torque. Biophys J 81: 1234–1244

Cherepanov DA, Mulkidjanian AY and Junge W (1999) Transient accumulation of elastic energy in proton translocating ATP synthase. FEBS Lett 449: 1–6

Chiadmi M, Navaza A, Miginiac-Maslow M, Jacquot J-P and Cherfils J (1999) Redox signalling in the chloroplast: structure of oxidized pea fructose-1,6-bisphosphate phosphatase. EMBO J 18: 6809–6815

Cingolani G and Duncan TM (2011) Structure of the ATP synthase catalytic complex (F_1) from *Escherichia coli* in an autoinhibited conformation. Nat Struct Mol Biol 18: 701–707

Cipriano DJ, Wood KS, Bi Y and Dunn SD (2006). Mutations in the dimerization domain of the *b* subunit from the *Escherichia coli* ATP synthase. Deletions disrupt function but not enzyme assembly. J Biol Chem 281: 12408–12413

Cui Q, Li G, Ma J and Karplus M (2004) A normal mode analysis of structural plasticity in the biomolecular motor F_1-ATPase. J Mol Biol 340: 345–372

Dautant A, Velours J and Giraud M-F (2010) Crystal structure of the Mg-ADP-inhibited state of the yeast F_1c_{10}-ATP synthase. J Biol Chem 285: 29502–29510

Del Rizzo PA, Bi Y, Dunn SD and Shilton BH (2002) The "second stalk" of *Escherichia coli* ATP synthase: Structure of the isolated dimerization domain. Biochemistry 41: 6875–6884

DeLeon-Rangel J, Zhang D and Vik SB (2003) The role of transmembrane span 2 in the structure and function of subunit *a* of the ATP synthase from *Escherichia coli*. Arch Biochem Biophys 418: 55–62

Dickson VK, Silvester JA, Fearnley IM, Leslie AG and Walker JE (2006) On the structure of the stator of the mitochondrial ATP synthase. EMBO J 25: 2911–2918

Dimroth P, Matthey U and Kaim G (2000) Osmomechanics of the *Propionigenium modestum* F_o motor. J Bioenerg Biomembr 32: 449–458

Dimroth P, von Ballmoos C and Meier T (2006) Catalytic and mechanical cycles in F-ATP synthases. Fourth in the Cycles Review Series. EMBO Rep 7: 276–282

Dimroth P, Wang H, Grabe M and Oster G (1999) Energy transduction in the sodium F-ATPase of *Propionigenium modestum*. Proc Natl Acad Sci USA 96: 4924–4929

Dittrich M, Hayashi S and Schulten K (2004) ATP hydrolysis in the β_{TP} and β_{DP} catalytic sites of F_1-ATPase. Biophys J 87: 2954–2967

Dmitriev O, Jones PC, Jiang W and Fillingame RH (1999) Structure of the membrane domain of subunit *b* of the *Escherichia coli* F_oF_1 ATP synthase. J Biol Chem 274: 15598–15604

Dmitriev OY, Freedman KH, Hermolin J and Fillingame RH (2008) Interaction of transmembrane helices in ATP synthase subunit *a* in solution as revealed by spin label difference NMR. Biochim Biophys Acta 1777: 227–237

Dong K, Ren H and Allison WS (2002) The fluorescence spectrum of the introduced tryptophans in the alpha $\alpha_3(\beta F155W)_3\gamma$ subcomplex of the F_1-ATPase from the thermophilic *Bacillus* PS3 cannot be used to distinguish between the number of nucleoside di- and triphosphates bound to catalytic sites. J Biol Chem 277: 9540–9547

Duncan TM (2004) The ATP synthase: Parts and properties of a rotary motor. In: Hackney DD and Tamanoi F (eds) The Enzymes, Vol 23: Energy Coupling and Molecular Motors, pp 203–275. Elsevier Academic Press, New York

Düser MG, Zarrabi N, Cipriano DJ, Ernst S, Glick GD, Dunn SD and Börsch M (2009) 36˚ step size of proton-driven *c*-ring rotation in F_oF_1-ATP synthase. EMBO J 28: 2689–2696

Elston T, Wang H and Oster G (1998) Energy transduction in ATP synthase. Nature 391: 510–513

Feniouk BA and Junge W (2009) Proton translocation and ATP synthesis by the F_oF_1-ATPase of purple bacteria. In : Hunter CN, Daldal F, Thurnauer MC and Beatty JT (eds) The Purple Phototropic Bacteria, Advances in Photosynthesis and Respiration, Vol 28, pp 475–493. Springer, Dordrecht

Feniouk BA, Suzuki T and Yoshida M (2007) Regulatory interplay between proton motive force, ADP, phosphate, and subunit ε in bacterial ATP synthase. J Biol Chem 282: 764–772

Ferguson SA, Keis S and Cook GM (2006) Biochemical and molecular characterization of a Na^+-translocating F_1F_o-ATPase from the thermoalkaliphilic bacterium *Clostridium paradoxum*. J Bacteriol 188: 5045–5054

Fillingame RH, Angevine CM and Dmitriev OY (2002) Coupling proton movements to *c*-ring rotation in F_1Fo ATP synthase: aqueous access channels and helix rotations at the *a-c* interface. Biochim Biophys Acta 1555: 29–36

Fillingame RH, Angevine CM and Dmitriev OY (2003) Mechanics of coupling proton movements to *c*-ring rotation in ATP synthase. FEBS Lett 555: 29–34

Fischer S, Gräber P and Turina P (2000) The activity of the ATP synthase from *Escherichia coli* is regulated by the transmembrane proton motive force. J Biol Chem 275: 30157–30162

Frasch WD (2000) The participation of metals in the mechanism of the F_1-ATPase. Biochim Biophys Acta 1458: 310–325

Furuike S, Hossain MD, Maki Y, Adachi K, Suzuki T, Kohori A, Itoh H, Yoshida M and Kinosita Jr K (2008) Axle-less F_1-ATPase rotates in the correct direction. Science 319: 955–958

Gao YQ, Yang W, Marcus RA and Karplus M (2003) A model for the cooperative free energy transduction and kinetics of ATP hydrolysis by F_1-ATPase. Proc Natl Acad Sci USA 100: 11339–11344

Gibbons C, Montgomery MG, Leslie AGW and Walker JE (2000) The structure of the central stalk in bovine F_1-ATPase at 2.4 Å resolution. Nat Struct Biol 7: 1055–1061

Girvin ME, Rastogi VK, Abildgaard F, Markley JL and Fillingame RH (1998) Solution structure of the transmembrane H^+-transporting subunit c of the F_1F_o ATP synthase. Biochemistry 37: 8817–2884

Greene MD and Frasch WD (2003) Interactions among γR268, γQ269, and the β subunit catch loop of *Escherichia coli* F_1-ATPase are important for catalytic activity. J Biol Chem 278: 51594–51598

Greie JC, Deckers-Hebestreit G and Altendorf K (2000) Secondary structure composition of reconstituted subunit *b* of the *Escherichia coli* ATP synthase. Eur J Biochem 267: 3040–3048

Hara K.Y., Kato-Yamada Y., Kikuchi Y., Hisabori T. and Yoshida M. (2001). The role of the βDELSEED motif of F_1-ATPase: Propagation of the inhibitory effect of the ε subunit. J Biol Chem 276: 23969–23973

Hausrath AC, Grüber G, Matthews BW and Capaldi RA (1999) Structural features of the γ subunit of the *Escherichia coli* F_1 ATPase revealed by a 4.4-Å resolution map obtained by x-ray crystallography. Proc Natl Acad Sci USA 96: 13697–13702

Hennig J and Herrmann RG (1986). Chloroplast ATP synthase of spinach contains nine nonidentical subunit species, six of which are encoded by plastid chromosomes in two operons in a phylogenetically conserved arrangement. Mol Gen Genet 203: 117–128

Hightower KE and McCarty RE (1996) Proteolytic cleavage within a regulatory region of the γ subunit of chloroplast coupling factor 1. Biochemistry 35: 4846–4851

Hirono-Hara Y, Noji H, Nishiura M, Muneyuki E, Hara KY, Yasuda R, Kinosita Jr K and Yoshida M (2001) Pause and rotation of F_1-ATPase during catalysis. Proc Natl Acad Sci USA 98: 13649–13654

Hisabori T, Kondoh A and Yoshida M (1999) The γ subunit in chloroplast F_1-ATPase can rotate in a unidirectional and counter-clockwise manner. FEBS Lett 463: 35–38

Hisabori T, Konno H, Ichimura H, Strotmann H and Bald D (2002) Molecular devices of chloroplast F_1-ATP synthase for the regulation. Biochim Biophys Acta 1555: 140–146

Hornung T, Ishmukhametov R, Spetzler D, Martin J and Frasch WD (2008a) Determination of torque generation from the power stroke of *Escherichia coli* F_1-ATPase. Biochim Biophys Acta 1777: 579–582

Hornung T, Volkov OA, Zaida TMA, Delannoy S, Wise JG and Vogel PD (2008b) Structure of the cytosolic part of the subunit *b*-dimer of *Escherichia coli* F_oF_1-ATP synthase. Biophys J 94: 5053–5064

Hunt AJ, Gittes F and Howard J (1994) The force exerted by a single kinesin molecule against a viscous load. Biophys J 67: 766–781

Ishmukhametov RR, Hornung T, Spetzler D and Frasch WD (2010) Direct observation of stepped proteolipid ring rotation in *E. coli* F_oF_1-ATP synthase. EMBO J 29: 3911–3923

Ishmukhametov RR, Pond JB, Al-Huqail A, Galkin MA and Vik SB (2008) ATP synthesis without R210 of subunit a in the *Escherichia coli* ATP synthase. Biochim Biophys Acta 1777: 32–38

Jault JM and Allison WS (1993) Slow binding of ATP to noncatalytic nucleotide binding sites which accelerates catalysis is responsible for apparent negative cooperativity exhibited by the bovine mitochondrial F_1-ATPase. J Biol Chem 268: 1558–1566

Jault JM, Dou C, Grodsky NB, Matsui T, Yoshida M and Allison WS (1996) The $\alpha_3\beta_3\gamma$ subcomplex of the F_1-ATPase from the thermophilic *Bacillus* PS3 with the βT165S substitution does not entrap inhibitory MgADP in a catalytic site during turnover. J Biol Chem 271: 28818–28824

Jiang W, Hermolin J and Fillingame RH (2001) The preferred stoichiometry of *c* subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10. Proc Natl Acad Sci USA 98: 4966–4971

Johansson K, Ramaswamy S, Saarinen M, Lemaire-Chamley M, Issakidis-Bourguet E, Miginiac-Maslow M and Eklund H (1999) Structural basis for light activation of a chloroplast enzyme: The structure of sorghum NADP-malate dehydrogenase in its oxidized form. Biochemistry 38: 4319–4326

Junge W, Lill H and Engelbrecht S (1997) ATP synthase: an electrochemical transducer with rotatory mechanics. Trends Biochem Sci 22: 420–423

Junge W, Pänke O, Cherepanov DA, Gumbiowski K, Müller M and Engelbrecht S (2001) Inter-subunit rotation and elastic power transmission in F_oF_1-ATPase. FEBS Lett 504: 152–160

Kabaleeswaran V, Puri N, Walker JE, Leslie AGW and Mueller DM (2006) Novel features of the rotary catalytic mechanism revealed in the structure of yeast F_1 ATPase. EMBO J 25: 5433–5442

Kato-Yamada Y, Yoshida M and Hisabori T (2000) Movement of the helical domain of the ε subunit is required for the activation of thermophilic F_1-ATPase. J Biol Chem 275: 35746–35750

Kinosita Jr K, Yasuda R, Noji H and Adachi K (2000) A rotary molecular motor that can work at near 100% efficiency. Philos Trans R Soc Lond B Biol Sci 355: 473–489

Kinosita Jr K, Adachi K and Itoh H (2004) Rotation of F1-ATPase: How an ATP-driven molecular machine may work. Annu Rev Biophys Biomol Struct 33: 245–268

Klionsky DJ, Brusilow WA and Simoni RD (1984) In vivo evidence for the role of the ε subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*. J Bacteriol 160: 1055–1060

Koga N and Takada S (2006) Folding-based molecular simulations reveal mechanisms of the rotary motor F_1-ATPase. Proc Natl Acad Sci USA 103: 5367–5372

Komatsu-Takaki M (1992) Energy-dependent changes in conformation and catalytic activity of the chloroplast ATP synthase. J Biol Chem 267: 2360–2363

Konno H, Yodogawa M, Stumpp MT, Kroth P, Strotmann H, Motohashi K, Amano T and Hisabori T (2000) Inverse regulation of F_1-ATPase activity by a mutation at the regulatory region on the γ subunit of chloroplast ATP synthase. Biochem J 352: 783–788

Konno H, Suzuki T, Bald D, Yoshida M and Hisabori T (2004) Significance of the ε subunit in the thiol modulation of chloroplast ATP synthase. Biochem Biophys Res Commun 318: 17–24

Konno H, Murakami-Fuse T, Fujii F, Koyama F, Ueoka-Nakanishi H, Pack C-G, Kinjo M and Hisabori T (2006) The regulator of the F_1 motor: inhibition of rotation of cyanobacterial F_1-ATPase by the ε subunit. EMBO J 25: 4596–4604

Lebowitz MS and Pedersen PL (1996) Protein inhibitor of mitochondrial ATP synthase: Relationship of inhibitor structure to pH-dependent regulation. Arch Biochem Biophys 330: 342–354

Lee LK, Stewart AG, Donohoe M, Bernal RA and Stock D (2010) The structure of the peripheral stalk of *Thermus thermophilus* H^+-ATPase/Synthase. Nat Struct Mol Biol 17: 373–378

Liu MS, Todd BD and Sadus RJ (2006) Dynamic and coordinating domain motions in the active subunits of the F_1-ATPase molecular motor. Biochim Biophys Acta 1764: 1553–1560

Long JC, DeLeon-Rangel J and Vik SB (2002) Characterization of the first cytoplasmic loop of subunit *a* of the *Escherichia coli* ATP synthase by surface labeling, cross-linking, and mutagenesis. J Biol Chem 277: 27288–27293

Ma J, Flynn TC, Cui Q, Leslie AGW, Walker JE and Karplus M (2002) A dynamic analysis of the rotation mechanism for conformational change in F_1-ATPase. Structure 10: 921–931

Matsui T, Muneyuki E, Honda M, Allison WS, Dou C and Yoshida M (1997) Catalytic activity of the $\alpha_3\beta_3\gamma$ complex of F_1-ATPase without noncatalytic nucleotide binding site. J Biol Chem 272: 8215–8221

McCarn DF, Whitaker RA, Alam J, Vrba JM and Curtis SE (1988) Genes encoding the alpha, gamma, delta, and four F_o subunits of ATP synthase constitute an operon in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 170: 3448–3458

McCarty RE (2005) ATP synthase of chloroplast thylakoid membranes: a more in depth characterization of its ATPase activity. J Bioenerg Biomembr 37: 289–297

McLachlin DT, Bestard JA and Dunn SD (1998) The *b* and δ subunits of the *Escherichia coli* ATP synthase interact via residues in their C-terminal regions. J Biol Chem 273: 15162–15168

McLachlin DT, Coveny AM, Clark SM and Dunn SD (2000) Site-directed cross-linking of *b* to the *a*, *b*, and *a* subunits of the *Escherichia coli* ATP synthase. J Biol Chem 275: 17571–17577

Meier T, Polzer P, Diederichs K, Welte W and Dimroth P (2005) Structure of the rotor ring of F-Type Na^+-ATPase from *Ilyobacter tartaricus*. Science 308: 659–662

Meier T, Krah A, Bond PJ, Pogoryelov D, Diederichs K and Faraldo-Gomez JD (2009) Complete ion-coordination structure in the rotor ring of Na^+-dependent F-ATP synthases. J Mol Biol 391: 498–507

Menz RI, Walker JE and Leslie AGW (2001) Structure of bovine mitochondrial F_1-ATPase with nucleotide bound to all three catalytic sites: Implications for the mechanism of rotary catalysis. Cell 106: 331–341

Mitome N, Suzuki T, Hayashi S and Yoshida M (2004) Thermophilic ATP synthase has a decamer *c*-ring: Indication of noninteger 10:3 H^+/ATP ratio and permissive elastic coupling. Proc Natl Acad Sci USA 101: 12159–12164

Moore KJ and Fillingame RH (2008) Structural interactions between transmembrane helices 4 and 5 of subunit *a* and the subunit *c* ring of *Escherichia coli* ATP synthase. J Biol Chem 283: 31726–31735

Moore KJ, Angevine CM, Vincent OD, Schwem BE and Fillingame RH (2008) The cytoplasmic loops of subunit *a* of *Escherichia coli* ATP synthase may participate in the proton translocating mechanism. J Biol Chem 283: 13044–13052

Motz C, Hornung T, Kersten M, McLachlin DT, Dunn SD, Wise JG and Vogel PD (2004) The subunit *b* dimer of the F_OF_1-ATP synthase: interaction with F_1-ATPase as deduced by site-specific spin-labeling. J Biol Chem 279: 49074–49081

Nadanaciva S, Weber J and Senior AE (2000) New probes of the F_1-ATPase catalytic transition state reveal that two of the three catalytic sites can assume a transition state conformation simultaneously. Biochemistry 39: 9583–9590

Nakamoto RK, Ketchum CJ and Al-Shawi MK (1999) Rotational coupling in the F_oF_1 ATP synthase. Annu Rev Biophys Biomol Struct 28: 205–234

Nakanishi-Matsui M and Futai M (2006) Stochastic proton pumping ATPases: from single molecules to diverse physiological roles. IUBMB Life 58: 318–322

Nalin CM and McCarty RE (1984) Role of a disulfide bond in the γ subunit in activation of the ATPase of chloroplast coupling factor-I. J Biol Chem 259: 7275–7280

Nath S (2008) The new unified theory of ATP synthesis/hydrolysis and muscle contraction, its manifold fundamental consequences and mechanistic implications and its applications in health and disease. Int J Mol Sci 9: 1784–1840

Nishio K, Iwamoto-Kihara A, Yamamoto A, Wada Y and Futai M (2002) Subunit rotation of ATP synthase embedded in membranes: *a* or β subunit rotation relative to the *c* subunit ring. Proc Natl Acad Sci USA 99: 13448–13452

Noji H, Yasuda R, Yoshida M and Kinosita Jr K (1997) Direct observation of the rotation of F_1-ATPase. Nature 386: 299–302

Noji H, Häsler K, Junge W, Kinosita Jr K, Yoshida M and Engelbrecht S (1999) Rotation of *Escherichia coli* F_1-ATPase. Biochem Biophys Res Commun 260: 597–599

Ogilvie I, Aggeler R and Capaldi RA (1997) Cross-linking of the δ subunit to one of the three α subunits has no effect on functioning, as expected if γ is a part of the stator that links the F_1 and F_o parts of the *Escherichia coli* ATP synthase. J Biol Chem 272: 16652–16656

Omote H, Sambonmatsu N, Saito K, Sambongi Y, Iwamoto-Kihara A, Yanagida T, Wada Y and Futai M (1999) The γ-subunit rotation and torque generation in F_1-ATPase from wild-type or uncoupled mutant *Escherichia coli*. Proc Natl Acad Sci USA 96: 7780–7784

Oster G (2002) Brownian ratchets: Darwin's motors. Nature 417: 25

Oster G and Wang H (1999) ATP synthase: two motors, two fuels. Structure 7: R67–72

Oster G and Wang H (2000) Reverse engineering a protein: the mechanochemistry of ATP synthase. Biochim Biophys Acta 1458: 482–510

Oster G and Wang H (2003). Rotary protein motors. Trends Cell Biol 13: 114–121

Oster G, Wang H and Grabe M (2000) How F_o-ATPase generates rotary torque. Philos Trans R Soc Lond B Biol Sci 355: 523–528

Pänke O, Gumbiowski K, Junge W and Engelbrecht S (2000) F-ATPase: specific observation of the rotating c subunit oligomer of EF_oEF_1. FEBS Lett 472: 34–38

Pänke O, Cherepanov DA, Gumbiowski K, Engelbrecht S and Junge W (2001) Viscoelastic dynamics of actin filaments coupled to rotary F-ATPase: angular torque profile of the enzyme. Biophys J 81: 1220–1233

Patterson AR, Wada T and Vik SB (1999) His^{15} of subunit *a* of the *Escherichia coli* ATP synthase is important for the structure or assembly of the membrane sector F_o. Arch Biochem Biophys 368: 193–197

Pilizota T, Bilyard T, Bai F, Futai M, Hosokawa H and Berry RM (2007) A programmable optical angle clamp for rotary molecular motors. Biophys J 93: 264–275

Pogoryelov D, Yu J, Meier T, Vonck J, Dimroth P and Muller DJ (2005) The c_{15} ring of the *Spirulina platensis* F-ATP synthase: F_1/F_o symmetry mismatch is not obligatory. EMBO Rep 6: 1040–1044

Pogoryelov D, Reichen C, Klyszeiko AL, Brunisholz R, Muller DJ, Dimroth P and Meier T (2007) The oligomeric state of c rings from cyanobacterial F-ATP synthases varies from 13 to 15. J Bacteriol 189: 5895–5902

Pogoryelov D, Nikolaev Y, Schlattner U, Pervushin K, Dimroth P and Meier T (2008) Probing the rotor subunit

interface of the ATP synthase from *Ilyobacter tartaricus*. FEBS J 275: 4850–4862

Pogoloryelov D, Yildiz O, Faraldo-Gomex JD and Meier T (2009) High resolution structure of the rotor ring of a proton-dependent ATP synthase. Nat Struct Mol Biol 16: 1068–1073

Preiss L, Yildiz O, Hicks DB, Krulwich TA and Meier T (2010) A new type of proton coordination in an F_1F_o-ATP synthase rotor. PLoS Biol 8: e1000443

Priya R, Tadwal VS, Roessle MW, Gayen S, Hunke C, Peng WC, Torres J and Grüber G (2008) Low resolution structure of subunit *b* ($b_{22\text{-}156}$) of *Escherichia coli* F_1F_O ATP synthase in solution and the *b*-δ assembly. J Bioenerg Biomembr 40: 245–255

Pu J and Karplus M (2008).How subunit coupling produces the γ-subunit rotary motion in F_1-ATPase. Proc Natl Acad Sci USA 105: 1192–1197

Ren H and Allison WS (2000) On what makes the γ subunit spin during ATP hydrolysis by F_1. Biochim Biophys Acta 1458: 221–233

Revington M, McLachlin DT, Shaw GS and Dunn SD (1999) The dimerization domain of the *b* subunit of the *Escherichia coli* F_1F_o-ATPase. J Biol Chem 274: 31094–31101

Richter ML, Patrie WJ and McCarty RE (1984) Preparation of the ε subunit and ε subunit-deficient chloroplast coupling factor I in reconstitutively active forms. J Biol Chem 259: 7371–7373

Richter ML, Samra HS, He F, Giessel AJ and Kuczera KK (2005) Coupling proton movement to ATP synthesis in the chloroplast ATP synthase. J Bioenerg Biomembr 37: 467–473

Rodgers AJW and Capaldi RA (1998) The second stalk composed of the b- and δ-subunits connects F_o to F_1 via an α-subunit in the *Escherichia coli* ATP synthase. J Biol Chem 273: 29406–29410

Rodgers AJ, Wilkens S, Aggeler R, Morris MB, Howitt SM and Capaldi RA (1997) The subunit δ-subunit b domain of the *Escherichia coli* F_1F_o ATPase. The b subunits interact with F_1 as a dimer and through the δ subunit. J Biol Chem 272: 31058–31064

Sabbert D, Engelbrecht S and Junge W (1996) Intersubunit rotation in active F-ATPase. Nature 381: 623–625

Sakaki N, Shimo-Kon R, Adachi K, Itoh H, Furuike S., Muneyuki E, Yoshida M, Kinosita Jr K (2005) One rotary mechanism for F_1-ATPase over ATP concentrations from millimolar down to nanomolar. Biophy J 88: 2047–2056

Sambongi Y, Iko Y, Tanabe M, Omote H, Iwamoto-Kihara A, Ueda I, Yanagida T, Wada Y and Futai M (1999) Mechanical rotation of the c subunit oligomer in ATP synthase (F_oF_1): Direct observation. Science 286: 1722–1724

Samra HS, Gao F, He F, Hoang E, Chen Z, Gegenheimer PA, Berrie CL and Richter M. (2006) Structural analysis of the regulatory dithiol-containing domain of the chloroplast ATP synthase γ subunit. J Biol Chem 281: 31041–31049

Schwem BE and Fillingame RH (2006) Cross-linking between helices within subunit *a* of *Escherichia coli* ATP synthase defines the transmembrane packing of a four-helix bundle. J Biol Chem 281: 37861–37867

Seelert H, Dencher NA and Müller DJ (2003) Fourteen protomers compose the oligomer III of the proton-rotor in spinach chloroplast ATP synthase. J Mol Biol 333: 337–344

Senior AE (2007) ATP synthase: Motoring to the finish line. Cell 130: 220–221

Senior AE, Nadanaciva S and Weber J (2002) The molecular mechanism of ATP synthesis by F_1F_o-ATP synthase. Biochim Biophys Acta 1553: 188–211

Shimabukuro K, Yasuda R, Muneyuki E, Hara KY, Kinosita Jr K and Yoshida M (2003) Catalysis and rotation of F_1 motor: cleavage of ATP at the catalytic site occurs in 1 ms before 40˚ substep rotation. Proc Natl Acad Sci USA 100: 14731–14736

Sielaff H, Rennekamp H, Engelbrecht S and Junge W (2008a). Functional halt positions of rotary F_oF_1-ATPase correlated with crystal structures. Biophys J 95: 4979–4987

Sielaff H, Rennekamp H, Wachter A, Xie H, Hilbers F, Feldbauer K, Dunn SD, Engelbrecht S and Junge W (2008b) Domain compliance and elastic power transmission in rotary F_oF_1-ATPase. Proc Natl Acad Sci USA 105: 17760–17765

Singh S, Turina P, Bustamante CJ, Keller DJ and Capaldi R (1996) Topographical structure of membrane-bound *Escherichia coli* F_1F_o ATP synthase in aqueous buffer. FEBS Lett 397: 30–34

Soong RK, Bachand GD, Neves HP, Olkhovets AG, Craighead HG and Montemagno CD (2000) Powering an inorganic nanodevice with a biomolecular motor. Science 290: 1555–1558

Sorgen PL, Bubb MR, McCormick KA, Edison AS and Cain BD (1998a). Formation of the *b* subunit dimer is necessary for interaction with F_1-ATPase. Biochemistry 37: 923–932

Sorgen PL, Caviston TL, Perry RC and Cain BD (1998b) Deletions in the second stalk of F_1F_o-ATP synthase in *Escherichia coli*. J Biol Chem 273: 27873–27878

Sorgen PL, Bubb MR and Cain BD (1999) Lengthening the second stalk of F_1F_o ATP synthase in *Escherichia coli*. J Biol Chem 274: 36261–36266

Soteropoulos P, Süss KH and McCarty RE (1992) Modifications of the γ subunit of chloroplast coupling factor 1 alter interactions with the inhibitory ε subunit. J Biol Chem 267: 10348–10354

Soteropoulos P, Ong AM and McCarty RE (1994) Alkylation of cysteine 89 of the γ subunit of chloroplast coupling factor 1 with *N*-ethylmaleimide alters nucleotide interactions. J Biol Chem 269: 19810–19816

Spetzler D, York J, Daniel D, Fromme R, Lowry D and Frasch W (2006) Microsecond time scale rotation measurements of single F_1-ATPase molecules. Biochemistry 45: 3117–3124

Spetzler D, Ishmukhametov R, Hornung T, Day LJ, Martin J and Frasch WD (2009) Single molecule measurements of F_1-ATPase reveal an interdependence between the power stroke and the dwell duration. Biochemistry 48: 7979–7985

Spudich JA (2006) Molecular motors take tension in stride. Cell 126: 242–244

Steed PR and Fillingame RH (2008) Subunit *a* facilitates aqueous access to a membrane-embedded region of subunit *c* in *Escherichia coli* F_1F_o ATP synthase. J Biol Chem 283: 12365–12372

Steed PR and Fillingame RH (2009) Aqueous accessibility to the transmembrane regions of subunit *c* of the *Escherichia coli* F_1F_o ATP synthase. J Biol Chem 284: 23243–23250

Steigmiller S, Borsch M, Graber P and Huber M (2005) Distances between the b-subunits in the tether domain of F_oF_1-ATP synthase from *E. coli*. Biochim Biophys Acta 1708: 143–153

Stock D, Leslie AGW and Walker JE (1999) Molecular architecture of the rotary motor in ATP synthase. Science 286: 1700–1705

Stock D, Gibbons C, Arechaga I, Leslie AGW and Walker JE (2000) The rotary mechanism of ATP synthase. Curr Opin Struct Biol 10: 672–679

Sun SX, Wang H and Oster G (2004) Asymmetry in the F_1-ATPase and its implications for the rotational cycle. Biophys J 86: 1373–1384

Suzuki T, Murakami T, Iino R, Suzuki J, Ono S, Shirakihara Y and Yoshida M (2003) F_oF_1-ATPase/synthase is geared to the synthesis mode by conformational rearrangement of ε subunit in response to proton motive force and ADP/ATP balance. J Biol Chem 278: 46840–46846

Takeyama M, Noumi T, Maeda M and Futai M (1988) F_o portion of *Escherichia coli* H^+-ATPase. Carboxyl-terminal region of the *b* subunit is essential for assembly of functional F_o. J Biol Chem 263: 16106–16112

Takeyama M, Ihara K, Moriyama Y, Noumi T, Ida K, Tomioka N, Itai A, Maeda M and Futai M (1990) The glycine-rich sequence of the β subunit of *Escherichia coli* H^+-ATPase is important for activity. J Biol Chem 265: 21279–21284

Takeyasu K, Omote H, Nettikadan S, Tokumasu F, Iwamoto-Kihara A and Futai M (1996) Molecular imaging of *Escherichia coli* F_oF_1-ATPase in reconstituted membranes using atomic force microscopy. FEBS Lett 392: 110–113

Tanabe M, Nishio K, Iko Y, Sambongi Y, Iwamoto-Kihara A, Wada Y and Futai M (2001) Rotation of a complex of the γ subunit and *c* ring of *Escherichia coli* ATP synthase. The rotor and stator are interchangeable. J Biol Chem 276: 15269–15274

Toei M, Gerle C, Nakano M, Gyobu N, Tamakoshi M, Sone N, Yoshida M, Fujiyoshi Y, Mitsuoka K and Yokoyama K (2007) Dodecamer rotor ring defines H^+/ATP ratio for ATP synthesis of prokaryotic V-ATPase from *Thermus thermophilus*. Proc Natl Acad Sci USA 104: 20256–20261

Tsunoda SP, Aggeler R, Noji H, Kinosita Jr K, Yoshida M and Capaldi RA (2000) Observations of rotation within the F_oF_1-ATP synthase: deciding between rotation of the F_oc subunit ring and artifact. FEBS Lett 470: 244–248

Tsunoda SP, Aggeler R, Yoshida M and Capaldi RA (2001a) Rotation of the c subunit oligomer in fully functional F_1F_o ATP synthase. Proc Natl Acad Sci USA 98: 898–902

Tsunoda SP, Rodgers AJW, Aggeler R, Wilce MCJ, Yoshida M and Capaldi RA (2001b) Large conformational changes of the ε subunit in the bacterial F_1F_o ATP synthase provide a ratchet action to regulate this rotary motor enzyme. Proc Natl Acad Sci USA 98: 6560–6564

Ueno H, Suzuki T, Kinosita Jr K and Yoshida M (2005) ATP-driven stepwise rotation of F_OF_1-ATP synthase. Proc Natl Acad Sci USA 102: 1333–1338

Ueoka-Nakanishi H, Nakanishi Y, Konno H, Motohashi K, Bald D and Hisabori T (2004) Inverse regulation of rotation of F_1-ATPase by the mutation at the regulatory region on the γ subunit of chloroplast ATP synthase. J Biol Chem 279: 16272–16277

Vik SB, Cain BD, Chun KT and Simoni RD (1988) Mutagenesis of the α subunit of the F_1F_o-ATPase from *Escherichia coli*. Mutations at Glu-196, Pro-190, and Ser-199. J Biol Chem 263: 6599–605

Vik SB, Long JC, Wada T and Zhang D (2000) A model for the structure of subunit *a* of the *Escherichia coli* ATP synthase and its role in proton translocation. Biochim Biophys Acta 1458: 457–466

Volkov OA, Zaida TM, Voeller P, Lill H, Wise JG and Vogel PD (2009) De-novo modeling and ESR validation of a cyanobacterial F_oF_1-ATP synthase subunit *bb'* left-handed coiled coil. Biochim Biophys Acta 1787: 183–190

Vollmar M, Schlieper D, Winn M, Buchner C and Groth G (2009) Structure of the c_{14} rotor ring of the proton translocating chloroplast ATP synthase. J Biol Chem 284: 18228–18235

Wachter A, Bi Y, Dunn SD, Cain BD, Sielaff H, Wintermann F, Engelbrecht S and Junge W (2011) Two rotary motors in F-ATP synthase are elastically coupled by a flexible rotor and a stiff stator stalk. Proc Natl Acad Sci USA 108: 3924–3928

Walker JE and Dickson VK (2006) The peripheral stalk of the mitochondrial ATP synthase. Biochim Biophys Acta 1757: 286–296

Walker JE, Cozens AL, Dyer MR, Fearnly IM, Powell SJ and Runswick MJ (1987) Structure and genes of ATP synthase. Biochem Soc Trans 15: 104–106

Wang H and Oster G (1998) Energy transduction in the F_1 motor of ATP synthase. Nature 396: 279–282

Wang H and Oster G (2002) The Stokes efficiency for molecular motors and its application. Europhys Lett 57: 134–140

Watt IN, Montgomery MG, Runswick MJ, Leslie AGW and Walker JE (2010) Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. Proc Natl Acad Sci USA 107: 16823–16827

Watts SD and Capaldi RA (1997) Interactions between the F_1 and F_o parts in the *Escherichia coli* ATP synthase. Associations involving the loop region of c subunits. J Biol Chem 272: 15065–15068

Watts SD, Tang C and Capaldi RA (1996) The stalk region of the *Escherichia coli* ATP synthase. Tyrosine 205 of the γ subunit is in the interface between the F_1 and F_o parts and can interact with both the ε and c oligomer. J Biol Chem 271: 28341–28347

Weber J and Senior AE (1996) F_1F_o-ATP synthase: development of direct optical probes of the catalytic mechanism. Biochim Biophys Acta 1275: 101–104

Weber J and Senior AE (1997) Catalytic mechanism of F_1-ATPase. Biochim Biophys Acta 1319: 19–58

Weber J and Senior AE (2000a) ATP synthase: what we know about ATP hydrolysis and what we do not know about ATP synthesis. Biochim Biophys Acta 1458: 300–309

Weber J and Senior AE (2000b) Features of F_1-ATPase catalytic and noncatalytic sites revealed by fluorescence lifetimes and acrylamide quenching of specifically inserted tryptophan residues. Biochemistry 39: 5287–5294

Weber J and Senior AE (2004) Fluorescent probes applied to catalytic cooperativity in ATP synthase. Methods Enzymol 380: 132–152

Weber J, Wilke-Mounts S, Lee RS-F, Grell E and Senior AE (1993) Specific placement of tryptophan in the catalytic sites of *Escherichia coli* F_1-ATPase provides a direct probe of nucleotide binding: Maximal ATP hydrolysis occurs with three sites occupied. J Biol Chem 268: 20126–20133

Weber J, Bowman C and Senior AE (1996) Specific tryptophan substitution in catalytic sites of *Escherichia coli* F_1-ATPase allows differentiation between bound substrate ATP and product ADP in steady-state catalysis. J Biol Chem 271: 18711–18718

Weber J, Muharemagic A, Wilke-Mounts S and Senior AE (2004) Analysis of sequence determinants of F_1F_o-ATP synthase in the N-terminal region of α subunit for binding of δ subunit. J Biol Chem 279: 25673–25679

Wilkens S (2005) Rotary molecular motors. Adv Protein Chem 71: 345–382

Wilkens S and Capaldi RA (1998a) ATP synthase's second stalk comes into focus. Nature 393: 29

Wilkens S and Capaldi RA (1998b). Electron microscopic evidence of two stalks linking the F_1 and F_o parts of the *Escherichia coli* ATP synthase. Biochim Biophys Acta 1365: 93–97

Xing J, Wang H, von Ballmoos C, Dimroth P and Oster G (2004) Torque generation by the F_o motor of the sodium ATPase. Biophys J 87: 2148–2163

Yasuda R, Noji H, Kinosita Jr K and Yoshida M (1998) F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. Cell 93: 1117–1124

Yasuda R, Noji H, Yoshida M, Kinosita Jr K and Itoh H (2001) Resolution of distinct rotational substeps by submillisecond kinetic analysis of F_1-ATPase. Nature 410: 898–904

Yoshida M, Muneyuki E and Hisabori T (2001) ATP synthase–a marvellous rotary engine of the cell. Nat Rev Mol Cell Biol 2: 669–677

Zaida TM, Hornung T, Volkov OA, Hoffman AD, Pandey SJ, Wise JG and Vogel PD (2008) Conformational changes in the *Escherichia coli* ATP synthase *b*-dimer upon binding to F_1-ATPase. J Bioenerg and Biomembr 40: 551–559

Zhang D and Vik SB (2003a) Helix packing in subunit *a* of the *Escherichia coli* ATP synthase as determined by chemical labeling and proteolysis of the cysteine-substituted protein. Biochemistry 42: 331–337

Zhang D and Vik SB (2003b) Close proximity of a cytoplasmic loop of subunit *a* with *c* subunits of the ATP synthase from *Escherichia coli*. J Biol Chem 278: 12319–12324

Zhang Y and Fillingame RH (1995) Subunits coupling H^+ transport and ATP synthesis in the *Escherichia coli* ATP synthase. Cys-Cys cross-linking of F_1 subunit ε to the polar loop of F_o subunit *c*. J Biol Chem 270: 24609–24614.

Chapter 23

Electron Transport in Leaves: A Physiological Perspective

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Summary

Light absorbed by photosystems I and II is used to drive linear electron transport, and associated proton transport, in the thylakoid membranes of leaves. In healthy leaves operating under non-stressful conditions and in which photorespiration is inhibited, photosynthetic electron transport is used primarily to reduce $NADP^+$ to NADPH, which is then used to drive the assimilation of CO_2 into carbohydrates with ca. 88% of electrons being consumed in this process. However, such a high quantum efficiency of CO_2 assimilation is frequently not observed in leaves. We examine the intrinsic physiological, metabolic and environmental factors that can modify photosynthetic electron transport in leaves. Electron transport is also required for the reduction and activation of key enzymes involved in photosynthetic metabolism and driving other metabolic processes, such as nitrogen and sulfur metabolism. Oxygen can act as an electron

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acceptor, being reduced by electrons from photosystem I via a Mehler reaction or by electrons from photosystem II via the plastid terminal oxidase. Although such photoreductions of oxygen do not appear to have a significant role in healthy, non-stressed leaves, there is evidence to support the contention that these processes can be important for photoprotection of photosystem II in leaves under light stress. Cyclic electron transport can occur around photosystem I; however, this process would also appear to only be of physiological importance when the ability of the leaf to assimilate CO_2 is severely restricted. It is concluded that leaves exhibit a high degree of plasticity in their ability to modify the pathways of photosynthetic electron transport in order to deal with fluctuations in metabolic demands and environmental stresses.

I. Introduction

Thylakoid membranes use light energy to produce ATP and NADPH. Light is captured by antennae comprising of light-harvesting complexes which are responsible for the absorption of light that drives photosynthesis in higher plants. Photosystem I (PS I) and II (PS II) are the sites of the primary photochemical reactions of photosynthesis, which involve a separation of a positively charged molecule from a negatively charged molecule. In the primary photochemical reaction an electron is transferred from an excited chlorophyll molecule, P700 in PS I and P680 in PS II, to an electron-accepting pigment creating positively and negatively charged molecules that are adjacent to each other. The electron cannot flow back to the positively charged molecule and this separation of charge "captures" the energy and drives all subsequent electron transfer reactions in the photosynthetic membrane (Fig. 23.1). In PS I, the energy captured in the primary charge separation drives the oxidation of plastocyanin, a copper-containing protein located in the thylakoid lumen, and the reduction of ferredoxin, an iron-containing protein located in the stroma (Fig. 23.1). Electrons can also be transferred from the iron-sulfur centers of PS I directly to O_2 resulting in the production of superoxide anion ($O_2^{\cdot-}$; Asada, 1999). In PS II excitation energy in P680 is used to remove electrons from water and to transfer electrons to plastoquinone. The photooxidation of water by PS II generates O_2 and releases protons into the thylakoid lumen. The transfer of electrons from water to ferredoxin, mediated by PS II and PS I, is frequently referred to as linear electron transfer (LET).

Ferredoxin plays a key role in distributing electrons transferred from PS I to a range of electron acceptors (Knaff, 1996). In leaves under optimal conditions the majority of electron flux through ferredoxin is used to reduce $NADP^+$ via a ferredoxin NADP oxidoreductase (FNR), thus providing the reductant required for CO_2 assimilation. However, reduced ferredoxin can also transfer electrons to a number of soluble enzymes involved in nitrogen metabolism, sulfur metabolism and the regulation of carbon metabolism (Knaff, 1996). Ferredoxin, like the iron-sulfur centers of PS I, can act as an electron donor to O_2, producing superoxide ($O_2^{\cdot-}$), in what is often termed the Mehler reaction (Asada, 1996). These redox reactions involving electron transfer from ferredoxin can play important roles in leaf physiology, not only being involved with assimilation of carbon, nitrogen and sulfur, but also in generation of signals which modulate the expression of many genes associated with a range cellular processes (Buchanan and Balmer, 2005).

The light-driven electron transfer reactions associated with PS I and PS II are interconnected through the activity of the cytochrome b_6f complex, which reduces plastocyanin. Plastoquinone serves as a mobile proton carrier within the

Abbreviations: C_a – Molar ratio of CO_2 in ambient air; C_i – Molar ratio of CO_2 concentration in intercellular leaf space; CET1 – Cyclic electron transfer around PS I; FBPase – Fructose 1,6-bisphosphatase; FNR – Ferredoxin NADP oxidoreductase; LET – Linear electron transfer; LHCII – Light-harvesting complexes associated with PS II; MDA – Monodehydroascorbate; MDH – Malate dehydrogenase; Ndh – NAD(P)H dehydrogenase complex; PPFD – Photosynthetically-active photon flux density; PS I – Photosystem I; PS II – Photosystem II; PTOX – Plastid terminal oxidase; Rubisco – Ribulose 1,5-bisphosphate carboxylase-oxygenase; ϕ_{CO_2max} – Maximum quantum yield of CO_2 assimilation; ϕ_{O_2max} – Maximum quantum yield of O_2 evolution

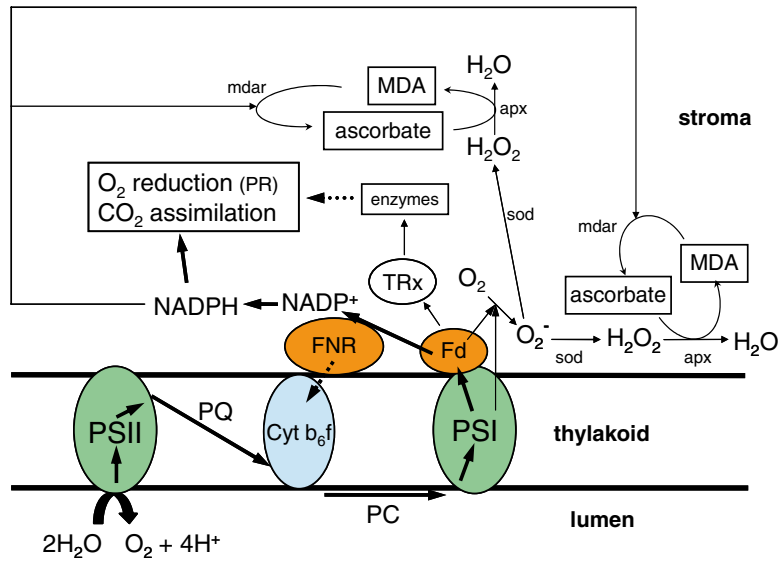

Fig. 23.1. Schematic drawing to show the major pathways of non-cyclic photosynthetic electron flux and the consumption of reductants in the stroma. Linear electron transport mediated by PS II and PS I occurs from water to ferredoxin (*Fd*). Plastoquinone (*PQ*) is reduced at PS II and plastoquinol carries two electrons and two protons to the cytochrome b_6f complex where it is oxidized to plastoquinone and the electrons transferred to plastocyanin (*PC*) and transported to PS I. Electrons are transferred through PS I to Fd although oxygen (O_2) can be directly reduced by electrons donated from the iron-sulfur centers of PS I to form the superoxide radical (O_2^-). Reduced Fd can also transfer electrons to O_2, producing O_2^-, or to thioredoxin (*TRx*), although under most physiological conditions the major electron flux from Fd is to $NADP^+$, mediated by a ferredoxin-$NADP^+$ reductase (*FNR*), with NADPH being formed. A large proportion of the NADPH formed is consumed in photosynthetic carbon assimilation and, in C_3 leaves, by photorespiration (*PR*); however, in situations where photoproduction of O_2^- occurs, NADPH will also be consumed in the reduction of monodehydroascorbate (*MDA*) by monodehydroascorbate reductase (mdar) to ascorbate. This reaction is essential to sustain an electron flux from PS I to O_2, as O_2^- is potentially damaging and must be rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (sod). H_2O_2 is then detoxified by ascorbate peroxidases (apx) which oxidize ascorbate to form MDA and water. Apx and sod enzymes are found both associated with the thylakoid membrane and in the stroma (Asada, 1999). It is essential to reduce the MDA back to ascorbate. TRx can transfer electrons to enzymes involved in carbon metabolism. Reduction of the enzymes by TRx is required for their activation (*dotted arrow*). Fd, via FNR, can transfer electrons back to the cytochrome b_6f complex (*dashed arrow*) and facilitate a cycling of electrons around PS I.

membrane, transporting electrons, and protons, from PS II to the cytochrome b_6f complex, while plastocyanin serves as a mobile electron carrier, transporting electrons from the cytochrome b_6f complex to PS I. In addition to linking the activity of PS II and PS I, the cytochrome b_6f complex plays a central role in energy transduction and storage by converting energy available in reduced plastoquinone (plastoquinol) into in a transmembrane pH difference as well as an electrical potential difference (Chapter 21). This is accomplished as the cytochrome b_6f complex oxidizes plastoquinol at a site near the inside of the membrane, resulting in the release of protons into the thylakoid lumen. In addition to oxidizing plastoquinone, the cytochrome b_6f complex also reduces plastoquinone at a second site that is near the outside of the membrane acquiring the protons from the stroma. The net result of these reactions, known as the cytochrome b_6f Q-cycle, is the transfer of protons from the stroma to the lumen, creating a transmembrane pH difference and, depending upon the extent of counter ion movement, an electrical potential difference. The energy stored in the pH and electrical potential differences is used for the energy-requiring reaction of converting ADP to ATP by the addition of a phosphate group by the ATP synthase in the thylakoid membrane. Operation of the Q cycle results in three protons being transferred from the stroma to the lumen for each electron transferred through the cytochrome b_6f complex from plastoquinol to plastocyanin; disengagement of the Q cycle would lower the H^+/e^- ratio to 2 (Cruz et al., 2005).

Besides LET mediated by PS II and PS I, electrons can cycle around PS I (Bendall and

Manasse, 1995). Electrons can be transferred from ferredoxin via FNR or ferredoxin quinone reductase to plastoquinone at the cytochrome b_6f complex (Johnson, 2005). Electrons are then transferred from plastoquinol through the cytochrome b_6f complex via PS I back to ferredoxin, as occurs in LET. This cyclic electron transport around PS I (CET1) will create transmembrane pH and electrical potential differences that can be used to drive ATP synthesis (Cruz et al., 2005). A pathway for the cycling of electrons around PS II (CET2) has also been proposed (Heber et al., 1979; Falkowski et al., 1986). PS II can both photoreduce and photooxidise cytochrome b_{559} (Stewart and Brudvig, 1998) and consequently cytochrome b_{559} is thought to play a key role in CET2. The physiological significance of CET2 is not known. It has been frequently speculated that operation of CET2 is a mechanism for protecting PS II from photoinactivation and damage (see Whithmarsh and Pakrasi, 1996). However, there is no convincing evidence that cytochrome b_{559} can turnover in vivo at sufficiently high rates to offer protection to PS II from photodamage at moderate and high light intensities.

It is not our intention to review in detail the structure and operation of the photosynthetic electron transport systems, as very many reviews have done this in the past. Our aim is to examine the operation of some of the possible pathways of electron transport to meet the metabolic requirements of leaves and how physiological and environmental factors might constrain these processes.

II. Linear Electron Transport for CO_2 Assimilation and Photorespiration

A. Quanta and ATP Requirements

For the efficient operation of LET a similar turnover of PS I and PS II reaction centers must occur. For each molecule of O_2 evolved as a result of LET, two molecules of water are oxidized. This requires the absorption and utilization of four photons by both PS I and PS II, which facilitate the transfer of four electrons from water via PS II and PS I to a terminal electron acceptor. Consequently, the maximum theoretical value for the quantum yield of O_2 evolution (ϕ_{O_2max}), assuming a 100% efficiency of the use of absorbed photons for reaction center turnover in both photosystems, is 0.125 O_2 photon^{-1}. However, quantum yields for charge separation and stabilization in PS I and PS II are considered to be 0.95 and 0.85–0.95, respectively (see Genty and Harbinson, 1996), and consequently the highest ϕ_{O_2max} that could be expected would be 0.119 O_2 photon^{-1}. If the lower value of 0.85 is taken for the PS II quantum yield, a value of 0.106 for ϕ_{O_2max} is obtained. In practice, observed values of ϕ_{O_2max} are very close to this value; Björkman and Demmig (1987) measured ϕ_{O_2max} for 47 C_3 species of differing taxonomic origins and from different habitats and reported a mean value of 0.106 ± 0.001 O_2 photon^{-1}. Measurements of the maximum quantum yield of CO_2 assimilation (ϕ_{CO_2max}) in a range of C_3 plants under non-photorespiratory conditions gave a mean value of 0.093 CO_2 photon^{-1} (Long et al., 1993). Comparison of these values of ϕ_{O_2max} and ϕ_{CO_2max} indicate that in C_3 leaves ca. 88% of electrons transferred from PS II as a result of LET are utilized for CO_2 assimilation. Consequently, under non-photorespiratory conditions when C_3 leaves are operating under light-limiting conditions, ca. 12% of electrons resulting from water oxidation by PS II are used in processes other than CO_2 assimilation, e.g., nitrogen metabolism, sulfur metabolism, and O_2 reduction.

In many situations the rate of photosynthetic electron transport can be transiently considerably greater than that required to sustain CO_2 assimilation. For example, when the photosynthetically-active photon flux density (PPFD) to which leaves of *Alocasia macrorhiza* were exposed was increased from 10.5 to 600 μmol m^{-2} s^{-1}, the rate of O_2 evolution was increased by up to ten times the rate of CO_2 assimilation (Pearcy et al., 1996). Using mass spectrometry Radmer and Kok (1976) demonstrated that during the first second of illumination of *Scenedesmus obliquus*, in the absence of CO_2 assimilation, the rate of photoreduction of O_2 was similar to the rate of CO_2 assimilation observed when steady-state CO_2 assimilation was achieved. Clearly, in both of these cases sinks for electrons, other than CO_2 assimilation, are operating to sustain high rates of LET.

ATP production from LET associated with given rates of CO_2 assimilation and ribulose 1,5-bisphosphate oxygenation can be estimated for C_3 leaves (von Caemmerer, 2000). Such

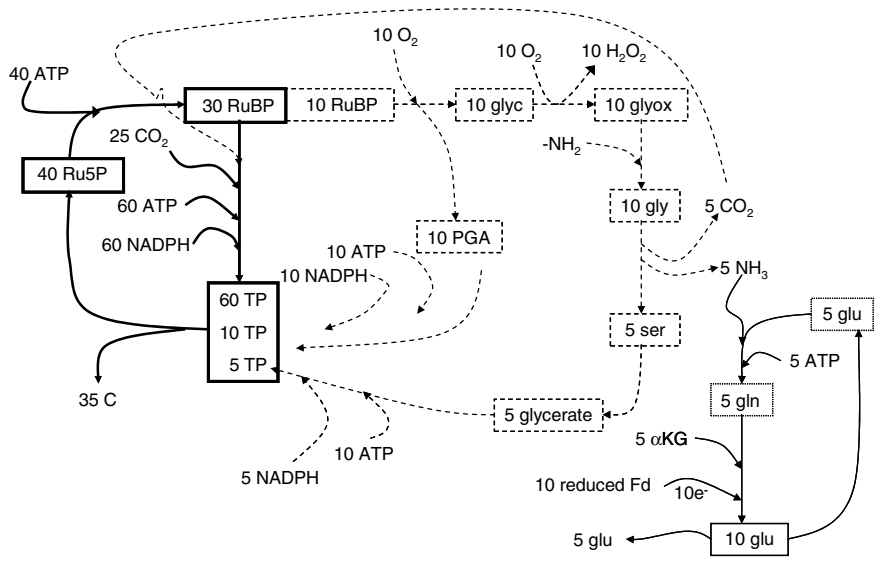

Fig. 23.2. Sites at which ATP and reductants are required to sustain the operation of the photosynthetic carbon reduction (carbon assimilation) and carbon oxidation (photorespiration) cycles and the associated assimilation of ammonia. The calculations are made for a non-stressed C_3 leaf in an atmospheric CO_2 molar ratio of 370 μmol mol^{-1}, which would give a CO_2 molar ratio of ca. 260 μmol mol^{-1} in the leaf intercellular spaces. Key: *Fd* ferredoxin, *gln* glutamine, *glu* glutamate, *gly* glycine, *glyc* glycollate, *glyox* glyoxalate, *PGA* 3-phosphoglycerate, *RuBP* ribulose 1,5-bisphosphate, *Ru5P* ribulose 5-phosphate, *ser* serine, *TP* triose phosphate, *αKG* 2-α-ketoglutarate.

calculations allow evaluation of whether the ATP produced is sufficient to sustain all of the metabolic reactions associated with CO_2 assimilation and photorespiration, which are shown in Fig. 23.2. We assume that (i) the Q cycle is obligatory giving a H^+/e^- of three, (ii) the specificity of Rubisco for CO_2 relative to O_2 (S) is 90, (iii) all of the CO_2 produced in the mitochondria from decarboxylation of glycine is re-assimilated, and (iv) half of the carbon not used in resynthesis of ribulose 1,5-bisphosphate is directed for starch synthesis. For a given rate of carboxylation (v_c), the rate of oxygenation (v_o) can be calculated from:

$$v_c / v_o = S(C / O) \qquad (23.1)$$

where C and O are the CO_2 and O_2 concentrations in the stroma, which are considered to be in equilibrium with the gas concentrations in the leaf intercellular space. For a C_3 leaf at 25°C the ATP production was calculated for operation in saturating atmospheric CO_2 and with CO_2 molar fractions within the leaf intercellular spaces (C_i) of 260 and 60 μmol mol^{-1}, which are representative of the situation for a leaf in ambient air (C_a = 370 μmol mol^{-1}, C_i = 260 μmol mol^{-1}) and subjected to drought (C_i = 60 μmol mol^{-1}), respectively (Table 23.1). The calculations were made for the ATP synthase having H^+/ATP coupling ratios of 4 and 4.67; a ratio of 4 has been frequently assumed in the past (Rumberg et al., 1990; Kobayashi and Heber, 1995), but structural data of Seelert et al. (2000) have shown that transport of 14 protons through the ATP synthase is associated with the synthesis of three ATP molecules giving a ratio of 4.67. These calculations indicate that insufficient ATP is generated by LET, associated with the carboxylation and oxygenation reaction of Rubisco, to sustain all the metabolic reactions associated with CO_2 assimilation, photorespiration and related nitrogen metabolism shown in Fig. 23.2 (Table 23.1). Since ATP is also used in many other metabolic processes, the deficits in ATP supply for all cell processes must be greater than those calculated here. As expected, the deficit of ATP supply by LET increases with decreasing C_i and increasing the H^+/ATP coupling ratio. Clearly, leaves must operate a mechanism(s) that generates ATP to meet such deficits. Cyclic electron transport around PS I (i.e., CET1) could result in additional ATP synthesis. However, operation of

Table 23.1. Deficit of ATP produced by linear electron transport associated with carboxylation and oxygenation reactions of Rubisco compared to the ATP that is required to sustain all of the metabolic reactions associated with CO_2 assimilation, photorespiration and associated nitrogen metabolism

	Leaf intracellular CO_2 molar ratio, Ci (μmol mol^{-1})		
H^+/ATP	Saturating	260	60
4.0	3.2	6.3	9.1
4.67	17.5	19.7	22.7

Calculations were made assuming an H^+/ATP coupling ratio of 4 and 4.67, and for saturating ambient CO_2 molar ratio and leaf intercellular CO_2 molar ratio, C_i, of 260 and 60 μmol mol^{-1}

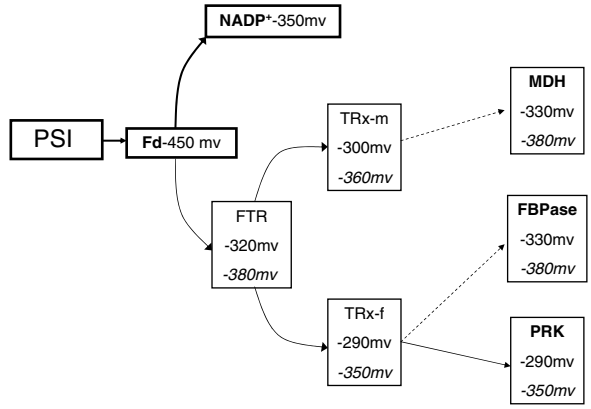

Fig. 23.3. Schematic representation of the electron transfer pathways from ferredoxin (Fd) to the stromal enzymes fructose 1,6-bisphospahatase (FBPase), malate dehydrogenase (MDH) and phosphoribulokinase (PRK). The midpoint redox potentials of the components of these pathways are given for pH 7 (*normal text*) and pH 8 (*italics*). Key: ferredoxin-thioredoxin reductase (*FTR*); thioredoxin-f (*TRx-f*); thioredoxin-m (*TRx-m*). *Dashed arrows* indicate that the disulfur bond on the target protein has a midpoint redox potential lower than that of the redox center in the donor protein, consequently reduction is not thermodynamically favored.

CET1 would require increased photon absorption by PS I which would result in a decrease in the quantum yield of O_2 evolution to below the maximum observed in leaves at low light (see above). Consequently, it is unlikely, at least at low light, that CET1 plays a major role in supplying ATP (see Section V). It is most likely that LET to acceptors other than CO_2, which is not linked to NADPH production, is the major candidate for providing the additional ATP required. Such acceptors could involve O_2 reduction via a Mehler reaction (see Section IV.A), nitrogen assimilation or enzymes involved with carbon metabolism which must be maintained in a reduced state by electron transport through the ferredoxin-thioredoxin system, although this would not be expected to provide a large sink for electrons (see Section II.B). In C_4 plants, particularly those using the $NADP^+$-malic enzyme pathway, CET1 would be expected to play an important role in providing energy for the shuttling of intermediates of the photosynthetic carbon reduction cycle between mesophyll and bundle sheath cells (Ivanov et al., 2005).

B. Electron Transport Through Ferredoxin and Thioredoxin

Ferredoxin can transfer electrons to thioredoxin via the enzyme ferredoxin-thioredoxin reductase (Fig. 23.3). Thioredoxin is involved in regulation of photosynthetic carbon metabolism (Buchanan and Balmer, 2005). Reduction of disulfide bonds on a number of stromal enzymes involved in photosynthetic carbon reduction, including fructose 1,6-bisphosphatase (FBPase), glyceraldehyde 3-phosphate dehydrogenase, phosphoribulokinase, Rubisco activase and sedoheptulose 1,7-bisphosphatase, by electron transfer from thioredoxin-f is required for the activation of the enzymes. Similarly thiol group reduction mediated by thioredoxin-m is required for activation of malate dehydrogenase (MDH), which is important for regulating the distribution of reductant between the chloroplast and cytoplasm. The thylakoid ATP synthase also requires reduction by thioredoxin for activation. The rate of electron flux through the ferredoxin-thioredoxin system involved in activation of enzymes is likely to be low, since enzyme activation is saturated at low light levels. In a reconstituted chloroplast system kept under nitrogen, light activation of ATP synthase, FBPase and MDH was saturated by ca. 30 μmol m^{-2} s^{-1} (Miginiac-Maslow et al., 1985). When the experiments were performed in a medium equilibrated with air, activation of these enzymes required a much higher light intensity of ca. 70 μmol m^{-2} s^{-1} and the maximum activity of the enzymes was lower than observed under nitrogen. This suggests that reduced thiol groups of the enzymes are being rapidly oxidized in the presence of O_2 and/or O_2-sensitive electron transport processes, such as the PS I-driven Mehler reaction, are competing with enzyme thiol group

reduction for electrons. Oxidation of enzyme thiol groups can result in the production of hydrogen peroxide, which must then be detoxified by ascorbate peroxidase forming monodehydroascorbate (MDA). The reduction of MDA back to ascorbate requires two electrons provided by NADPH or reduced ferredoxin, which will also contribute to the number of electrons required to maintain the thiol groups of regulatory enzymes in a reduced state. Although the total flux of electrons required to keep the regulatory enzymes activated when leaves are in the light will be a relatively small proportion of the electron flux through PS II, it is essential for the efficient operation of photosynthetic carbon reduction.

An important aspect of regulation by thioredoxin is that the redox potentials of some of the target enzymes are lower than that of reduced thioredoxin (Fig. 23.3). For example, the mid-point redox potential of MDH is −330 mV whereas that of thioredoxin-m is −300 mV, making reduction of MDH by thioredoxin-m thermodynamically unfavourable. However, reduction of MDH will occur when 80% of thioredoxin-m is reduced (Hirasawa et al., 2000). This allows control of MDH activation by the reducing equivalent status of the stroma. MDH plays a major role in the export of reducing equivalents out of the chloroplast when the stroma is highly reduced. This redox control of export of reductants from the chloroplast has been termed the 'malate valve' (Bachhausen et al., 1994). Activation of FBPase by thioredoxin-f is similarly thermodynamically unfavourable with the mid-point redox potential of FBPase being −330 mV compared to −290 mV for thioredoxin-f (Fig. 23.3; Balmer et al., 2001). However, this is not the case for phosphoribulokinase, which has a mid-point redox potential of −290 mV, similar to that of thioredoxin-f (Fig. 23.3; Hirasawa et al., 1998). Consequently, reduction of phosphoribulokinase is favored in the absence of a high level of reductants in the stroma. There appears to be the possibility of a differential redox control on different steps of carbon flux through the photosynthetic carbon reduction cycle. The situation is potentially made even more complex by the fact that the mid-point redox potentials of regulatory enzymes and thioredoxin will all be pH sensitive.

C. Partitioning of Electrons Between Carboxylation and Oxygenation of Ribulose 1,5-Bisphosphate

Fluxes of electrons through the photosystems and the redox state of components of electron transfer pathways are strongly dependent upon the availability of substrates that consume reducing equivalents. Since the majority of electrons produced by PS II-driven photo-oxidation of water are used for CO_2 reduction (see Section II.A), the rate of LET can often be determined by the availability of CO_2 to the chloroplast, and consequently by environmental constraints that bring about stomatal closure. An additional factor that can influence LET flux is the rate of oxygenation of RuBP by Rubisco, since O_2 can compete with CO_2 for the use of RuBP. At a high molar ratio of CO_2 in ambient air (C_a) no oxygenation of RuBP occurs, while at low C_a oxygenation is stimulated. The effects of changes in C_a on LET in C_3 plants are dependent on the light intensity at which leaves are operating (Badger, 1985; Cornic and Briantais, 1991). At light levels that are limiting for photosynthesis, changing C_a does not result in significant changes in the rate of LET as a repartitioning of electrons between CO_2 assimilation and photorespiration occurs to maintain a similar LET rate. In contrast, under saturating light decreasing C_a below that of the ambient atmosphere results in a decrease in the LET rate due to the rate of regeneration of RuBP being limiting and insufficient to sustain high rates of RuBP oxygenation.

In leaves of C_3 plants subjected to mild drought the molar ratio of CO_2 concentration in the intercellular leaf space (C_i) can be low as a result of stomatal closure (Cornic and Briantais, 1991; Tourneux and Peltier, 1995). At limiting light levels decreasing C_i can result in an increase in the absolute rate of RuBP oxygenation, whereas in saturating light decreasing C_i will increase the rate of RuBP oxygenation relative to the rate of LET (Cornic and Massacci, 1996). Consequently, at both limiting and saturating light levels oxygenation of RuBP will enable leaves with low C_i to maintain substantial rates of LET, thereby preventing reduction of components of the electron transport system and the occurrence of PS II photoinactivation and damage (Cornic, 1976; Powles, 1984). Clearly, photorespiration has the potential, by acting as a significant sink for reducing

equivalents, to protect the photosynthetic apparatus from photoinhibition and photodamage. However, photorespiration does not appear to be the main mechanism used by leaves to protect against the potentially damaging effects of excess light. In leaves exposed to drought there are other efficient protective mechanisms involving non-photochemical quenching processes, which can fully protect PS II from photoinactivation and damage when photorespiration is inhibited (Brestic et al., 1995).

III. Some Structural Considerations

A. Distribution of Thylakoid Complexes

The structure and organisation of thylakoid membranes is important in determining the distribution of excitation energy and rate of electron transport. Thylakoids contain two distinct membrane domains; appressed regions which are often in stacks comprising the grana and non-appressed regions termed stroma thylakoids (Staehelin and Van der Staay, 1996). There is a distinct heterogeneity in the distribution of the complexes involved in electron transport and ATP synthesis. PS I complexes are mainly located in the non-appressed domains of the grana margins and stromal thylakoids, with PS II and its light-harvesting complexes (LHCII) being primarily found in appressed regions of the grana. Cytochrome b_6f complexes are evenly distributed between appressed and non-appressed domains. ATP synthase is only found in non-appressed domains. However, the specific distribution of complexes can change in response to changes in photosynthetic metabolism and the light environment, and this can have consequences for LET and CET1 (Staehelin and Van der Staay, 1996; see below). The spatial separation of PS I and PS II complexes might suggest that long distance electron transfer must occur within the membranes. This would have to be facilitated by diffusion of plastoquinol within the lipid bilayer between PS II and cytochrome b_6f complexes and by plastocyanin between cytochrome b_6f and PS I complexes in the lumen. However, modeling studies have suggested that at the density of protein complexes found in the granal thylakoid membranes long distance diffusion of plastoquinol would be unlikely (Tremmel et al., 2003) and support the suggestion that plastoquinol diffusion is primarily restricted to microdomains of PS II and cytochrome b_6f complexes (Lavergne and Joliot, 1991). This being the case, diffusion of plastocyanin from the cytochrome b_6f complexes in the granal thylakoids to PS I complexes in the stromal thylakoids would have to occur over large distances, or such plastocyanin-mediated electron transfers might only occur between cytochrome b_6f complexes and closely associated PS I complexes located in the grana margins (Albertsson, 2001). Although electron transfer from cytochrome b_6f complexes to PS I is not rate limiting for LET under normal conditions, it can become significantly inhibited when cells experience hyperosmotic stress (Cruz et al., 2001). It is widely accepted that the limiting step in LET in non-stressed thylakoids is the transfer of electrons from plastoquinol to the cytochrome b_6f complex (Haehnel, 1984; Hauska et al., 1996), and this is a general feature of the regulation of LET in leaves operating at steady state at light levels that are not limiting for photosynthesis (Genty and Harbinson, 1996). Under many physiological conditions a major factor regulating plastoquinol oxidation by the cytochrome b_6f complex is the pH of the lumen (Kramer et al., 1999, 2003).

B. State Transitions and LHCII Phosphorylation

State transitions have long been considered to be a mechanism by which the distribution of excitation energy to PS I and PS II can be regulated (Myers, 1971; Fork and Satoh, 1986; Williams and Allen, 1987). State 1 is achieved by equilibration of thylakoids in light absorbed preferentially by PS I and results in an increased proportion of excitation energy being directed to PS II. State 2 results from equilibration in light absorbed preferentially by PS II and increases the proportion of excitation energy delivered to PS I at the expense of PS II. The mechanistic basis of the state 1-state 2 transition is the phosphorylation of a proportion of the apoproteins of LHCII, which results in a diffusion of the phosphorylated LHCII out of the appressed grana thylakoids and away from PS II complexes into non-appressed stroma thylakoids (Allen, 1992). It has been proposed that such LHCII diffusion is due to an increased negative electrical charge on the complex (Allen, 1992), although more recently it has been speculated that

conformational changes occurring within the complex as a result of phosphorylation may be the cause and may also be involved in docking the phosphorylated LHCII to PS I in the stroma thylakoids (Nilsson et al., 1997). The thylakoid kinase responsible for phosphorylating LHCII is activated by reduction of the plastoquinone pool (Allen, 1992) and the cytochrome b_6f complex appears to play a key role in transducing the redox signal from the plastoquinone pool to the kinase (Finazzi, 2004). Dephosphorylation of the phosphorylated LHCII by a phosphatase results in diffusion of the complex back into appressed granal thylakoids (Allen, 1992).

Whilst there is no doubt that state transitions can occur in green algae and leaves of higher plants and result in changes in the rates of excitation of the reaction centers of PS I and PS II, it is not clear what the physiological relevance of such changes is. It is unlikely that state transitions play a role in optimising light use for carbon assimilation under light-limiting conditions. Although large changes in the quantum efficiencies of PS I and PS II photochemistry were observed during state transitions in leaves, these were not accompanied by changes in the quantum yield of CO_2 assimilation (Andrews et al., 1993). It has been suggested that in *Chlamydomonas reinhardtii* state transitions do not act to balance the distribution of excitation energy between PS I and PS II, but serve to act as mechanism to modulate CET1 relative to LET (Vallon et al., 1991; Finazzi, 2004). This does not appear to be the case in higher plants. Changes in CET1 relative to LET would be expected to result in changes in the quantum yield of CO_2 assimilation under limiting light conditions, and this was not found to be the case for state transitions in leaves (Andrews et al., 1993). State transitions may play different roles in different groups of organisms. In higher plants only up to 25% of the LHCII is thought to be involved with this process (Allen, 1992), but 85% of LHCII has been implicated in *C. reinhardtii.* The dissociation of LHCII from PS II complexes on phosphorylation would facilitate migration of damaged PS II complexes from appressed to non-appressed membranes where they can be repaired. However, this is not considered to be a key factor in PS II repair as the major LHCII proteins become phosphorylated at low light levels and the kinase responsible for their phosphorylation is inhibited at moderate light intensities by thiols (Aro et al., 2004).

IV. Photoreduction of Oxygen

A. Water-Water Cycle

Oxygen reduction by PS I via the Mehler reaction results in the production of superoxide radicals, which must rapidly be dismutated to hydrogen peroxide by CuZn-superoxide dismutase to prevent oxidative damage to cell components (Asada, 1999). The hydrogen peroxide is then detoxified to form water by ascorbate peroxidases which convert ascorbate to MDA. In order to sustain a linear electron flux from water through PS II and PS I to O_2, MDA must be reduced back to ascorbate; this is achieved by electron donation from reduced ferredoxin (Asada, 1996). Consequently, operation of the water-water cycle involves not only electron flux from PS I to O_2, but also a similar flux to MDA.

If the electron flux to O_2 in leaves can be as great as 30% of the total LET, as has been suggested (Asada, 1999), then operation of the water-water cycle provides an effective mechanism for increasing the ATP:NADPH ratio resulting from LET. The fact that chloroplasts contain high levels of ascorbate (20–300 mM; Smirnoff et al., 2001) and glutathione (25 mM; Asada, 1999), which is required for the regeneration of ascorbate from MDA, would suggest that rapid operation of the water-water cycle could be sustained.

When leaves are exposed to environmental stresses their ability for CO_2 assimilation is often reduced, thus reducing the sink for consumption of ATP and NADPH. In such situations operation of the water-water cycle could protect PS II reaction centers from overexcitation, photoinactivation and damage (Ort and Baker, 2002). Drought stress has been shown to increase O_2 uptake in wheat leaves, which has been attributed to increases in the rate of the Mehler reaction (Biehler and Fock, 1996); however, it has been suggested that this conclusion may not be correct as photorespiratory oxidation of carbohydrate was not taken into account (Heber, 2002). When C_4 plants experience chilling temperatures, increases in the ratio of electron

flux through PS II to the rate of CO_2 assimilation have been observed (Fryer et al., 1998; Farage et al., 2006). In field-grown maize leaves which had experienced chilling during development, up to 21 electrons were transported through PS II for each CO_2 molecule assimilated, compared to six electrons in non-stressed leaves (Fryer et al., 1998). Occurring simultaneously with these increases in PS II electron flux to CO_2 assimilation were increases in ascorbate and the activities of many of the enzymes involved in scavenging of the reactive oxygen species resulting from photoreduction of O_2 and in ascorbate regeneration (Fryer et al., 1998). Such changes are consistent with an increased rate of operation of the water-water cycle. A similar situation has been reported for mangrove leaves growing in the field in tropical Australia, where electron fluxes were three times greater than could be accounted for by CO_2 assimilation (Cheeseman et al., 1997). Consequently, although there are few direct measurements demonstrating increased O_2 photo-reduction in stressed leaves, there is an increasing body of indirect evidence to support the contention that O_2, via the Mehler reaction, does play an important role as an alternative electron acceptor to CO_2. However, a recent study using mass spectrometry to measure O_2 uptake and evolution in leaves in which CO_2 assimilation had been severely compromised surprisingly demonstrated that water-water cycle activity did not significantly increase when CO_2 assimilation was restricted (Driever and Baker, 2011). These observations suggest that in higher plants the water-water cycle does not act as a protective photochemical quenching mechanism, as is does in some algae (Badger et al., 2000; Waring et al., 2010).

In order for operation of the water-water cycle to effectively protect PS II from photoinactivation and damage during periods when CO_2 assimilation is restricted by the imposition of an environmental stress, ATP produced from LET to O_2 must be consumed or a mechanism for uncoupling LET from ATP synthesis must operate (Ort and Baker, 2002). If this were not the case, a high transthylakoid proton electrochemical difference would result and restrict LET, which in turn would result in photoinactivation and damage to PS II. There are reports that at high transthylakoid proton electrochemical differences a large leakage of protons can occur through the ATP synthase which is not associated with ATP formation (Ort and Baker, 2002).

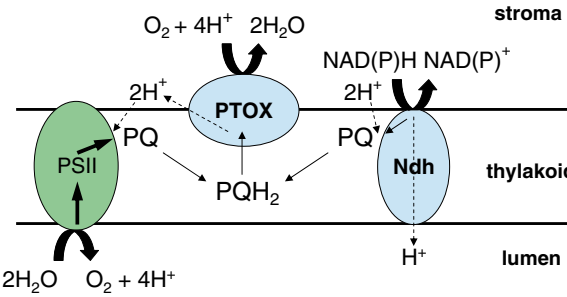

Fig. 23.4. Schematic representation of the electron transfer pathways involved in chlororespiration and O_2 reduction by the plastid terminal oxidase (PTOX) using electrons resulting from PS II photochemistry. *Solid arrows* indicate electron transfers; *dashed arrows* show H^+ movements associated with the electron transfer reactions. Key: *Ndh* NAD(P)H dehydrogenase complex, *PQ* plastoquinone.

B. Chlororespiration

The observation by Bennoun (1982) that the redox state of the plastoquinone pool could change in the dark led him to suggest an NAD(P)H dehydrogenase was responsible for reduction of plastoquinone and that oxidation of the plastoquinol was by electron transfer to O_2 mediated by an oxidase. It is now well established that such a chlororespiratory pathway operates in chloroplasts (Fig. 23.4; Peltier and Cournac, 2002). The identification of a NAD(P)H dehydrogenase (Ndh) complex in thylakoids, which has sequence similarities to sub-units of the mitochondrial and eubacterial respiratory NADH:ubiquinone oxidoreductase, and of a plastid terminal oxidase (PTOX, which is a plastoquinol oxidase, is also sometimes referred to as IMMUTANS since it was identified in the *immutans Arabidopsis thaliana* mutant) provided biochemical evidence for a chlororespiratory electron transport system (Nixon, 2000; Peltier and Cournac, 2002). Both Ndh and PTOX are located on stroma exposed regions of the thylakoid membrane (Peltier and Cournac, 2002). It is generally thought, since the Ndh has structural similarities to the mitochondrial complex one, that electron flux through Ndh to plastoquinone is associated with a transfer of protons from the stroma to the lumen.

The physiological significance of chlororespiration is not clear. Chlororespiration could generate a transthylakoid proton electrochemical difference which could be used to make ATP in the dark. The electron flux through the chlororespiratory pathway is thought to be very low, being estimated in sunflower leaves at only 0.3% of the light-saturated flux involved in LET (Field et al., 1998), consequently this being the case, it is unlikely to make a significant contribution to ATP synthesis in the light. However, in the dark it may be important in helping meet demands for ATP.

The oxidation of plastoquinol by PTOX is a potential sink for electrons transferred from PS II to plastoquinone that could act to contribute to the protection of PS II from photoinhibition. If the electron flux through PTOX can only be 0.3% of light-saturated LET flux, then it is unlikely that PTOX plays a significant role in such photoprotection. However, it has recently been found that considerably elevated levels of PTOX occur in leaves of the alpine plants *Arabis alpina, Geum montanum, G. reptans* and *Ranunculus glacialis* which were collected from a field site at an altitude of ca. 2,600 m in the French Alps (Streb et al., 2005). Interestingly, when plants of *R. glacialis* were transferred from this field site and grown for 3 weeks at low elevation their PTOX content decreased dramatically. Alpine-grown *R. glacialis* exhibited electron fluxes through PS II that considerably exceeded CO_2 assimilation rates when photorespiration was inhibited. Since this plant has low capacities for antioxidant scavenging of reactive oxygen species it is unlikely that the water-water cycle can account for high electron flux through PS II (Streb et al., 2005). It has been suggested that the elevated PTOX levels could be associated with a large electron flux from plastoquinol to O_2, which acts to protect PS II from overexcitation and consequent photoinactivation (Streb et al., 2005). If this is the case, then increased PTOX levels resulting from acclimation to high light and low temperature environments would appear to be an important mechanism transferring electrons from PS II to O_2 to form water, and thus protecting PS II from photodamage.

Chlororespiratory electron flux from NADH to O_2 mediated by Ndh and PTOX may have a regulatory role in determining the redox state of the plastoquinone pool (Peltier and Cournac, 2002), which in turn is involved in regulating the operation of activities such as CET1 and state transitions.

V. Cyclic Electron Transport Around Photosystem I

While it is well established that there is a pathway for cyclic electron transport driven by PS I (CET1), which can generate a proton motive force to drive ATP synthesis (Fork and Herbert, 1993; Bendall and Manasse, 1995; Johnson, 2005), its operation in vivo and its physiological significance are still matters of conjecture. There is no doubt that CET1 can occur in leaves under non-steady state conditions, e.g., after treatment with far-red light or DCMU and on illumination of dark-adapted leaves (Johnson, 2005). What is not clear is whether CET1 occurs at significant rates in leaves operating at steady-state photosynthesis over a range of light intensities. In leaves at limiting light levels it is unlikely that significant rates of CET1 can be occurring since the observed maximum quantum yields of CO_2 assimilation are close to the theoretical maximum (see Section II). Also, many studies have shown that in C_3 leaves operating over a wide range of light intensities under different conditions a strong linear correlation is observed between the quantum yields of PS I and PS II photochemistry (Harbinson et al., 1989, 1990; Foyer et al., 1990, 1992; Genty et al., 1990; Habash et al., 1995; Klughammer and Schreiber, 1994). This linearity between PS I and PS II quantum efficiencies indicates that either CET1 does not occur at significant rates in vivo or, if it does, then the CET1 flux must always be proportional to the LET flux. This conclusion is supported by the observation that LET and electron fluxes through PS II and from cytochrome *f* to PS I were closely correlated over a wide range of light intensities (Sacksteder and Kramer, 2000).

Although there is little compelling evidence for modulations in the rate of CET1 relative to LET in leaves operating under a wide range of physiological conditions, there is no doubt that CET1 can be induced by perturbing leaves. Operation of CET1 has been found to be induced on depletion of CO_2 in the leaf (Harbinson and Foyer, 1991; Golding and Johnson, 2003). Similarly, introducing drought conditions into

leaves was also found to induce CET (Golding and Johnson, 2003; Golding et al., 2004), presumably as a result of depletion of CO_2 within the leaves (Cornic and Massacci, 1996). Exposure of dark-adapted leaves to saturating light induces CET1 (Joliot and Joliot, 2002); however, this decreases at the expense of LET with time (Joliot and Joliot, 2005). It was concluded that on initial exposure of dark-adapted leaves to light, a very large proportion of PS I complexes are involved in CET1, and this decreases with time of illumination until almost all of the electrons transferred through PS I are involved in LET to $NADP^+$ (Joliot and Joliot, 2005).

It can be concluded that CET1 will be induced when CO_2 assimilation is severely reduced or the rate of PS I reaction center turnover exceeds the rate of utilization of NADPH in CO_2 assimilation. Consequently, CET1 would only appear to be of physiological significance in leaves which have had CO_2 assimilation significantly perturbed or are exposed to large increases in light intensity.

VI. Concluding Remarks

In non-stressed leaves when photosynthesis is light limited it is evident that CO_2 is the acceptor for the majority (ca. 88%) of the electrons transferred through the LET pathway. A similar situation appears to be the case in light-saturated, non-stressed leaves, as demonstrated by the empirically observed constancy of relationship between the quantum efficiencies of PS II photochemistry and CO_2 assimilation. However, chloroplasts exhibit an impressive plasticity in their ability to modify the pathways of electron transport when leaves experience rapid fluctuations in light intensity or environmental stresses which result in restrictions in the ability to assimilate CO_2. Such changes in the pathways of electron transfer will match the changing metabolic requirements and play a role in protecting the photosynthetic apparatus from photoinactivation and damage when the supply of CO_2 to the chloroplasts or the capacity to assimilate CO_2 becomes limiting for photosynthesis. Under such conditions PS I-mediated photoreduction of O_2 via the Mehler reaction or by PTOX and CET1 can become important and result in changes in the redox state of electron transport components. Operation of a Mehler reaction results in the production of superoxide and hydrogen peroxide and the cell must have the capacity to rapidly detoxify the resulting superoxide and hydrogen peroxide otherwise damage to cell components will result and potentially lead to cell death. Changes in the redox state of electron transport components (Buchanan and Balmer, 2005) and the production of superoxide and hydrogen peroxide (Apel and Hirt, 2004) have been implicated as triggers of signaling pathways which result in changes in gene expression that allow the leaf to acclimate to the changed metabolic or environmental situation. Consequently, such changes in electron transport pathways not only have a role in protecting the photosynthetic apparatus from photodamage and meeting changing metabolic demands, but they are also important in triggering changes in gene expression that enable the leaf to acclimate to such changes. Clearly, a major future challenge is not only to accurately determine the changes induced in electron transport pathways in leaves with changing metabolic demands and exposed to environmental stresses, but to resolve their role in modifying gene expression to enable leaves to adapt to their changed condition.

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References

Albertsson P-Å (2001) A quantitative model of the domain structure of the photosynthetic membrane. Trends Plant Sci 6: 349–354

Allen JF (1992) Protein phosphorylation in regulation of photosynthesis. Biochim Biophys Acta 1098: 275–335

Andrews JR, Bredenkamp GJ and Baker NR (1993) Evaluation of the role of state transitions in determining the efficiency of light utilisation for CO_2 assimilation in leaves. Photosynth Res 38: 15–26

Apel K and Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–99

Aro E-M, Suorsa M, Rokka A, Allahverdiyeva Y, Paakkarinen V, Saleem A, Battchikova N and Rintamäki E (2004)

Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. J Exp Bot 56: 347–356

Asada K (1996) Radical production and scavenging in the chloroplasts. In: Baker NR (ed) Photosynthesis and the Environment, Advances in Photosynthesis, Vol 5, pp 123–150. Kluwer Academic Publishers, Dordrecht

Asada K (1999) The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50: 601–639

Bachhausen JE, Kitzmann C and Scheibe R (1994) Electron acceptors in photosynthesis – regulation of the malate valve during CO_2 fixation and nitrite reduction. Photosynth Res 42: 75–86

Badger MR (1985) Photosynthetic oxygen exchange. Annu Rev Plant Physiol 36: 27–53

Badger MR, von Caemmerer S, Ruuska S and Nakanao H (2000) Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and rubisco oxygenase. Phil Trans Roy Soc London B 355: 1433–1446

Balmer Y, Stritt-Etter A-L, Hirasawa M, Jacquot J-P, Keryer E, Knaff DB and Schürmann P (2001) Oxidation-reduction and activation properties of chloroplast fructose 1,6-bisphosphatase with mutated regulatory site. Biochemistry 40: 15444–15450

Bendall DS and Manasse RS (1995) Cyclic photophosphorylation and electron transport. Biochim Biophys Acta 1229: 23–28

Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. Proc Natl Acad Sci USA 79: 4352–4356

Biehler K and Fock H (1996) Evidence for the contribution of the Mehler-peroxidase reaction in dissipating excess electrons in drought-stressed wheat. Plant Physiol 112: 265–272

Björkman O and Demmig B (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta 170: 489–504

Brestic M, Cornic G, Fryer MJ and Baker NR (1995) Does photorespiration protect the photosynthetic apparatus in French bean leaves from photoinhibition during drought stress? Planta 196: 450–457

Buchanan BB and Balmer Y (2005) Redox regulation: A broadening horizon. Annu Rev Plant Biol 56: 187–220

Cheeseman JM, Herendeen LB, Cheeseman AT and Clough BF (1997) Photosynthesis and photoprotection in mangroves under field conditions. Plant Cell Environ 20: 579–588

Cornic G (1976) Effet exercé sur l'activité photosynthétique du Sinapis alba L. par une inhibition temporaire de la photorespiration se déroulant dans un air sans CO_2. C R Acad Sc Paris série D 282: 1955–1958

Cornic G and Briantais JM (1991) Partitioning of photosynthetic electron flow between CO_2 and O_2 reduction in a C3 leaf (*Phaseolus vulgaris* L.) at different CO_2 concentrations and during drought stress. Planta 183: 178–184

Cornic G and Massacci A (1996) Leaf photosynthesis under drought stress. In: Baker NR (ed) Photosynthesis and the Environment, Advances in Photosynthesis, Vol 5, pp 347–366. Kluwer Academic Press, Dordrecht

Cruz JA, Salbilla BA, Kanazawa A and Kramer DM (2001) Inhibition of plastocyanin to P_{700}^+ electron transfer in *Chlamydomonas reinhardii* by hyperosmotic stress. Plant Physiol 127: 1167–1179

Cruz JA, Avenson TJ, Kanazawa K, Edwards GE and Kramer DM (2005) Plasticity in light reactions of photosynthesis for energy production and photoprotection. J Exp Bot 56: 395–406

Driever SM and Baker NR (2011) The water-water cycle in leaves is not a major alternative electron sink for dissipation of excess excitation energy when CO_2 assimilation is restricted. Plant Cell Environ 34: 837–846

Falkowski PG, Fujita Y, Ley A and Mauzerall D (1986) Evidence for cyclic electron flow around photosystem-II in *Chlorella pyrenoidosa*. Plant Physiol 81: 310–312

Farage PK, Blowers D, Long SP and Baker NR (2006) Low growth temperatures modify the efficiency of light use by photosystem II for CO_2 assimilation in leaves of two chilling-tolerant C4 species, *Cyperus longus* L. and *Miscanthus* x *giganteus*. Plant Cell Environ 29: 720–728

Field TS, Nedbal L and Ort DR (1998) Nonphotochemical reduction of the plastoquinone pool in sunflower leaves originates from chlororespiration. Plant Physiol 116: 1209–1218

Finazzi G (2004) The central role of the green alga *Chlamydomonas reinhardtii* in revealing the mechanism of state transitions. J Exp Bot 56: 383–388

Fork DC and Herbert SK (1993) Electron transport and photophosphorylation by photosystem I *in vivo* in plants and cyanobacteria. Photosynth Res 36: 149–168

Fork DC and Satoh K (1986) The control by state transitions of the distribution of excitation energy in photosynthesis. Annu Rev Plant Physiol 37: 335–361

Foyer C, Furbank R, Harbinson J and Horton P (1990) The mechanisms contributing to control of electron transport by carbon assimilation in leaves. Photosynth Res 25: 83–100

Foyer C, Lelandais M and Harbinson J (1992) Control of quantum efficiencies of Photosystems I and II electron flow and enzyme activation following dark-to-light transitions in pea leaves. Relationship between NADP/NADPH ratios and NADP-malate dehydrogenase activation state. Plant Physiol 99: 979–986

Fryer MJ, Andrews JR, Oxborough K, Blowers DA and Baker NR (1998) Relationship between CO_2 assimilation, photosynthetic electron transport, and active O_2 metabolism in leaves of maize in the field during periods of low temperature. Plant Physiol 116: 571–580

Genty B and Harbinson J (1996) Regulation of light utilisation for photosynthetic electron transport. In: Baker N R (ed) Photosynthesis and the Environment, Advances

in Photosynthesis, Vol 5, pp 67–99. Kluwer Academic Publishers, Dordrecht

Genty B, Harbinson J and Baker NR (1990) Relative quantum efficiencies of the two photosystems of leaves in photorespiratory and non-photorespiratory conditions. Plant Physiol Biochem 28: 1–10

Golding AJ and Johnson GN (2003) Down-regulation of linear and activation of cyclic electron transport during drought. Planta 218: 107–114

Golding AJ, Finazzi G and Johnson GN (2004) Reduction of the thylakoid electron transport chain by stromal reductants – evidence for activation of cyclic electron transport upon dark adaptation or under drought. Planta 220: 356–363

Habash D, Paul M, Parry MAJ, Keys AJ and Lawlor DW (1995) Increased capacity for photosynthesis in wheat grown at elevated CO_2: the relationship between electron transport and carbon metabolism. Planta 197: 482–489

Haehnel W (1984) Photosynthetic electron transport in higher plants. Annu Rev Plant Physiol 35: 659–693

Harbinson J and Foyer CH (1991) Relationships between the efficiencies of photosystems I and II and stromal redox state in CO_2-free air. Evidence for cyclic electron flow *in vivo*. Plant Physiol 97: 41–49

Harbinson J, Genty B and Baker NR (1989) The relationship between the quantum efficiencies of photosystems I and II in pea leaves. Plant Physiol 90: 1029–1034

Harbinson J, Genty B and Baker NR (1990) The relationship between CO_2 assimilation and electron transport in leaves. Photosynth Res 25: 213–224

Hauska G, Schütz M and Büttner M (1996) The cytochrome b_6f complex-composition, structure and function. In: Ort DR and Yocum CF (eds) Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, Vol 4, pp 377–398. Kluwer Academic Publishers, Dordrecht

Heber U (2002) Irrungen, Wirrungen? The Mehler reaction in relation to cyclic electron transport in C3 plants. Photosynth Res 73: 223–231

Heber U, Kirk MR and Boardman NK (1979) Photoreactions of cytochrome *b*-559 and cyclic electron flow in Photosystem II of intact chloroplasts. Biochim Biophys Acta 546: 292–306

Hirasawa M, Brandes HK, Hartman FC and Knaff DB (1998) Oxidation-reduction properties of the regulatory site of spinach phosphoribulokinase. Arch Biochem Biophys 350: 127–131

Hirasawa M, Ruelland E, Schepens I, Issakidis-Bourget E, Miginiac-Maslow M and Knaff DB (2000) Oxidation-reduction properties of the regulatory disulfides of sorghum chloroplast nicotinamide adenine dinucleotide phosphate-malate dehydrogenase. Biochemistry 39: 3344–3350

Ivanov B, Asada K, Kramer DM and Edwards G (2005) Characterization of photosynthetic electron transport in bundle sheath cells of maize. I. Ascorbate effectively stimulates cyclic electron flow around PSI. Planta 220: 572–581

Johnson GN (2005) Cyclic electron transport in C_3 plants: fact or artifact? J Exp Bot 56: 407–416

Joliot P and Joliot A (2002) Cyclic electron transfer in plant leaf. Proc Natl Acad Sci USA 99, 10209–10214

Joliot P and Joliot A (2005) Quantification of cyclic and linear flows in plants. Proc Natl Acad Sci USA 102: 4913–4918

Klughammer C and Schreiber U (1994) An improved method, using saturating pulses, for the determination of Photosystem I quantum yield via $P700^+$-absorbance changes at 830 nm. Planta 192: 261–268

Knaff DB (1996) Ferredoxin and ferredoxin-dependent enzymes. In : Ort DR and Yocum CF (eds) Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, Vol 4, pp 333–361. Kluwer Academic Publishers, Dordrecht

Kobayashi Y and Heber U (1995) Bioenergetics of carbon assimilation in intact chloroplasts: coupling of proton to electron transport at the ratio $H^+/ATP = 4$ in ATP synthesis. Plant Cell Physiol 36: 1629–1637

Kramer DM, Sacksteder CA and Cruz JA (1999) How acidic is the lumen? Photosynth Res 60: 151–163

Kramer DM, Cruz JA and Kanazawa A (2003) Balancing the central roles of the thylakoid proton gradient. Trends Plant Sci 8: 27–32

Lavergne J and Joliot P (1991) Restricted diffusion in photosynthetic membranes. Trends Biochem Sci 16: 129–134

Long SP, Postl WF and Bolhar-Nordenkampf HR (1993) Quantum yield for uptake of carbon dioxide in C3 vascular plants of contrasting habitats and taxonomic groupings. Planta 189: 226–234

Miginiac-Maslow M, Jacquot JP and Droux M (1985) Energetic aspects of the light activation of two chloroplast enzymes: fructose-1,6-bisphosphatase and NADP-malate dehydrogenase. Photosynth Res 6:201–213

Myers J (1971) Enhancement studies in photosynthesis. Annu Rev Plant Physiol 22: 289–312

Nilsson A, Stys D, Drakenberg T, Spangfort MD, Forsen S and Allen JF (1997) Phosphorylation controls the three-dimensional structure of plant light-harvesting complex II. J Biol Chem 272: 18350–18357

Nixon PJ (2000) Chlororespiration. Phil Trans Royal Soc Lond B 355: 1541–1547

Ort DR and Baker NR (2002) A photoprotective role for O_2 as an alternative electron sink in photosynthesis? Curr Opin in Plant Biol 5: 193–198

Pearcy RW, Krall JP and Sassenrath-Cole GF (1996) Photosynthesis in fluctuating light environments. In: Baker NR (ed) Photosynthesis and the Environment, Advances in Photosynthesis, Vol 5, pp 321–346. Kluwer Academic Publishers, Dordrecht

Peltier G and Cournac L (2002) Chlororespiration. Annu Rev Plant Biol 53: 523–550

Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol 35: 15–44

Radmer RJ and Kok B (1976). Photoreduction of O_2 primes and replaces CO_2 assimilation. Plant Physiol 58: 336–340

Rumberg B, Schubert K, Strelow F and Tran-Anh T (1990) The H+/ATP coupling ratio at the H^+-ATP-synthase of spinach chloroplasts is four. In: Baltscheffsky M (ed) Current Research in Photosynthesis III, pp 125–128. Kluwer Academic Publishers, Dordrecht

Sacksteder C and Kramer DM (2000) Dark-interval relaxation kinetics (DIRK) of absorbance changes as a quantitative probe of steady-state electron transfer. Photosynth Res 66: 145–158

Seelert H, Poetsch A, Dencher NA, Engel A, Stahlberg H, Muller DJ (2000) Proton-powered turbine of a plant motor. Nature 405: 418–419

Smirnoff N, Conklin PL and Loewus FA (2001) Biosynthesis of ascorbic acid in plants: A renaissance. Annu Rev Plant Physiol Plant Mol Biol 52: 437–467

Staehelin LA and Van der Staay GWM (1996) Structure, composition, functional organisation and dynamic properties of thylakoid membranes. In: Ort DR and Yocum CF (eds) Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, Vol 4, pp 11–30. Kluwer Academic Publishers, Dordrecht

Stewart DH and Brudvig GW (1998) Cytochrome b_{559} of photosystem II. Biochim Biophys Acta 1367: 63–87

Streb P, Josse E-M, Gallouët E, Baptist F, Kuntz M and Cornic G (2005) Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*. Plant Cell Environ 28: 1123–1135

Tourneux C and Peltier G (1995) Effect of water deficit on photosynthetic oxygen exchange measured using $^{18}O_2$ and mass spectrometry in *Solanum tuberosum* L. leaf discs. Planta 195: 570–577

Tremmel IG, Kirchhoff H, Weis E and Farquhar GD (2003) Dependence of plastoquinol diffusion on the shape, size, and density of integral thylakoid proteins. Biochim Biophys Acta 1607: 97–109

Vallon O, Bulte L, Dainese, Olive J, Bassi R and Wollman F-A (1991) Lateral redistribution of cytochrome b_6f complexes along thylakoid membranes upon state transitions. Proc Natl Acad Sci USA 88: 8262–8266

von Caemmerer S (2000) Biochemical models of leaf photosynthesis. CSIRO publishing, Collingwood, Australia

Waring J, Klenell M, Underwood GJC and Baker NR (2010) Light-induced responses of oxygen photoresuction, reactive oxygen species production and scavenging in two diatom species. J Phycol 46: 1206–1217

Whithmarsh J and Pakrasi HB (1996) Form and function of cytochrome *b*-559. In: Ort DR and Yocum CF (eds) Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, Vol 4, pp 249–264. Kluwer Academic Publishers, Dordrecht

Williams WP and Allen JF (1987) State 1/State 2 changes in higher plants and algae. Photosynth Res 13: 19–45

Chapter 24

Towards Artificial Photosynthesis[1]

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Summary

Artificial photosynthesis in the broadest sense encompasses human attempts to convert sunlight, water and carbon dioxide into carbohydrates, other organic compounds and oxygen, to split water into hydrogen and oxygen using sunlight, or to convert sunlight directly into electricity. Although largely still in its infancy, artificial photosynthesis is being researched on various fronts. Moderately-high-efficiency silicon photovoltaic generators are the most established and durable devices, but their efficiencies are still very much below thermodynamic limitation; thus, further improvements are feasible. Of great concern is how to lower the costs of manufacturing silicon photovoltaic devices. Dye-sensitized photovoltaic cells, when designed to absorb a broad spectrum of solar radiation, seem to offer great promise, especially in terms of lower manufacturing costs. The field of photon-harvesting for non-biological hydrogen production has advanced steadily, ever since the discovery of light-induced splitting of water into

[1] Dedicated to the memory of Sir Rutherford N (Bob) Robertson (1913–2001)

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DOI 10.1007/978-94-007-1579-0_24,

hydrogen and oxygen at a semiconductor electrode (Fujishima A, Honda K, Nature 238:37–38, 1972). A deficiency that needs to be addressed, however, is the limited absorption of the solar spectrum by a semiconductor. A tandem device with two semiconductor electrodes, absorbing complementary portions of the solar spectrum and working in series, partially overcomes the poor absorption, in a way that is analogous to the "Z Scheme" of natural photosynthesis in which two photosystems operate in series. Photoconversion efficiency, however, has to be further improved. Artificial reaction centers capable of light-induced charge separation have been constructed, based on porphyrin-fullerene complexes, ruthenium complexes or synthetic proteins holding redox cofactors. The longer-term application of such artificial reaction centers is their coupling to chemical/biochemical reactions that produce useful products/devices. Another spin-off from research on artificial reaction centers is the basic understanding of the mechanisms of forward and back charge transfers that can be gained from a comparison of natural and artificial reaction centers. Perhaps the most ambitious goal in artificial photosynthesis is the in vitro making of carbon-based end products, especially foodstuffs. Such a supplementation of the natural process may be necessitated by a gradual loss of agricultural land, a shortage of fresh water and a need to feed an increasing world population. Artificial photosynthesis for food production may present one of the greatest challenges to future scientists and technologists.

I. Introduction

"*The ultimate possibility of in vitro photosynthesis is beckoning*" (Rabinowitch and Govindjee, 1969, p. 255). Life is dependent on a supply of chemical energy that has been met, directly or indirectly, almost entirely by natural photosynthesis. Human life, in particular, requires: (1) food in the form of plant material or animals we eat; (2) fuel in the form of stored end products of photosynthesis – peat, lignite, coal, oil and natural gas, etc.; and (3) fiber materials that ultimately originate in photosynthesis. Human civilization can be considered to have advanced through the utilization or transformation of energy. Indeed, as society becomes more and more complex, the energy flow through unit mass increases, a trend that is true for cosmic evolution in general (Chaisson, 2005).

The amount of fossil fuel is finite. For example, the entire oil supply would last 2 years if the world population were to consume the liquid fuel at the same rate per person as in Australia (Lowe, 2005). Coal is more abundant, but there are environmental problems associated with the use of non-renewable fossil fuel, e.g., acid rain and human-induced alteration of the climate. As for nuclear power, to produce 10 TW of additional carbon-free power (projected to be required by 2050), we would need to construct 10,000 nuclear plants, i.e., one 1-gigawatt-electric power plant every 1.6 days somewhere in the world for the next 45 years (Lewis and Nocera, 2006). Adding to the current number of ca. 400 nuclear plants at such a rate presents formidable difficulties, as do sustaining a supply of relatively high-grade uranium ore beyond one to three decades and storage of nuclear waste safely for very long periods. On the other hand, the energy supplied from the Sun to the Earth is about 10,000 times the rate of current energy consumption by the world population. In fact, "the large gap between our present use of solar energy and the enormous undeveloped potential defines a compelling imperative for science and technology in the 21st century" (Lewis and Nocera, 2006).

A concept in solar energy utilization that easily captures our imagination is artificial photosynthesis. One definition of artificial photosynthesis is a man-made process that attempts to replicate the natural process, converting sunlight, water and carbon dioxide into carbohydrates and oxygen. A broader definition includes the use of sunlight to produce oxygen and organic compounds from water and carbon dioxide, or to split water into hydrogen and oxygen. A still broader definition includes the conversion of light into electricity.

Abbreviations: BChl – Bacteriochlorophyll; Car – Carotenoid; Chl – Chlorophyll; C_{60} – Fullerenes; DSSC – Dye-sensitized solar cells; HV^{2+} – 1,1′-dihexyl-4,4′-dipyridium diperchlorate; IPCE – Incident photon conversion efficiency; ITO – Indium tin oxide; MV – Methyl viologen; NADPH – Reduced nicotinamide adenine dinucleotide phosphate; P – Porphyrin; P680 – The primary electron donor in the PS II reaction center; PS – Photosystem

A broad concept of artificial photosynthesis was first expressed by Jules Verne in his 1874 novel *The Mysterious Island* (quoted by Hoffmann, 2001):

> Yes, my friends, I believe that water will one day be employed as fuel, that hydrogen and oxygen which constitute it, used singly or together, will furnish an inexhaustible source of heat and light…. As long as the earth is inhabited, it will supply the wants of its inhabitants, and there will be no want of either light or heat as long as the production of the vegetable, mineral or animal kingdoms do not fail us.

Ciamician (1912), considering the finite availability of "fossil solar energy", put forward serious proposals for what could be classified as artificial photosynthesis. He asked: "whether there are not other methods of production which may rival the photochemical processes of the plants", citing as possible examples the use of sunlight to transform a "mixture of water and carbon dioxide into oxygen and methane" and to produce "photoelectrical batteries or batteries based on photochemical processes". He even envisaged that "in the desert regions, unadapted to any kind of cultivation, photochemistry will artificially put their solar energy to practical use".

Almost a century on, much is known about the details of the natural photosynthetic processes, and research is proceeding on artificial photosynthesis as broadly defined above. This chapter attempts to survey areas of artificial photosynthesis research to indicate progress that has been made in the harvesting of photons for photovoltaics and for non-biological hydrogen production. (For biological hydrogen production see, for example, Melis, 2007; Hankamer et al., 2007 and Ghirardi et al., 2009.) It will also attempt to review progress made in bio-mimetic reaction centers for charge separation, as researchers learn from nature and adapt reactions coupled to such reaction centers for specific purposes useful to human needs. Finally, in a futuristic fashion, this chapter will outline desirable carbon-based end products of artificial photosynthesis – end products that one may have in mind when talking about artificial photosynthesis. This survey is necessarily sketchy, as the research is still in its infancy. For a more complete collection of recent papers covering various topics in artificial photosynthesis, from basic biology to industrial application including engineering, social and political issues, please refer to Collings and Critchley (2005). Further, a special issue of Chemical Society Reviews (see Nocera, 2009) covers the latest developments in renewable energy research, including ideas from biology that can inspire technology for photochemical fuel production (Hambourger et al., 2009).

II. Photon-Harvesting for Photovoltaics

A. High Efficiency Silicon Photovoltaics

The silicon photovoltaics industry has been growing at 30–40% per year over the past several years (Green, 2005), albeit from a small base. It has benefited from the much larger microelectronics industry, taking advantage of scrap silicon and production technology from this industry, particularly during its down-turn periods. Silicon photovoltaic products at the same time have enjoyed steadily decreasing costs of production while maintaining reasonable energy conversion efficiency.

The "first generation" silicon photovoltaic products (Green, 2005) consist of silicon wafers, either the traditional monocrystalline wafers as used in the microelectronics industry, multicrystalline wafers cut from a large ingot, or wafers prepared directly as sheets or ribbons. Approximately 90% of recent photovoltaic sales are of products based on silicon wafers. Typical energy conversion efficiency of commercial modules range from 10% to 16% (von Aichberger, 2004). These silicon wafers are highly durable, warranties of 25 years being common. They have one drawback, however. A considerable thickness of about 0.3 mm is needed during manufacture of silicon wafers, which also require elaborate encapsulation to maintain a working life of 25 years, thereby adding to the total cost of production.

To reduce costs, "second generation" photovoltaic products are based on a thin-film approach. Photovoltaically active material is deposited on a supporting substrate or superstrate. Thin-film poly-crystalline silicon on glass has energy conversion efficiencies approaching 10% (Green et al., 2004), with excellent durability and stability both in the field and in accelerated testing (Green, 2005).

The energy conversion efficiency of 10–16% for "first generation" and ~10% for "second

generation" photovoltaics is not due to any thermodynamic limitation. Indeed the limiting efficiency is likely to be as high as 74% (Green, 2005). Such a high limit for energy conversion efficiency has driven the search for "third generation" photovoltaic products, some possible routes to which are being explored (Green, 2005). For example, one possibility is to employ a stack of tandem cells, where different components of the stacked unit have different bandgaps (energy differences between the valence and conduction bands) which together utilize the wide solar spectrum. Another is to use a single target cell, but to manipulate the incoming spectrum itself by up- or down-conversion of the wavelength for matching with the target cell. Other as yet less explored possibilities also exist, and in the very long run, "third generation" photovoltaic products will aim for efficiencies approaching the thermodynamic limiting efficiency; then they will realize their full commercial potential (Green, 2005).

B. Dye-Sensitized Solar Cells

To date, photovoltaic products are predominantly solid-state p-n junction devices in which the semiconductor, usually doped silicon, performs both tasks of photon absorption and charge separation. This contrasts with natural photosynthesis in which light is harvested by an antenna complex and is transferred to a reaction center where charge separation and charge stabilization occur. It is now possible, however, to mimic this "division of labour" in photovoltaic devices known as "dye-sensitized solar cells" (DSSCs). Such products have achieved remarkably high solar to power conversion efficiencies, reaching 10.4% in one case (Nazeeruddin et al., 2001) and offering a serious alternative to solid-state p-n junction devices.

The principle of operation of a DSSC, a prototype of which was reported by O'Regan and Grätzel (1991), is illustrated by the scheme in Fig. 24.1. It consists of a mesoporous layer of nano-sized crystals of oxide, e.g., TiO_2, which have been sintered together to allow for electronic conduction. Bound to the surface of the nanocrystalline film is a monolayer of a dye. Photon absorption by the dye leads to an electron being injected from the dye to the conduction band of the oxide, the oxide and dye together making up the photo-anode. The injected electrons are

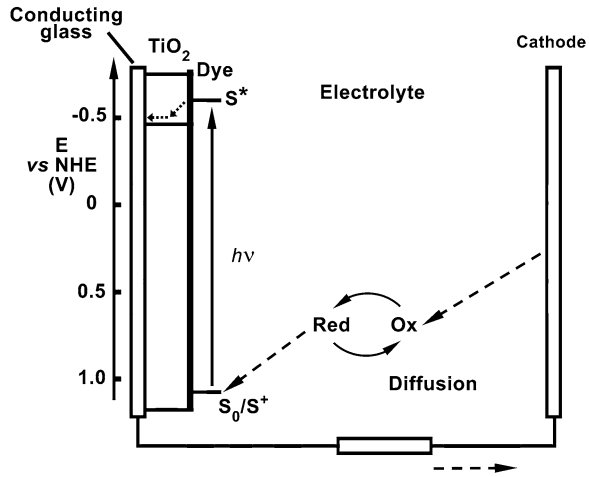

Fig. 24.1. A diagram showing the operation of a dye-sensitized solar cell. The photoanode, made of mesoporous semiconductor material (e.g., TiO_2) receives electrons from the excited state of a dye sensitizer (S*) that coats the semiconductor material; the electrons injected into the conduction band of the semiconductor material are transferred to a conducting glass plate, and then to the cathode via an external conductor/load. The oxidized dye (S^+) retrieves an electron donated by a reductant present in the electrolyte, the reductant having received its electron from the cathode. An approximate reduction potential scale (*E*) vs the normal hydrogen electrode (*NHE*) is displayed to indicate the position of the conduction band of the semiconductor (Re-drawn from Grätzel, 2001).

conducted to a transparent conducting glass and then via an external circuit to the cathode, which is made of a thin film of low work-function material such as platinum. Oxidized dye molecules are replenished by electron donation from a redox couple such as the I^-/I_3^- couple in an electrolyte, followed by diffusion of the oxidant (hole transport) to the cathode where reduction occurs.

For 8 years since its discovery by M.K. Nazeeruddin and co-workers (Nazeeruddin et al., 1993), the ruthenium complex, *cis*-$RuL_2(NCS)_2$, (where L = 4,4′-COOH-2,2′-bipyridine), was unmatched in its photovoltaic performance. Then Nazeeruddin et al. (2001) introduced the "black dye" (L = 4,4′,4″-COOH-2,2′-terpyridine), and extended the photocurrent onset wavelength to 920 nm (by comparison, TiO_2 does not absorb above ca. 420 nm), achieving a 10.4% solar to power conversion efficiency.

Such conversion efficiencies are comparable with those of silicon-based solid-state devices. Furthermore, the DSSCs are made from low- to

medium-purity materials through low-cost processes, and the ruthenium complexes seem to be stable. For example, *cis*-$RuL_2(NCS)_2$ survived 10^8 redox cycles during illumination, corresponding to 20 years of operation in natural sunlight (Grätzel, 2003). The DSSC was further stabilized by using a quasi-solid-state polymer gel electrolyte, achieving unprecedented thermo- and photostability in a solar simulator for at least 1,000 h (Wang et al., 2003). When the dye is maintained in the oxidized state over a long time, however, degradation occurs via the loss of sulfur. Therefore, re-reduction of the oxidized dye molecules should occur quickly to avoid undesirable reactions. One disadvantage of the ruthenium dyes, however, is that they are expensive to manufacture. Therefore, other dyes are being sought.

Various organic dyes have been investigated, including porphyrins and pthalocyanines, the most successful being coumarin or polyene type sensitisers. Hara et al. (2003a) reported a solar energy-to-electricity conversion efficiency of 6.0% obtained using a novel coumarin dye (NKX-2311) in a DSSC, for which the incident photon conversion efficiency (IPCE) spectrum extends to far-red wavelengths. The authors succeeded in obtaining 7,000 h of cell stability using strong light with a UV cut-off filter, which corresponds to 6 years of outdoor use. NKX-2311 also appears to have good thermal stability at temperatures below 240°C.

Hara et al. (2003b) investigated novel polyene dyes for highly efficient DSSCs. Their best solar energy-to-electricity conversion efficiency is 6.8%, obtained with the novel polyene NKX-2569. The IPCE spectrum of this poly extends to 800 nm. Although the dye was stable over several days under strong white light (>420 nm), its long-term stability has yet to be investigated.

Another photovoltaic system that has an IPCE spectrum spanning both visible and near-infrared wavelengths has been reported by Hasobe et al. (2004). It is the first organic photovoltaic cell that uses supramolecular porphyrin dendrimers for light-harvesting and for effective electron transfer from the excited porphyrin to the fullerene located within the dendritic matrix. The use of a dendritic structure could provide a new way of developing more efficient photon energy conversion, as different generation dendritic structures could be explored.

Meredith et al. (2005) investigated melanins as a possible dye in DSSCs. Melanins are an important class of pigmented macromolecules found throughout nature: they absorb UV and visible light broadly, are chemically and photochemically very stable, and can be synthesized cheaply. The authors demonstrated that under illumination eumelanin, the predominant form of melanin in human skin and eyes, was able to inject electrons into the conduction band of TiO_2. However, the energy conversion efficiency (0.1%) has a long way to go to match that of silicon cells (10–15%).

The above examples demonstrate that dye-sensitized nanocrystalline solar cells show great promise of reasonable efficiency, low cost of production and long-term stability. No doubt, new variants of DSSCs will be found with further improvements, including lower cost of production and simplification in manufacture. To greatly improve energy conversion efficiency, it will be necessary to harvest a broad portion of the solar spectrum. In this respect, there is much that can be learned from nature. Light-harvesting in natural photosynthesis is mainly achieved by diverse pigment-protein complexes, in which the chromophores are held at optimal distances and orientations from one another. Obviously, this arrangement is too complicated to mimic in an artificial system. Therefore, attention has been turned to the light-harvesting architecture of evolutionarily ancient green photosynthetic bacteria (Balaban, 2005). Green photosynthetic bacteria evolved a unique antenna, the chlorosome, which consists of agglomerates of BChl *c*, *d* or *e* molecules surrounded by a monolipid membrane. The most remarkable property of chlorosomes is that while the BChls self-assemble into stacks in a crystal lattice without the aid of proteins, they nevertheless manage to avoid concentration quenching, which otherwise dissipates excitation energy as heat when the inter-chromophore distance becomes too small (Beddard and Porter, 1976). By avoiding concentration quenching, the chlorosomes efficiently collect light and transfer the excitation energy to the reaction center for charge separation (Balaban, 2005). If this property of light-harvesting without concentration quenching can be exploited with a number of pigments which absorb complementary parts of the solar spectrum, light-harvesting will be much

improved in dye-sensitized solar cells in which a self-assembled antenna is used with a semiconductor anode.

III. Photon-Harvesting for Non-biological Hydrogen Production

A. Photoelectrolysis of Water at a Semiconductor Electrode

Fujishima and Honda (1972) used a TiO_2 photoelectrode to split water into O_2 and H_2. At the TiO_2 electrode, excitation of TiO_2 by light results in the production of electrons and holes (p^+); the holes react with water to yield O_2:

$$TiO_2 + 4h\nu \rightarrow 4e^- + 4p^+$$

$$4p^+ + 2H_2O \rightarrow TiO_2 + O_2 + 4H^+$$

At the platinum black electrode, protons are reduced to H_2:

$$4e^- + 4H^+ \rightarrow 2H_2$$

The overall reaction is:

$$2H_2O + 4h\nu \rightarrow O_2 + 2H_2$$

Unfortunately, because of its large band gap, TiO_2 absorbs only radiation below about 420 nm. Numerous attempts to shift the absorption spectrum of TiO_2 had not been successful until Khan et al. (2002) modified an n-type TiO_2 by substituting carbon for some of the lattice oxygen atoms. The chemically modified TiO_2 absorbs light of wavelengths up to 535 nm. The lowering of the band gap did not affect the stability of the semiconductor over a period of illumination of 6 months. The reported conversion efficiency was quite good, 8.35%, obtained with a xenon arc lamp. However, this efficiency was subsequently considered to be much over-estimated (Fujishima, 2003; Hägglund et al., 2003; Lackner, 2003). In any case, Fujishima et al. (1975) had used a similar flame-thermal treatment of TiO_2, but obtained a much lower efficiency of only 0.4% using sunlight. Fujishima (2003) considered that perhaps a significant amount of ultraviolet light in the xenon arc lamp could have contributed to an over-estimation of the efficiency reported by Khan et al. (2002).

To increase the absorption of visible light (at green wavelengths), Abe et al. (2000) chemically fixed the dye Eosin Y onto platinised TiO_2 particles in a dye-sensitized photocatalytic system in an aqueous triethanolamine solution. During 520 nm illumination, steady H_2 production was demonstrated, with a quantum yield of about 10%. The dye was able to survive more than 10^4 turnovers. In other dye-sensitized systems, Abe et al. (2004) found that the addition of iodide gave a significant improvement on water splitting into H_2 and O_2 by suppressing the back reaction.

Recently Kanan and Nocera (2008) reported a self-assembling catalyst formed from earth-abundant elements, cobalt and phosphorus, which can produce molecular oxygen from water at neutral pH with a low over-potential akin to that which operates in the oxygen-evolving complex in Photosystem II (PS II) in natural photosynthesis. The next step will be to couple this oxygen production system to another catalyst which will use the electron and protons derived from water to produce hydrogen gas or reduce carbon dioxide (see Barber, 2009). For example, the stable and metal-free photocatalyst of Wang et al. (2009) for hydrogen production from water under visible light, when further improved in efficiency, could be coupled to the self-assembling, oxygen-evolving catalyst of Kanan and Nocera (2008) for more efficient oxygen-evolution and hydrogen production. Currently, the photocatalyst of Wang et al. (2009), made from an abundant material (polymeric carbon nitride), is coupled to RuO_2 (3 wt.%) which gave a rather low rate of oxygen evolution.

Another system (Brimblecombe et al., 2008), also akin to the oxygen-evolving complex in PS II, is a bio-inspired manganese cluster, a $\{Mn_4O_4\}$ cubane complexed with a suitable ligand, reminiscent of the "cube-like" $\{CaMn_3O_4\}$ core in the oxygen-evolving complex of PS II. The authors showed that a Nafion membrane doped with their cubane complex gave sustained electro-oxidation of water at potentials between 0.8 and 0.12 V (vs Ag/AgCl). However, light was also required, implying that there was an additional limiting reaction, which effectively contributed to a larger over-potential. If the requirement for light proves to be associated only with overcoming an energy

barrier rather than with the oxidation, then future work should focus on lowering the barrier (Brimblecombe et al., 2008).

B. Photoelectrolysis of Water for Hydrogen Production by Mimicking the Z Scheme of Natural Photosynthesis

Grätzel (1999) achieved the cleavage of water into hydrogen and oxygen using a low-cost tandem device based on an analogy with the two photosystems working in series in natural photosynthesis. A schematic of the tandem cell is shown in Fig. 24.2. A thin film of nanocrystalline tungsten trioxide, WO_3, serves as an electrode absorbing in the blue part of the spectrum. On excitation of electrons into the conduction band, the holes left in the valence band of WO_3 oxidize water to oxygen. The electrons in the conduction band are fed to the other "photosystem", a Ru dye-sensitized nanocrystalline TiO_2 electrode, where protons are reduced to hydrogen. The dye-sensitized TiO_2 electrode utilizes the green and red part of the solar spectrum. The absorption of complementary parts of the spectrum by the two electrodes enhances the overall efficiency of conversion from standard solar radiation into chemical energy, which at present is 4.5%. This efficiency is comparable with the 7% in the "brute force" approach using a set of four silicon photovoltaic cells in series to generate electricity which is subsequently used in a commercial water electrolyzer.

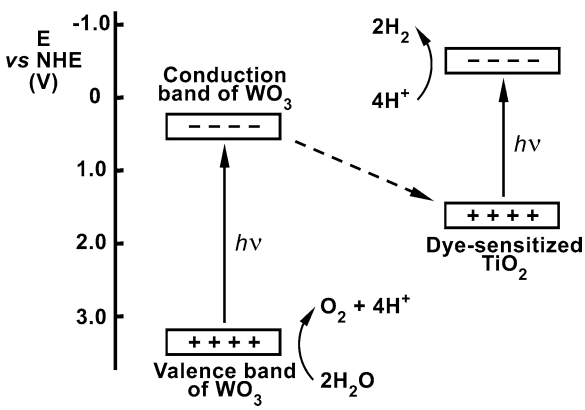

Fig. 24.2. A mimetic Z Scheme for the photoelectrolysis of water using a tandem cell. On excitation with blue light, nanocrystalline tungsten trioxide, WO_3, forms holes in its valence band while the electrons are injected into the conduction band. The holes oxidize water to O_2. Excitation with green and red light injects electrons into the conduction band of dye-sensitized TiO_2; the holes on TiO_2 receive electrons from the conduction band of WO_3. The electrons in the conduction band of TiO_2 reduce H^+ to H_2 (Re-drawn from Grätzel, 2001).

Mimicking the Z-scheme for hydrogen production was also achieved in a mixture of Pt-WO_3 and doped Pt-$SrTiO_3$ powders in an aqueous NaI solution (Sayama et al., 2002). However, because of the absence of a dye sensitizer, the Pt-WO_3 could not work at wavelengths above 460 nm, and even at 421 nm, the quantum efficiency of this photocatalytic mixture was only 0.1%. Therefore, it seems that future improvements will come from the Grätzel tandem cell, perhaps with dye-sensitized WO_3 for a more balanced excitation of the two "photosystems" or with acceleration of the rate-limiting steps.

IV. Bio-Mimetic Reaction Centers for Charge Separation

The aim of converting light into useful chemical energy is to first bring about a light-induced electron transfer (charge separation), and then stabilize the charge separation for long enough to allow useful chemical reactions to be coupled to the electron transfer. The site where charge separation and stabilization occurs constitutes a reaction center, as distinct from an antenna that harvests light.

A. Non-protein Based Reaction Centers

1. Porphyrin – Fullerene-Based Reaction Centers

The synthesis of a molecule that mimics the reaction center in natural photosynthesis was achieved by Moore et al. (1984). The molecule consists of three parts in a "triad". The porphyrin in the middle traps the light, much like P680, the PS II reaction center Chl dimer, in the natural system; the excited porphyrin donates an electron to a quinone group, becoming a hole. Before the electron can recombine with the hole, a β-carotene at the other end of the molecule speedily donates an electron to the porphyrin cation. The result is a

relatively long-lived charge separation across two ends of the triad molecule, allowing further reactions to occur, e.g., coupling electron transport to proton deposition in the internal aqueous phase of liposomes, and utilization of the proton gradient to synthesize ATP (Steinberg-Yfrach et al., 1988).

Compared with a quinone acceptor group, fullerenes (C_{60}) have been found to have features well suited to act as an electron acceptor group (see, for example, Guldi, 2000; Moore et al., 2002; Imahori et al., 2003). For instance, fullerene-based reaction centers have faster charge separation and slower charge recombination compared with planar electron acceptors such as quinones and diimides. Imahori et al. (1996, 2003) were the first to propose and demonstrate that faster charge separation and slower charge recombination are attributable to the small reorganization energy of fullerenes. Their proposal is based on the extensive π-electron delocalization of C_{60} together with the rigid structure. The reorganization energy is the sum of a solvent term and a vibrational term: the highly delocalized charge over the three-dimensional framework of C_{60}^- means that the charge density on each carbon atom is small, rendering the solvent term small, while the rigid structure of C_{60} results in a small vibrational term. The lower reorganization energy of fullerenes relative to that of a flat electron acceptor means that the forward electron transfer is faster, while charge recombination is slower, as explained by Imahori et al. (2003) in terms of the Marcus theory of electron transfer (Marcus and Sutin, 1985; Marcus, 1993; Gray and Winkler, 1996). A further advantage of using fullerenes as electron acceptors is that they can accept electrons in both the ground state and the excited state. Taking advantage of these properties of fullerenes, Gust et al. (1997) produced a triad consisting of a carotenoid (Car), porphyrin (P) and fullerene (C_{60}). Further refinement of the triad consisted of modifying the carotenoid-to-porphyrin linkage to increase the rate of electron donation from Car to P (Kuciauskas et al., 2000) and changing P to a tetraphenyl-based porphyrin to retard charge recombination of C_{60}^- with P^+ (Bahr et al., 2000); these modifications resulted in a quantum yield for the formation of $Car^+–P–C_{60}^-$ of ca. 100%.

Imahori and colleagues made a systematic study of porphyrin-fullerene-linked systems (reviewed by Imahori et al., 2003). As a spectacular example, they achieved an extremely long-lived charge-separated state using a ferrocene – zincporphyrin – freebaseporphyrin – fullerene tetrad; the lifetime of the resulting charge-separated state (i.e., ferricenium cation-C_{60} anion pair) was 380 ms, an order of magnitude greater than any other intramolecular charge recombination process in a synthetic system (Imahori et al., 2001). In another spectacular example, they linked P–C_{60} to an indium-tin oxide (ITO) electrode (instead of a carotenoid), the P–C_{60} forming a self-assembled monolayer on ITO. The photoinduced electrons on C_{60} were transferred to a Pt counter electrode via 1,1′-dihexyl-4,4′-dipyridinium diperchlorate (HV^{2+}). Thus, the cell is represented by ITO/P–C_{60}/HV^{2+} / Pt, where P is a free-base porphyrin. The quantum yield of photocurrent generation was 6.4% in this system, enhanced to a large extent by the incorporation of the C_{60} moiety (Yamada et al., 2002). Further improvements of the photocurrent generation would be expected from the enhancement of light absorption at >450 nm. For a review of the supramolecular design of photocurrent devices using fullerenes, see Konishi et al. (2005).

2. Ruthenium-Based Reaction Centers

Photosystem II is the only natural photosynthetic reaction center that uses water as a source of electrons, able to provide plants (and indirectly almost all other organisms) with an unlimited supply of electrons and protons. Therefore, an important goal in artificial photosynthesis has been to mimic the PS II reaction center with model systems. Because of its photostability and suitability in synthetic reactions, ruthenium(II), usually in the form of a *tris*-bipyridyl complex, has been the favourite sensitizer that takes the place of P680, the primary electron donor in PS II. In building a bio-mimetic reaction center from $[Ru(bpy)_3]^{2+}$, much of what is known about the function and structure of PS II has been applied, while conversely, the model systems have been used to test current ideas about the interactions of components within PS II.

Since the Mn cluster is integral to the water-splitting capability of PS II reaction centers, the

first Ru-Mn sensitizer-donor complex was reported by Sun et al. (1997). In this binuclear complex, a ruthenium(II) *tris*-bipyridyl was covalently linked to a manganese(II) ion through bridging ligands. When the binuclear complexes were given flash illumination in the presence of an electron acceptor such as methyl viologen (MV), an initial electron transfer occurred from the excited state of Ru(II), forming Ru(III) and $MV^{+\bullet}$. An intramolecular electron transfer from the ligated Mn(II) to Ru(III) then occurred with a first-order rate coefficient of $1.8 \times 10^5 \cdot s^{-1}$, regenerating Ru(II). Importantly, this is the first demonstration of an intramolecular system in which a manganese complex donates an electron to a photo-oxidized sensitizer. A drawback of this system, however, was that the Mn also quenched the excited state of Ru, thereby shortening the lifetime of the excited state and resulting in inefficient electron donation to an added electron acceptor. To minimize the quenching and for other reasons, a tyrosine was covalently-linked to the ruthenium(II) *tris*-bipyridyl complex (Magnuson et al., 1999). Flash excitation of the Ru(II) complex in the presence of the electron acceptor $[Co(NH_3)_5Cl]^{2+}$ resulted in Ru(III) which was then re-reduced by tyrosine. On adding a suitable Mn(III/III) dimer in solution, a large fraction of the flash-induced oxidized tyrosine was in turn re-reduced with a fast time constant, via electron donation from Mn(III/III). This demonstrates that a synthetic redox "triad" should be able to mimic the donor "triad" in PS II. Further, the introduction of hydrogen bonds greatly accelerated electron donation from tyrosine to Ru(III) in the synthetic system (Sun et al., 1999).

In order to oxidize water, it is necessary to accumulate four positive charges. With this in mind, a Mn(II/II) dimer was prepared and covalently linked to a ruthenium(II) *tris*-bipyridyl complex via a tyrosine (Sun et al., 2000). These investigators were able to observe flash-induced electron transfer from the excited state of the ruthenium *tris*-bipyridyl moiety to MV, followed by retrieval of an electron from the manganese (II/II) dimer with a rate coefficient $>1 \times 10^7\ s^{-1}$. Further work by the same group using multiple flashes showed a stepwise extraction of three electrons by Ru(III) *tris*-bipyridyl, from the manganese (II/II) dimer, giving the Mn(III/IV) oxidation state (Huang et al., 2002). Thus, this trinuclear complex closely resembles the oxidizing side of PS II in its capacity for charge accumulation, though catalytic water oxidation has not been achieved. Similarly, Burdinski et al. (1999, 2000) prepared a complex containing a linear cluster of three manganese (II) ions; again photo-oxidation of Ru(II) to Ru(III) was followed rapidly (rate coefficient $>5 \times 10^7\ s^{-1}$) by re-reduction via intramolecular electron transfer from the manganese cluster, but no water oxidation has yet been achieved. For reviews of ruthenium-manganese chemistry mimicking the PS II reaction, see Sun et al. (2001), Hammarström et al. (2001) and Hammarström (2003).

As distinct from the accumulation of positive charge in the systems described above, Konduri et al. (2002) reported a ruthenium dimer system capable of storing up to four electrons in a single acceptor ligand. Interestingly, each photoinduced electron-transfer step is coupled with protonation of the reduced state. In this way, excessive build-up of negative charge can be avoided. The studies described above show that much is learnt from comparing natural and synthetic reaction centers, and that progress is being made towards the ultimate catalytic oxidation of water as an abundant source of electrons and protons.

B. Protein-Based Reaction Centers

Natural photosynthetic reaction centers consist of redox components held in a protein scaffolding that provides both the optimal distances and orientation between the redox components and the microenvironment that fine-tunes the appropriate redox potentials. Protein-based reaction centers offer model systems to explore the effects of different redox cofactors, protein environments and spatial relationships on the function of biomimetic reaction centers. For reviews, see Razeghifard and Wydrzynski (2005), Razeghifard (2008) and Conlon (2008).

A first step in assembling an artificial reaction center is to design a helical structure that has hydrophobic residues on one side and hydrophilic residues on the other side of the helix. Then four helices in the same or separate peptide(s) will spontaneously form a bundle in such a way that the interior is hydrophobic and the exterior is hydrophilic, thereby providing stability and water solubility (Robertson et al., 1994;

Degrado et al., 1999; Gibney and Dutton, 2001; Discher et al., 2003). Next, organic cofactors or transition metals can be incorporated in the interior of the helix bundle with a view to facilitating light-induced electron transfer. For example, heme maquettes have been constructed to study how the redox potential can be modulated to a considerable extent by altering the environment of the heme group (for a review see Gibney and Dutton, 2001).

Several studies have incorporated electron donors and acceptors into synthetic peptides with a view to generating light-induced electron transport. Some studies involved electron donors and acceptors at the exterior of the helix bundle; these are, therefore, located in a hydrophilic environment. For example, flash-induced electron transfer occurs from MV (reduced by triethanolamine) at one end of a three-helix bundle to Co(III) $(bipyridine)_3$ at the other end (Muntz et al., 1996); further, the rate coefficient of electron transfer increased with the helical content of the bundle, consistent with changes in donor-acceptor distances. Later, a long-range (24 Å) electron transfer from a Ru(II) complex (in a hydrophilic environment), across a non-covalent peptide-peptide interface, to a Ru(III) complex (also in a hydrophilic environment) with a rate coefficient of 380 s^{-1} was demonstrated (Kornilova et al., 2000). Other studies have involved donors and/or acceptors located in a hydrophobic environment. For example, a *de novo*-designed metalloprotein was constructed from a four-helix protein; it contained a ruthenium bipyridyl complex covalently bound at the hydrophilic side of one of the helices while a heme was ligated by two histidines in the hydrophobic interior of the four-helix bundle (Rau et al., 1998). Laser-induced electron transfer from Ru, through the helix carrying the Ru complex, to the heme was demonstrated (Rau et al., 1998).

Les Dutton's research group designed a photosynthetic reaction center as a dimer of two synthetic peptides, each of which is a helix-loop-helix structure (Rabanal et al., 1996). At the N-terminus of each synthetic helix-loop-helix peptide, and therefore in a hydrophilic environment, a coproporphyrin I was covalently bound. A Ru(II) porphyrin was incorporated into the heme-binding site (i.e., in a hydrophilic environment). A heme was bound close to the Ru(II) porphyrin to act as a secondary electron acceptor, thereby minimizing charge recombination. In this way, when two peptides assembled to form a dimer with a four-helix bundle, there was a pair of coproporphyrin I (which could be metallated with Cu(II) or Zn(II)) at the N-terminus, a pair of Ru(II) porphyrins in the interior of the helix bundle and a pair of hemes further along the interior of the helix bundle to help stabilize the charge separation. Razeghifard and Wydrzynski (2003) carried this design further by binding a zinc chlorin, Zn-Ce_6, to a histidine in the interior of such a four-helix bundle system (instead of a coproporphyrin I in a hydrophilic environment); they demonstrated light-induced electron transfer to quinones added in solution.

Yet a further step was to incorporate a quinone covalently into a four-helix bundle. This was achieved by Hay et al. (2004) by using a modified cytochrome b_{562} (a small, water-soluble, four-helix bundle system) as the protein base. Various quinones could be covalently attached to the protein via cysteine, each quinone acting as an analogue of the acceptor side of PS II. The heme-binding site of the cytochrome was occupied by a zinc chlorin, an analogue of P680 on the donor side of PS II. The authors were able to study light-induced electron transfer from the bound chlorin to the bound quinone molecule, both in a hydrophobic environment. For a closer bio-mimetic version of PS II, future studies may be extended to proteins that bind manganese. In this regard, a promising start has been made by DeGrado et al. (2003) who designed a dimer of helix-loop-helix motifs with a di-Mn(II) site near the center of the four-helix bundle. Further progress was achieved by Thielges et al. (2005) who modified proteins in the reaction center of *Rhodobacter sphaeroides* by mutagenesis; in this way they introduced a manganese-binding site that contains three carboxylate groups, a histidine residue and a bound water molecule. They further showed that the bound manganese complex had a dissociation constant of approximately 1 μM, and that manganese donated an electron to the oxidized bacteriochlorophyll dimer with a halftime of 12 ms. The above studies demonstrate that synthetic peptides and/or modified natural proteins offer a promising avenue for elucidation of effects of the protein environment on reaction-center function. In particular, the ability to bind (preferably more than one) manganese, and to modify the midpoint

redox potential of the primary electron donor in a reaction center, such as by the introduction of hydrogen bonds (Lin et al., 1994), will be an important tool for elucidating the mechanism of action of PS II and for eventual adaptation of a bio-mimetic reaction center that can split water into oxygen and hydrogen.

V. Carbon-Based End Products of Artificial Photosynthesis

A. Oils and Terpenes

As we have seen, photocurrent is one of the desirable products of artificial photosynthesis; hydrogen is another. What would others be? Sharkey (2005), in addressing this question, considered several criteria for choosing a carbon-based end product of artificial photosynthesis. One is that the compound should be relatively easy to make from a biochemical perspective, and should allow for continuous production; another is that the end-product should be a fuel. An oil that resembles soybean oil, for example, would be a useful end-product, since it can be used in place of diesel fuel, although requiring methylation by addition of methanol (Sharkey, 2005). It can, of course, also be used as a high-value food. Another useful product is the plant-derived polyhydroxybutyrate, which can be used as feedstock in the manufacture of plastics. However, the biochemical synthesis of both oils and polyhydroxybutyrate is relatively complex, and will require a great deal of research and development.

Plants make more isoprene (C_5H_8) than any other hydrocarbon (Guenther et al., 1995; Fuentes et al., 2000). Isoprene is made in the chloroplast with a surprisingly large capacity (Sharkey and Yeh, 2001). At present, it is produced industrially from petroleum for the manufacture of rubber and pharmaceuticals. Conveniently, isoprene is a liquid that boils at 32°C, so it is easily amenable to continuous production.

Related to isoprene are the terpenes, hydrocarbons made from precursors whose lengths are multiples of five carbon atoms. Less volatile than isoprene, terpenes are used in high-value cosmetic products in high purity. Required in less purity, terpenes are also used in rubber manufacture. Their potential as a fuel has yet to be explored.

B. Carbohydrates

On the face of it, artificial photosynthesis may not be a reasonable alternative to traditional agriculture for food production. There is a strong case, however, for artificial photosynthesis to eventually supplement food production via natural photosynthesis. Apart from the ever-dwindling suitable land, traditional agriculture requires a great deal of fresh water, a limited resource in many areas. Indeed, if one conservatively takes the ratio of water transpired to the carbon fixed in photosynthesis as 300 (it may be higher), and the energy released on complete oxidation of glucose as 2,879 kJ mol^{-1}, then one estimates that each person consuming metabolism energy at 120 W requires a large swimming pool of fresh water to grow plants to produce enough carbohydrates to live on for 1 year. Given that water stress is the greatest factor limiting crop yield, artificial photosynthesis may offer a form of "dry agriculture" with minimum water input.

Pace (2005) has offered an integrated view of how food production by artificial photosynthesis may be achieved. Solar energy is harvested at places that cannot be used for agriculture (e.g., a desert). Photovoltaic electricity is transmitted from such remote areas to a plant that houses bioreactors, in which ATP and NADPH are produced for the conversion of CO_2 to carbohydrates and other food products via a suite of ~20 enzymes (Fig. 24.3). This vision essentially separates light-harvesting and the biochemical reactions in space, such that the light-harvesting occurs over a large area, while the biochemistry takes place in an enclosed bio-reactor to conserve water, and under anaerobic conditions to suppress undesirable side reactions with oxygen. In addition, the photovoltaic electricity can be use for hydrogen production. This vision, therefore, involves the conversion of electricity into useful products such as food. Although a semi-artificial system for generating electricity from glucose has been devised (Park and Zeikus, 2000), the reverse of the biofuel cell, namely, the synthesis of carbohydrates from electricity, will be undoubtedly much more difficult.

In vitro food production requires ATP and NADPH. A bio-electrode could involve ATP synthase (denoted as ATPase in Fig. 24.4) complexes embedded in a lipid membrane tethered at an anode surface. Hydrogen is oxidized at the

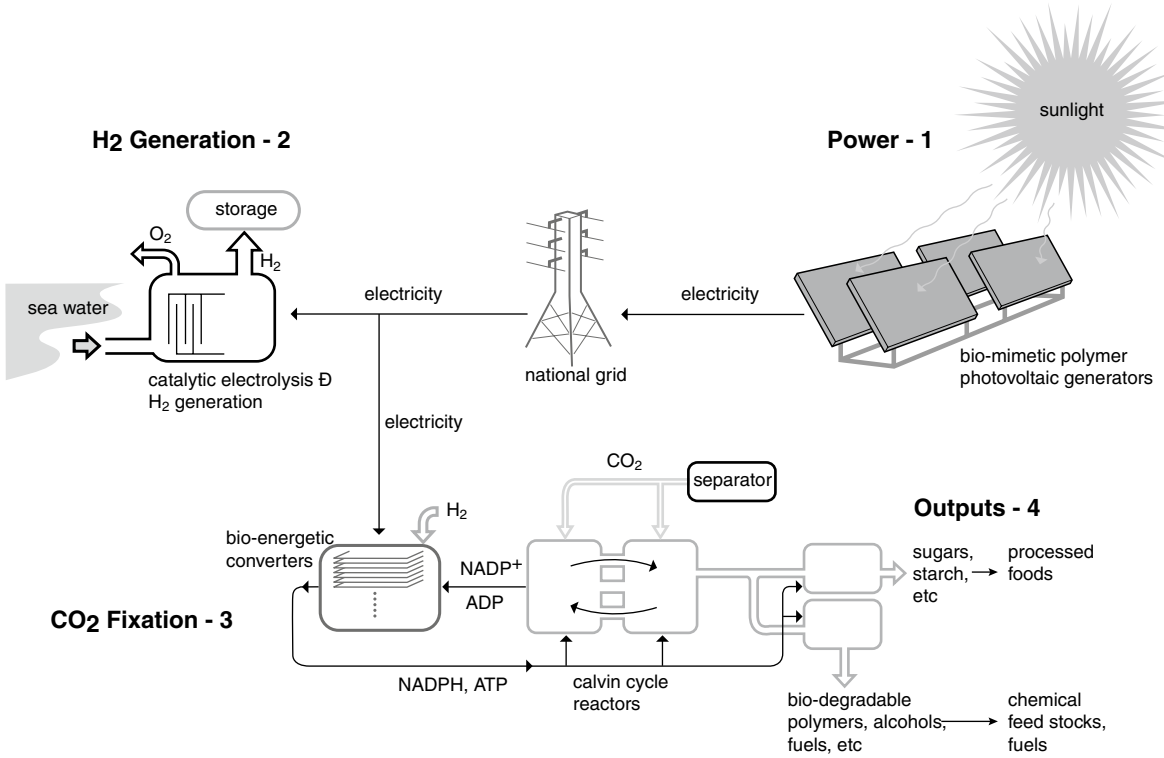

Fig. 24.3. An integrated artificial photosynthesis model. Bio-mimetic photovoltaic generators convert sunlight on, for example, otherwise unproductive land (Program 1). The electricity is fed to the national grid and transmitted to plants that generate either hydrogen (Program 2) or energy-storage compounds, ATP and NADPH, (Program 3) for use in bio-reactors that make useful end products (Program 4). Since the bio-reactor in Program 4 is a closed, anaerobic system, minimum water is required in this form of "dry agriculture", an important feature when fresh water is in short supply. Note that light-harvesting and utilization of light energy are spatially separated, allowing light absorption over a large area, while the "artificial thylakoids" (Program 3) and the "artificial stroma" (Program 4) are compact bio-reactors (Reproduced from Pace (2005). With permission from the publisher).

anode surface, releasing protons which drive the formation of ATP. At the cathode, protons are reduced to hydrogen by a natural or bio-mimetic hydrogenase (Hase). Also at the cathode, it is envisaged that a complex (Rh) such as the pentamethylcyclopentadienyl rhodium complex investigated by Hollmann et al. (2003) catalyzes the non-enzymatic reduction of $NADP^+$. The input to the bio-energetic electrode is ADP, inorganic phosphate and $NADP^+$, while the output is ATP and NADPH. The next stage is to use a Calvin-Benson cycle reactor to convert CO_2 into sugars, starch and other products with the help of ~20 enzymes. Such a process is by no means easy. For one thing, maintaining enzyme stability will be difficult, although anaerobic operation will help. Secondly, there is a need to separate the end products to avoid "end product inhibition". In this respect, starch may be more amenable to continuous production than most other products if the granules are big enough to be easily separated out. Thirdly, current rates of NADPH formation catalyzed by the pentamethylcyclopentadienyl rhodium (Hollmann et al., 2003) are too low to adequately match rates of carbon assimilation that will be required to occur. All these and many other problems pose a great challenge to future generations of researchers.

VI. Future Prospects

Natural oxygenic photosynthesis has been in existence for about half of the Earth's age. In contrast, artificial photosynthesis in all its various forms is in its infancy. Some of the subfields of artificial photosynthesis may take decades to produce practical outcomes. Probably,

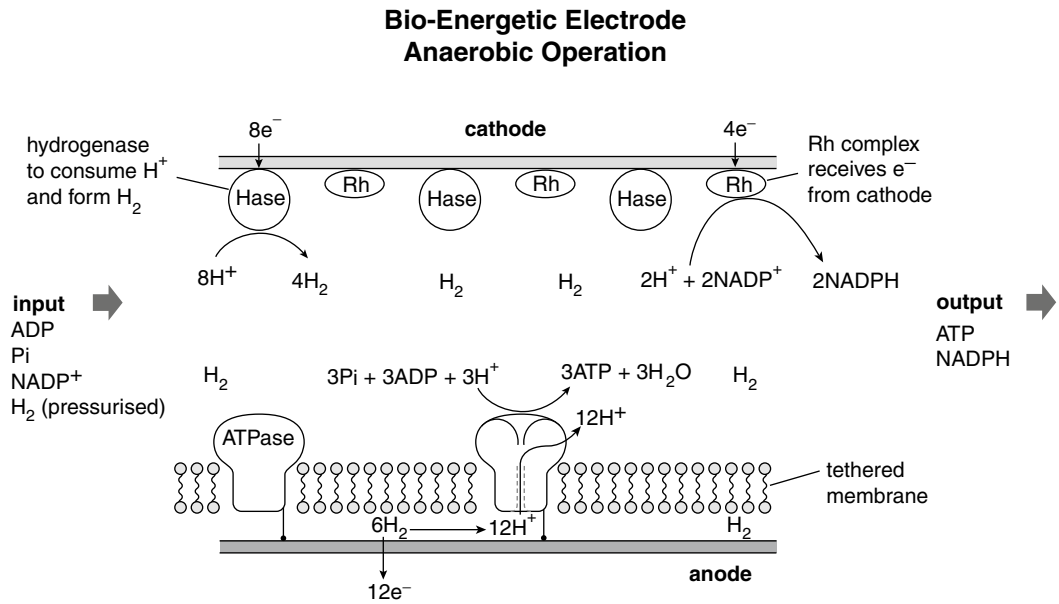

Fig. 24.4. A conceptual bio-energetic electrode (Program 3 in Fig. 24.3) for converting electricity into chemical energy stored in ATP and NADPH, compounds that are normally made via thylakoid function and are needed for a Calvin-Benson cycle bioreactor. Pressurised H_2 is oxidized at an anode, releasing protons which are confined by a tethered lipid membrane to efflux through an ATP synthase (denoted as ATPase). The protons are reduced at a cathode to H_2 at a hydrogenase (Hase) or used, together with $NADP^+$, for the formation of NADPH (Hollmann et al., 2003) catalyzed by a rhodium (Rh) complex (Modified from Pace, 2005).

the rate-limiting steps will be largely determined by research funding priorities. Because artificial photosynthesis is a relatively long-term goal, it is easy for funding agencies to neglect it in favor of other shorter-term objectives. Yet, one senses the urgency for humans to adopt a policy of sustainable living, since they will have to confront a future without cheap fossil fuels, with shortages of agricultural water, and with continual degradation of agricultural land. The driving force for artificial photosynthesis research may come from a necessity to feed an increasing world population, and to meet the increasing demand for renewable energy and fiber. Indeed, artificial photosynthesis, when carried out by a concerted effort from many interested parties and disciplines, may provide the opportunity that "combines scientific excellence and social relevance" (Lowe, 2005). For future generations of researchers, artificial photosynthesis will present a worthwhile challenge and an opportunity to exercise human ingenuity. In the words of R.N. Robertson (1992), it would be one facet of science that "can play a vital role, helping us to leave the world a better place for having lived".

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References

Abe R, Hara K, Sayama K, Domen K and Arakawa H (2000) Steady hydrogen evolution from water on Eosin Y-fixed TiO_2 photocatalyst using a silane-coupled reagent under visible light irradiation. J Photochem Photobiol A: Chem 137: 63–69

Abe R, Sayama K and Arakawa H (2004) Dye-sensitized photocatalysts for efficient hydrogen production from aqueous I^- solution under visible light irradiation. J Photochem Photobiol A: Chem 166: 115–122

Bahr JL, Kuciauskas D, Liddell PA, Moore AL, Moore TA and Gust D (2000) Driving force and electronic coupling effects on photoinduced electron transfer in a fullerene-based molecular triad. Photochem Photobiol 72: 598–611

Balaban TS (2005) Tailoring porphyrins and chlorins for self-assembly in biomimetic artificial antenna systems. Acc Chem Res 38: 612–623

Barber J (2009) Photosynthetic energy conversion: natural and artificial. Chem Soc Rev 38: 185–196

Beddard GS and Porter G (1976) Concentration quenching in chlorophyll. Nature 260: 366–367

Brimblecombe R, Swiegers GF, Dismukes GC and Spiccia L (2008) Sustained water oxidation photocatalysis by a bioinspired manganese cluster. Angew Chem 120: 7445–7448

Burdinski D, Wieghardt K and Steenken S (1999) Intramolecular electron transfer from Mn or ligand phenolate to photochemically generated Ru^{III} in multinuclear Ru/Mn complexes. Laser flash photolysis and EPR studies on photosystem II models. J Am Chem Soc 121: 10781–10787

Burdinski D, Bothe E and Wieghardt K (2000) Synthesis and characterization of tris(bipyridyl)ruthenium(II)-modified mono-, di-, and trinuclear manganese complexes as electron-transfer models for photosystem II. Inorg Chem 39: 105–116

Chaisson EJ (2005) Non-equilibrium thermodynamics in an energy-rich universe. In: Kleidon A and Lorenz RD (eds) Non-equilibrium Thermodynamics and the Production of Entropy: Life, Earth and Beyond, pp. 21–31, Springer, Berlin

Ciamician G (1912) The photochemistry of the future. Science 36: 385–394.

Collings AF and Critchley C (2005) Artificial Photosynthesis: From Basic Biology to Industrial Application. Wiley-VCH, Weinheim

Conlon B (2008) Designing photosystem II: molecular engineering of photo-catalytic proteins. Photosynth Res 98: 687–700

DeGrado WF, Summa CM, Pavone V, Nastri F and Lombardi A (1999) *De novo* design and structural characterisation of proteins and metalloproteins. Annu Rev Biochem 68: 779–819

DeGrado WF, Costanzo LD, Geremia S, Lombardi A, Pavone V and Randaccio L (2003) Sliding helix and change of coordination geometry in a model di-MnII protein. Angew Chem Int Ed 42: 417–420

Discher BM, Koder RL, Moser CC and Dutton PL (2003) Hydrophilic to amphiphilic design in redox protein maquettes. Curr Opin Chem Biol 7: 741–748

Fuentes JD, Lerdau M, Atkinson R, Baldocchi D, Botteneheim JW, Ciccioli P, Lamb B, Geron C, Gu L, Guenther A, Sharkey TD, and Stockwell W. (2000) Biogenic hydrocarbons in the atmospheric boundary layer: A review. Bull Amer Met Soc 81: 1537–1575

Fujishima A (2003) Comment on "Efficient photochemical water splitting by a chemically modified n-TiO_2" (I). Science 301: 1673a

Fujishima A and Honda K (1972) Electrochemical photophotolysis of water at a semiconductor electrode. Nature 238: 37–38

Fujishima A, Kohayakawa K and Honda K (1975) Hydrogen production under sunlight with an electrochemical photocell. J Electrochem Soc 122: 1487–1489

Ghirardi ML, Dubini A, Yu J and Maness P-C (2009) Photobiological hydrogen-producing systems. Chem Soc Rev 38: 52–61

Gibney BR and Dutton PL (2001) *De novo* design and synthesis of heme proteins. Adv Inorg Chem 51: 409–455

Grätzel M (1999) The artificial leaf: bio-mimetic photocatalysis. Cattech 3: 3–17

Grätzel M (2001) Photoelectrochemical cells. Nature 414: 338–344

Grätzel M (2003) Dye-sensitized solar cells. J Photochem Photobiol C: Photochem Rev 4: 145–153

Gray HB and Winkler JR (1996) Electron transfer in proteins. Annu Rev Biochem 65: 537–561

Green MA (2005) Very high-efficiency *in silico* photovoltaics. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 169–185. Wiley-VCH, Weinheim

Green MA, Basore PA, Chang N, Clugston D, Egan R, Evans R, Hogg, D, Jarnason S, Keevers M, Lasswell P, O'Sullivan J, Schuberg U, Turner A, Wenham SR and Young T (2004) Crystalline silicon on glass (CSG) thin-film solar cell modules. Solar Energy 77: 857–863

Guldi DM (2000) Fullerenes: three dimensional electron acceptor materials. Chem Commun 5: 321–327

Guenther A, Hewitt CN, Erickson D, Fall R, Geron C, Graedel T, Harley P, Klinger L, Lerdau M, McKay WA, Pierce T, Scholes B, Steinbrecher R, Tallamraju R, Taylor J and Zimmerman P (1995) A global model of natural volatile organic compound emissions. J Geophys Res 100: 8873–8892

Gust D, Moore TA, Moore AL, Liddell PA, Kuciauskas D, Sumida JP, Nash B and Nguyen D (1997) A carotene-porphyrin-fullerene triad: photoinduced charge separation and charge recombination to a triplet state. In: Kadish KM and Ruoff RS (eds) Recent Advances in the Chemistry and Physics of Fullerenes and Related Materials, Vol 4, pp. 9–24, The Electrochemical Society, Pennington, NJ

Hägglund C, Grätzel M and Kasemo B (2003) Comment on "Efficient photochemical water splitting by a chemically modified n-TiO_2" (II). Science 301: 1673b

Hambourger M, Moore GF, Kramer DM, Gust D, Moore A and Moore TA (2009) Biology and technology for photochemical fuel production. Chem Soc Rev 38: 25–35

Hammarström L (2003) Towards artificial photosynthesis: ruthenium-manganese chemistry mimicking photosystem II reactions. Curr Opin Chem Biol 7: 666–673

Hammarström L, Sun L, Åkermark B and Styring S (2001) A biomimetic approach to artificial photosynthesis: Ru(II)-polypyridine photo-sensitizers linked to tyrosine and manganese electron donors. Spectrochimica Acta 37: 2145–2160

Hankamer B, Lehr F, Rupprecht J, Mussgnug JH, Posten C and Kruse O (2007) Photosynthetic biomass and

H_2 production by green algae: from bioengineering to bioreactor scale-up. Physiol Plant 131: 10–21

Hara K, Tachbana Y, Ohga Y, Shinpo H, Suga S, Sayama K. Sugihara H and Arawaka H (2003a) Dye-sensitized nanocrystalline TiO_2 solar cells based on novel coumarin dyes. Solar Energy Materials and Solar Cells 77: 89–103

Hara K, Kurashige M, Ito S, Shinpo A, Suga S, Sayama K and Arawaka H (2003b) Novel polyene dyes for highly efficient dye-sensitized solar cells. Chem Commun 2: 252–253

Hasobe T, Kashiwagi Y, Absalom MA, Sly J, Hosomizu K, Crossley MJ, Imahori H, Kamat PV and Fukuzumi S (2004) Supramolecular photovoltaic cells using porphyrin dendrimers and fullerenes. Adv Mater 16: 975–979

Hay S, Wallace BB, Smith TA, Ghiggino KP and Wydrzynski T (2004) Protein engineering of cytochrome b_{562} for quinone binding and light-induced electron transfer. Proc Natl Acad Sci USA 101: 17675–17680

Hoffmann P (2001) Tomorrow's Energy: Hydrogen, Fuel Cells, and the Prospects for a Cleaner Planet. The MIT Press, Cambridge, Massachusetts

Hollmann F, Witholt B and Schmid A (2003) [Cp* Rh (bpy) $H_2O]^{2+}$: a versatile tool for efficient and non-enzymatic regeneration of nicotinamide and flavin coenzymes. J Mol Catalysts B: Enzymes 19–20: 167–176

Huang P, Magnuson A, Lomoth R, Abrahamson M, Tamm M, Sun L, Van Rotterdam B, Park J, Hammarström L, Åkermark B and Styring S (2002) Photo-induced oxidation of a dinuclear $Mn_2^{II,II}$ complex to the $Mn_2^{III,IV}$ state by inter- and intramolecular electron transfer to Ru^{III} *tris*-bipyridne. J Inorg Biochem 91: 159–172

Imahori H, Hagiwara K, Aoki M, Akiyama T, Taniguchi S, Okada T, Shirakawa M and Sakata Y (1996) J Am Chem Soc 118: 11771

Imahori H, Guldi DM, Tamaki K, Yoshida Y, Luo C, Sakata Y and Fukuzumi S (2001) J Am Chem Soc 123: 6617–6628

Imahori H, Mori Y and Matano Y (2003) Nanostructured artificial photosynthesis. J Photochem Photobiol C: Photochem Rev 4: 51–83

Kanan MW and Nocera DG (2008) In situ formation of an oxygen-evolving catalyst in neutral water containing phosphate and Co^{2+}. Science 321: 1072–1075

Khan SUM, Al-Shahry M and Ingler WB (2002) Efficient photochemical water splitting by a chemically modified n-TiO_2. Science 297: 2243–2245

Konduri R, Ye H, MacDonnell FM, Serroni S, Campagna S and Rajeshwar K (2002) Ruthenium photocatalysts capable of reversibly storing up to four electrons in a single acceptor ligand: a step closer to artificial photosynthesis. Angew Chem Int Ed 41: 3185–3187

Konishi T, Ikeda A and Shinkai S (2005) Supramolecular design of photocurrent devices using fullerenes aimed at modelling artificial photosynthesis. Tetrahedron 61: 4881–4899

Kornilova A, Wishart J, Xiao W, Lasey R, Fedorova A, Shin Y and Ogawa M (2000) Design and characterization of a synthetic electron-transport protein. J Am Chem Soc 122: 7999–8006

Kuciauskas D, Liddell PA, Lin S, Stone SG, Moore AL, Moore TA and Gust D (2000) Photoinduced electron transfer in carotenoporphyrin-fullerene triads: temperature and solvent effects. J Phys Chem 140: 4307–4321

Lackner KS (2003) Comment on "Efficient photochemical water splitting by a chemically modified n-TiO_2" (III). Science 301: 1673c

Lewis NS and Nocera DG (2006) Powering the planet: Chemical challenges in solar energy utilization. Proc Natl Acad Sci USA 103: 15729–15735

Lin X, Murchison HA, Nagarajan V, Parson WW, Allen JP and Williams JC (1994) Specific alterations of the oxidation potential of the electron donor in reaction centers from *Rhodobacter sphaeroides*. Proc Natl Acad Sci USA 91: 10265–10269

Lowe I (2005) Artificial Photosynthesis: social and political issues. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 3–12. Wiley-VCH, Weinheim

Magnuson A, Frapart Y, Abrahamsson M, Horner O, Åkermark B, Sun S, Girerd J-J, Hammarström L, and Styring S (1999) A biomimetic model system for the water oxidizing triad in photosystem II. J Am Chem Soc 121: 89–96

Marcus RA (1993) Electron transfer reactions in chemistry: Theory and experiment. Angew Chem Int Ed Engl 32: 1111–1121

Marcus RA and Sutin N (1985) Electron transfers in chemistry and biology. Biochim Biophys Acta 811: 265–322

Melis A (2007) Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). Planta 226: 1075–1086

Meredith P, Powell BJ, Riesz J, Vogel R, Blake D, Kartini I, Will G and Subiato S (2005) Broadband photo-harvesting biomolecules for photovoltaics. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 37–65. Wiley-VCH, Weinheim

Moore TA, Gust D, Mathis P, Mialocq, J-C, Chachaty C, Bensasson RV, Land EJ, Doizi D, Liddell PA, Lehman WR, Nemeth GA and Moore AL (1984) Photodriven charge-separation in a carotenoporphyrin-quinone triad. Nature 307: 630–632

Moore TA, Moore AL and Gust D (2002) The design and synthesis of artificial photosynthetic antennas, reaction centers and membranes. Phil Trans R Soc Lond B 357: 1481–1498

Muntz MW, McLendon GL, Wishart, JF, Gaillard ER and Corin AF (1996) Conformational dependence of electron transfer across *de novo* designed metalloproteins. Proc Natl Acad Sci USA 93: 9521–9526

Nazeeruddin MK, Kay A, Rodicio A, Humphery-Baker R, Müller E, Liska P, Vlachopoulos N and Grätzel M (1993) Conversion of light to electricity by *cis*-X_2Bis(2,2′-bipyridyl-4,4′-dicarboxylate)ruthenium(II) charge-transfer

sensitizers (X=CL$^-$, Br$^-$, I$^-$, CN$^-$, and SCN$^-$) J Am Chem Soc 115: 6382–6390

Nazeeruddin MK, Péchy P, Renouard T, Nazeeruddin SM, Humphery-Baker P, Comte P, Liska P, Cevey L, Costa E, Shklover V, Spiccia L, Deacon GB, Bignozzi CA and Grätzel M (2001) Engineering of efficient panchromatic sensitizers for nanocrystalline TiO_2-based solar cells. J Am Chem Soc 123: 1613–1624

Nocera DG (2009) Living healthy on a dying planet. Chem Soc Rev 38: 13–15

O'Regan and Grätzel M (1991) A low-cost, high-efficiency solar cell based on dye-sensitized colloidal TiO_2 films. Nature 253: 737–740

Pace RJ (2005) An integrated artificial photosynthesis model. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 13–34. Wiley-VCH, Weinheim

Park DH and Zeikus JG (2000) Electricity generation in microbial fuel cells using neutral red as an electronophore. Appl Environ Microbiol 66: 1292–1297

Rabanal F, Gibney BR, DeGrado WF, Moser CC and Dutton PL (1996) Engineering photosynthesis: synthetic redox proteins. Inorg Chim Acta 243: 213–218

Rabinowitch E and Govindjee (1969) Photosynthesis. John Wiley, New York

Rau HK, DeJonge N and Haehnel W (1998) Molecular synthesis of *de novo*-designed metalloproteins for light-induced electron transfer. Proc Natl Acad Sci USA 95: 11526–11531

Razeghifard R (2008) Artificial photoactive proteins. Photosynth Res 98: 677–685

Razeghifard R and Wydrzynski T (2003) Binding of Zn-chlorin to a synthetic four-helix bundle peptide through histine ligation. Biochem 42: 1024–1030

Razeghifard R and Wydrzynski T (2005) Protein-based artificial reaction centers. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 127–145. Wiley-VCH, Weinheim

Robertson DE, Farid RS, Moser CC, Urbauer JL, Mulholland SE, Pidikiti R, Lear JD, Wand AJ, DeGrado WF and Dutton PL (1994) Design and synthesis of multi-haem proteins. Nature 368: 425–431

Robertson RN (1992) A dilettante Australian plant physiologist. Annu Rev Plant Physiol Plant Mol Biol 43: 1–24

Sayama K, Mukasa K, Abe R, Abe Y and Arakawa H (2002) A new catalytic water splitting system under visible light irradiation mimicking a Z-scheme mechanism in photosynthesis. J Photochem Photobiol A: Chem 148: 71–77

Sharkey TD (2005) Carbon-based end products of artificial photosynthesis. In: Collings AF and Critchley C (eds) Artificial Photosynthesis: From Basic Biology to Industrial Application, pp. 283–289. Wiley-VCH, Weinheim

Sharkey TD and Yeh S (2001) Isoprene emission from plants. Annu Rev Plant Physiol Plant Mol Biol 52: 407–436

Steinberg-Yfrach G, Rigaud J-L, Duratini EN, Moore AL, Gust D and Moore TA (1988) Light-driven production of ATP catalyzed by FoF1-ATP synthase in an artificial photosynthesis membrane. Nature 392: 479–482

Sun L, Berglund H, Davydov R, Norrby T, Hammarström L, Korall P, Börje A, Philouze C, Berg K, Tran A, Andersson M, Stenhagen G, Mårtensson J, Almgren M, Styring S and Åkermark B (1997) Binuclear ruthenium-manganese complexes as simple artificial models for photosystem II in green plants. J Am Chem Soc 119: 6996–7004

Sun L, Burkitt M, Tamm M, Raymond MK, Abramhamsson M, LeGourriérec D, Frapart Y, Magnuson A, Kenéz PH, Brandt P, Tran A, Hammarström L, Styring S and Åkermark B (1999) Hydrogen-bond promoted intramolecular electron transfer to photogenerated Ru(III): A functional mimic of tyrosine$_z$ and histidine 190 in photosystem II. J Am Chem Soc 121: 6834–6842

Sun L, Raymond MK, Magnuson A, LeGourriérec D, Tamm M, Abramhamsson M, Kenéz PH, Mårtensson J, Stenhagen G, Hammarström L, Styring S and Åkermark B (2000) Towards an artificial model for photosystem II: a manganese(II.II) dimer covalently linked to ruthenium(II) tris-bipyridine via a tyrosine derivative. J Inorg Biochem 78: 15–22

Sun L, Hammarström L, Åkermark B and Styring S (2001) Towards artificial photosynthesis: ruthenium-manganese chemistry for energy production. Chem Soc Rev 30: 36–49

Thielges M, Uyeda G, Cámara-Artigas A, Kálmán L, Williams JC and Allen JP (2005) Design of a redox-linked active metal site: manganese bound to bacterial reaction centers at a site resembling that of Photosystem II. Biochemistry 44: 7389–7394

von Aichberger S (2004) Strong east wind: market survey on photovoltaic modules. Photon International, February 46–55

Wang P, Zakeeruddin SM, Moser JE, Nazeeruddin MK, Sekiguchi T and Grätzel M (2003) A stable quasi-solid-state dye-sensitized solar cell with an amphiphilic ruthenium sensitizer and polymer gel electrolyte. Nature Materials 2: 402–407

Wang X, Maeda K, Thomas A, Takanabe K, Xin G, Carlsson JM, Domen K, Antonietti M (2009) A metal-free polymeric photocatalyst for hydrogen production from water under visible light. Nature Materials 8: 76–80

Yamada H, Imahori H, Nishimura Y, Yamasaki I and Fukuzumi S (2002) Enhancement of photocurrent generation by ITO electrodes modified chemically with self-assembled monolayers of porphyrin-fullerene dyads. Adv Mater 14: 892–895

Part V

Carbon Assimilation, Sucrose Synthesis and Transport

Chapter 25

The Uptake of CO_2 by Cyanobacteria and Microalgae

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Summary

Cyanobacteria and eukaryotic algae possess a CO_2-concentrating mechanism (CCM), which involves the transport of inorganic carbon (C_i) driven by light energy and the fixation of CO_2 in the subcellular compartments (carboxysomes in cyanobacteria and pyrenoids in green algae) where most of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is confined. Physiological and molecular analysis identified five C_i uptake systems in cyanobacteria. Two of them are CO_2-uptake systems driven by the thylakoid membrane-located NAD(P)H dehydrogenase (NDH-1) complexes. Three bicarbonate transporters, BCT1 (an ABC-type transporter composed of Cmp proteins), SbtA and BicA, are localized on the cytoplasmic membranes. One of the main features of the CCMs is a marked rise in the ability to take up C_i observed when high-CO_2-grown cells are transferred to CO_2-limiting conditions in the light. Many low-CO_2 (LC)-inducible genes including those involved in C_i uptake have been identified in cyanobacteria and green algae. *Chlamydomonas reinhardtii* is a model eukaryotic alga and has been used extensively for the study of the CCM. Candidate genes responsible for C_i uptake in *C. reinhardtii,* and that encode proteins homologous to transporters in other organisms, were found among LC-inducible genes identified by DNA microarray analysis. These genes include *LciA* and *LciB*, whose transcripts are not accumulated in the *ccm1 (cia5)* mutant defective in C_i-transport upon exposure to LC. The CCM1 (CIA5) is essential for the control of CCM induction and the expression of CO_2-responsive genes through putative LC signal transduction pathways. We present recent studies on the mechanisms of CO_2-sensing and of induction of gene expression by LC. Other microalgae such as coccolithophorids, diatoms and dinoflagellates also possess CCMs. We summarize the present state of the art on the CCMs of these major aquatic primary producers and other CCM-related topics such as cycling of C_i, CO_2-mediated interspecies communication, stable carbon isotope fractionation and biotechnological implications.

I. Introduction

Cyanobacteria and eukaryotic algae possess a CO_2-concentrating mechanism (CCM) that enables them to cope with both the low affinity of their ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) to CO_2 and the competition between CO_2 and O_2 for the active sites of this enzyme. The cellular components involved in the operation of the CCM include those engaged in inorganic carbon (C_i) uptake and the subcellular compartments (carboxysomes in cyanobacteria and pyrenoids in green algae) where most of the Rubisco is confined. Both CO_2 and HCO_3^- are taken up and accumulated within the cells mostly as HCO_3^-. Conversion of the latter to CO_2 within the carboxysomes or pyrenoids is facilitated by carbonic anhydrase (CA) located in these bodies, while the CO_2 produced is fixed by Rubisco (cf. Fig. 25.1 showing a model of the CCM in cyanobacteria). Apparently, the cyanobacterial and algal CCMs have developed independently and, with the exception of CAs, the proteins involved in this process do not show significant homologies. One of the main features of the CCMs is a marked rise in the ability to take up C_i, observed when high-CO_2 (HC)-grown cells (grown under 1–5% CO_2 in air) are transferred to low-CO_2 (LC) conditions (CO_2 concentration as in air, or lower) in the light. Many LC-inducible genes have been identified in cyanobacteria and green algae. Significant progress was recently made in our understanding of the C_i-uptake systems and the mechanism involved in LC-induced gene expression in these organisms.

In this chapter we discuss recent progress in research on cyanobacterial and microalgal CCMs, focusing on C_i-uptake systems, the mechanism of acclimation to LC, and the ecological role of the CCM. For earlier research on molecular biology of cyanobacteria, see Bryant (1994) and for research on molecular biology of *C. reinhardtii,* see Rochaix et al. (1998).

Abbreviations: CA – Carbonic anhydrase; CCM – CO_2-Concentrating mechanism; C_i – Inorganic carbon; HC – High CO_2; HCR – High-CO_2-requring; LC – Low CO_2; NDH-1 – NAD(P)H dehydrogenase; PS – Photosystem; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase

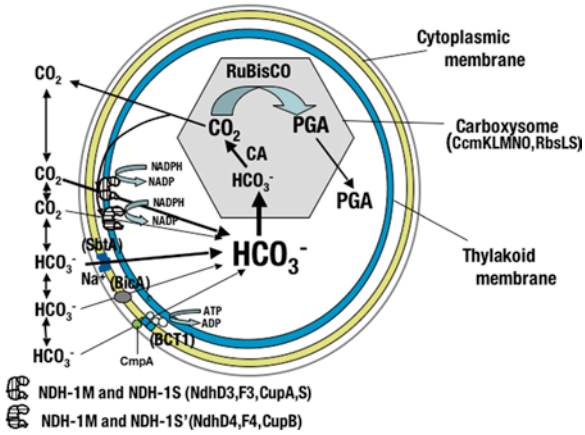

Fig. 25.1. A schematic model for the CCM in a *Synechocystis* sp. PCC 6803 cell. Two CO_2 uptake systems on the thylakoid membrane and three HCO_3^- transporters on the cytoplasmic membrane are shown. The *thick arrows* on the high affinity CO_2-uptake system and SbtA HCO_3^- transporter indicate their major contribution in carbon acquisition in *Synechocystis* sp. PCC 6803.

II. High-CO_2-Requiring Mutants

These mutants are often used as a major research tool. Mutants defective in their CCM require high concentrations of CO_2 for growth. Isolation and analysis of such mutants led to identification of the genes involved and clarification of many of the physiological processes leading to the efficient operation of the CCM. Since the first high CO_2-requiring (HCR) mutant, E1, was isolated from *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* 7942) over 25 years ago (Marcus et al., 1986), a number of HCR mutants have been isolated from this strain and from *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803). Many of the HCR mutants are impaired in the structure and/or function of the carboxysomes and will not be discussed in this Chapter (but see Marcus et al., 1986; Kaplan et al., 1988, 2008; Fukuzawa et al., 1992; Ogawa, 1993; Price et al., 1993; Kaplan and Reinhold, 1999; Badger and Spalding, 2000; Badger and Price, 2003; Badger et al., 2006; Price et al., 2008). Other HCR mutants are unable to accumulate C_i within the cells due to impaired uptake of C_i (see below). In these mutants, which do not possess a normal CCM, the HCR phenotype is due to very high apparent photosynthetic $K_{1/2}$ (C_i) in the range of 20–40 mM, as compared to the wild type where the $K_{1/2}$ (C_i) is typically 0.1 or 0.01 mM in HC- and LC-grown cells (Kaplan and Reinhold, 1999). Searching for HCR mutants also uncovered another mutant where the phenotype did not emerge from low apparent photosynthetic affinity but rather from the inability to produce purins (Schwarz et al., 1992) or to metabolize 2-phosphoglycolate (Eisenhut et al., 2008).

III. Inorganic Carbon Acquisition Systems in Cyanobacteria

The CO_2-uptake systems and bicarbonate transporters play a central role in the operation of the CCM. It is well established that CO_2 and HCO_3^- are actively taken up by separate, independent systems (Volokita et al., 1984; Espie et al., 1989; Miller et al., 1990, 1991; Badger et al., 1994). The presence of multiple C_i acquisition systems made it difficult to isolate mutants defective in these systems. In 1990 a break-through occurred in the molecular analysis of cyanobacterial CO_2-uptake systems with the isolation of *Synechocystis* 6803 mutants (RKa and RKb) defective in CO_2 uptake (Ogawa, 1990, 1991a, b). The first identification of a bicarbonate transporter followed the recognition of a 42 kDa protein in the cytoplasmic membrane of *Synechococcus* 7942 that was induced under low CO_2 (Omata and Ogawa, 1985, 1986). Cloning and analysis of the gene encoding this protein (*cmpA*) revealed its homology to genes encoding ABC-type transporters (Omata et al., 1990). Inactivation of *cmpA*, however, did not show an HCR phenotype because of the presence of multiple C_i acquisition systems and it took nearly 10 years before CmpA was demonstrated to be a substrate binding protein of the BCT1 bicarbonate transporter (see Fig. 25.1; Omata et al., 1999; Maeda et al., 2000). Genome sequencing of *Synechocystis* 6803 and other cyanobacterial strains opened a new era in the identification of genes involved in C_i acquisition.

In the following sections we briefly describe the identification of five C_i acquisition systems in cyanobacteria (see Fig. 25.1); two CO_2-uptake systems involving NAD(P)H dehydrogenase (NDH-1) complexes, NDH-1MS and NDH-1MS' on the thylakoid membranes (Shibata et al., 2001;

Maeda et al., 2002; Zhang et al., 2004, 2005; Folea et al., 2008; Xu et al., 2008b) and three bicarbonate transporters operated by SbtA, BCT1 and BicA on the cytoplasmic membranes (Omata et al., 1999; Shibata et al., 2002; Price et al., 2004; Xu et al., 2008a). The reader may also refer to earlier reviews (Ogawa and Kaplan, 2003; Giordano et al., 2005; Price et al., 2008; Kaplan et al., 2008).

A. Two CO_2 Uptake Systems Involving NAD(P)H Dehydrogenase Complexes

Mutants RKa and RKb, defective in CO_2 uptake, bear point mutations in *ndhB* and *ndhL* (renamed from *ictA*) encoding subunits of NDH-1 complexes (Ogawa, 1990, 1991a, b, 1992). A Δ*ndhB* mutant of *Synechococcus* 7942 also showed an HCR phenotype (Marco et al., 1993). Mutants M55 and M9 were constructed by inserting a kanamycin-resistance cassette into *ndhB* and *ndhL*, respectively. Studies on M55 revealed that NDH-1 is an essential component of cyclic electron transport (Mi et al., 1992, 1995). These results, together with the observation that CO_2 uptake is driven by Photosystem (PS) I, suggested that PS I-cyclic electron transport plays a role in CO_2 uptake. The observation that NDH-1 complexes are localized on the thylakoid membrane (Ohkawa et al., 2001; Xu et al., 2008b) supported the hypothesis that CO_2 enters the cells by diffusion followed by conversion to HCO_3^- by a thylakoid-located mechanism (Kaplan and Reinhold, 1999). This is consistent with the mass spectrometric observation that light-dependent ^{18}O exchange between CO_2 and water was strongly impaired in the RKa and RKb mutants (Ogawa, 1990). Tchernov et al. (2001) suggested that CO_2 is likely to diffuse into cells via water channels since application of a water-channel blocker strongly inhibited CO_2 uptake in *Synechococcus* 7942.

Cyanobacteria contain multiple copies of *ndhD* and *ndhF* whereas most of the other *ndh* genes are present as a single copy (Cyanobase; http://www. kazusa.or.jp/cyano/). The NdhD and NdhF polypeptides are both members of a larger family and may be related to an ancient gene duplication event. In *Synechocystis* 6803, at least four genes belong to the *ndhD* (*ndhD1-D4*) and three genes to the *ndhF* (*ndhF1, F3* and *F4*) families. In addition, two genes named *ndhD5* and *ndhD6* belong to the *ndhD/F* group (Shibata et al., 2001). The *ndhD1* and *ndhD4* genes are constitutively expressed in HC-grown cells whereas expression of *ndhD2* and *ndhD3* is induced under LC conditions (Ohkawa et al., 1998). Among the 4 *ndhD* genes, *ndhD1* and *ndhD3* are very similar to *ndhD2* and *ndhD4*, respectively. The double mutant, Δ*ndhD1/ndhD2*, was unable to grow under photoheterotrophic conditions, even though it took up CO_2 in the light and grew normally under air containing normal levels of CO_2. In contrast, the double mutant Δ*ndhD3/ndhD4* grew under photoheterotrophic conditions but was unable to take up CO_2 and could not grow photoautotrophically in the presence of air at pH 7.0 (Ohkawa et al., 2000). Thus it became evident that *Synechocystis* 6803 possesses two functionally distinct NDH-1 complexes, one essential to photoheterotrophic growth of cells and the other essential to CO_2 uptake. These studies led to the identification of two CO_2 uptake systems; a LC inducible system dependent on *ndhD3* and a constitutive system dependent on *ndhD4* (see Fig. 25.1; Shibata et al., 2001; Maeda et al., 2002). The constitutive system is characterized by a relatively low affinity and low V_{max} for CO_2 uptake, regardless of the CO_2 concentrations during growth. In contrast, the LC-inducible system is characterized by higher affinity to CO_2 and V_{max} typical of LC-grown cells (Shibata et al., 2001; Maeda et al., 2002).

Analysis of cyanobacterial membrane proteins revealed the presence of three complexes; NDH-1L (large size), NDH-1M (medium size) and NDH-1S (small size) (Herranen et al., 2004). The NDH-1M and NDH-1S are present as a larger complex, NDH-1MS (Zhang et al., 2005). Recently, single particle analysis of thylakoid proteins from *Thermosynechococcus elongatus* and *Synechocystis* 6803 solubilized by digitonin identified U-shaped NDH-1 complex in addition to L-shaped complexes (Folea et al., 2008). The U-shaped complexes were induced under LC and were absent in the *ΔcupA* mutant. It is evident that the NDH-1MS complex forms U-shaped structures with the CupA protein constituting one of the peripheral arms whereas the NDH-1L complex forms an L-shaped structure like the NDH-1 complex from *E. coli*. Figure 25.2 depicts the structure of NDH-1MS (a) and NDH-1L (b) observed by single particle electron microscopy

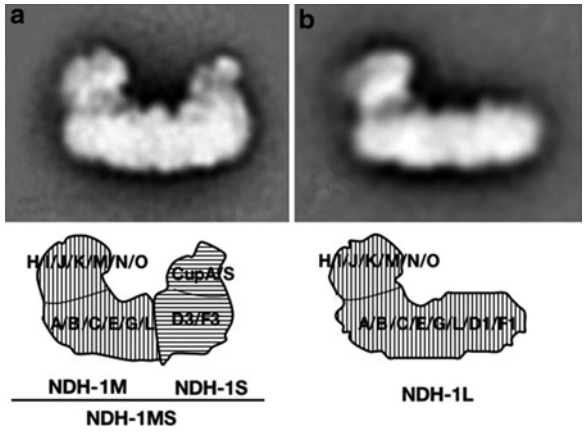

Fig. 25.2. Structure of NDH-1MS (**a**) and NDH-1L (**b**), as observed by single electron microscopy of the thylakoid membrane of *Thermosynechococcus elongatus* solubilized by digitonin. Models of these complexes showing the Ndh subunits are shown under the electronmicrographs taken from Folea et al. (2008).

(Folea et al., 2008), together with the structural models of these complexes. The NDH-1L complex contains NdhD1 and NdhF1 in addition to NdhA, B, C, E, G, H, I, J, K, L, M, N and O. All these subunits, except NdhD1 and NdhF1, were present in NDH-1M. The NDH-1S complex contains NdhD3, NdhF3, CupA and CupS (Prommeenate et al., 2004; Herranen et al. 2004; Zhang et al., 2004, 2005; Battchikova et al., 2005). The NDH-1L complex was absent in the Δ*ndhD1/D2* mutant while NDH-1S was missing in the Δ*ndhD3* and Δ*ndhD3/D4* mutants (Zhang et al., 2004). A complex (NDH-MS') containing CupB, NdhD4 and NdhF4 was recently identified in the thylakoid membrane of *Synechocystis* 6803 (Xu et al., 2008b). HC-grown cells contained predominantly NDH-1L while NDH-1M and NDH-1S were the major complexes in LC-adapted cells. The M55 mutant defective in *ndhB* did not contain either NDH-1L or NDH-1M complexes but, when grown in air at pH 8.3 and presumably relying on HCO_3^- uptake as a C_i source, it contained a large amount of the NDH-1S complex. However, since mutant M55 was unable to take up CO_2, it appears that NDH-1S is not functional in the absence of NDH-1M. Interestingly, mutant M9 defective in *ndhL* possessed NDH-1L, NDH-1M and NDH-1S but, for an unknown reason, these complexes are not functional in the absence of NdhL (Battchikova et al., 2005).

The reader may also refer to earlier reviews (Ogawa and Mi, 2007; Battchikova and Aro, 2007; Kaplan et al., 2008).

B. An ABC-Type HCO_3^- Transporter (BCT1)

The *cmpA* that encodes the LC-inducible 42 kDa protein (found in the cytoplasmic membrane of *Synechococcus* 7942, (Omata and Ogawa, 1985, 1986)) forms an operon with *cmpB, cmpC and cmpD* (Omata et al., 1990; Omata, 1992). Sequence data suggested that these genes encode subunits of an ABC-type transporter, CmpA being a substrate-binding subunit (see Fig. 25.1). When expressed constitutively in HC cells, where most of the other C_i-acquisition systems do not function (Omata et al., 1999), the $K_{1/2}$ (HCO_3^-) value for the BCT1 was 15 μM. The CmpA protein isolated after expression of *cmpA* in *Escherichia coli* possessed HCO_3^- binding capability (Maeda et al., 2000). Recently, the CmpA protein expressed in *E. coli* was crystalized and its structure was determined in the absence and presence of bicarbonate and carbonic acid (Koropatkin et al., 2007). CmpA binds carbonic acid and bicarbonate. Bicarbonate binding is accompanied by Ca^{2+}, which suggests that CmpA may co-transport bicarbonate and calcium or that calcium acts as a cofactor in bicarbonate transport.

C. A Sodium-Dependent HCO_3^- Transporter (SbtA)

Sodium ions are essential for HCO_3^- uptake by cyanobacteria (Reinhold et al., 1984; Miller et al., 1984; Espie and Kandasamy, 1994; So et al., 1998) and hence for their growth, particularly at alkaline pH values. A gene, *slr1512*, designated *sbtA* (sodium-bicarbonate transport) encodes a transporter involved in Na^+-dependent HCO_3^- uptake (Shibata et al., 2002). Inactivation of this gene in a Δ*ndhD3/ndhD4* mutant (impaired in CO_2 acquisition) abolished C_i uptake almost completely and the cells grew very slowly under air containing a normal level of CO_2. The SbtA-type HCO_3^- transporter was essential for growth at alkaline pH values in air when both CO_2-uptake systems were inactivated. It is possible that SbtA functions as a Na^+/HCO_3^- symport system, but the nature of the primary pump that provides the free energy for accumulation of HCO_3^- against its

electrochemical potential is not known. A sodium pump has not yet been identified in *Synechocystis* 6803 and $\Delta\mu H^+$ is too low to drive HCO_3^- accumulation, particularly at alkaline pH values (Kaplan and Reinhold, 1999).

D. SulP-Type Low Affinity HCO_3^- Transporter (BicA)

Inactivation of one of the *sul* genes annotated to encode low affinity sulfate transporters in *Synechococcus* sp. PCC 7002 (hereafter *Synechococcus* 7002), showed a lower rate of O_2 evolution at high HCO_3^- concentrations as compared with the wild type (Price et al., 2004). The gene, designated *bicA*, has homologues in other cyanobacterial strains. Expression of *bicA* of *Synechococcus* 7002, *Synechocystis* 6803 (*sll0834*) and *Synechococcus* sp. WH8102 (W1524) in HC-grown cells of *Synechococcus* 7942 resulted in a faster rate of O_2 evolution. Since BicA shows low affinity towards HCO_3^- but relatively high V_{max}, its role in C_i-limiting conditions is not known.

E. Role of IctB

Application of an inactivation library resulted in a *Synechococcus* 7942 mutant impaired in the ability to accumulate C_i within the cells (Bonfil et al., 1998). The mutation is within a gene encoding a transmembrane protein most likely involved in transport. Despite numerous attempts in three different laboratories, it was not possible to isolate a fully segregated mutant of *Synechocystis* 6803 where the *ictB* homologue, *slr1515*, was inactivated. The role of IctB, highly conserved in all cyanobacteria examined so far, is not known but expression in *Arabidopsis thaliana*, tobacco (Lieman-Hurwitz et al., 2003) and wheat resulted in transgenic plants that performed a higher photosynthetic rate under a limiting CO_2 level than did the respective wild types.

F. Phylogeny of the Inorganic Carbon Acquisition Systems in Cyanobacteria

The presence or absence of the C_i acquisition systems in various cyanobacterial strains were investigated by searching for the genes homologous to *ndhD3, ndhD4, ndhF3*, *ndhF4, cupA, cupB, cupS, sbtA*, *cmp* and *bicA* (see Fig. 12.2 in Kaplan et al. (2008) and Table 1 in Price et al. (2008)). This may provide a better understanding of C_i uptake mechanisms in phytoplankton species and of their phylogenetic relationship. Inducible and/or constitutive CO_2-uptake systems are present in all the cyanobacterial species except *Prochlorococcus* strains, for which genome sequences are available. The phylogenetic analysis of NdhD/NdhF proteins suggested an evolutionary relationship between cyanobacterial *ndhD1/ndhD2* and *ndhD* genes in chloroplast genomes and that the cyanobacterial *ndhF1* is related to the chloroplast *ndhF* (Shibata et al., 2001). Among the cyanobacterial strains of which genomes have been sequenced, BCT1 encoded by the *cmp* operon is present in all of the fresh water strains but is absent in the marine strains (marine *Synechococcus* and *Prochlorococcus* strains) except *Synechococcus* sp. WH 5701. Homologues of SbtA consisting of 370–374 amino acids have been identified in nine strains including *Synechococcus* sp. PCC 6301, *Synechococcus* 7002 and *Anabaena* sp. PCC 7120. Distant homologues of SbtA consisting of 324–339 amino acids are present in *Prochlorococcus* strains and several other strains (Shibata et al., 2002). The sequence similarity between the two types of SbtA is relatively weak but both types contain ten membrane-spanning domains that are structurally highly conserved. It is yet to be examined whether the distant homologues of SbtA function as a HCO_3^- transporter. The BicA protein is most widely distributed. All of the cyanobacterial strains for which genome sequences are available, except for *G, violaceus*, possess this type of HCO_3^- transporter.

G. Energization of CO_2 Uptake and HCO_3^- Transport

Both CO_2 uptake and HCO_3^- transport are energized by light. Action spectra for CO_2 uptake suggested that it is energized by PS I (Ogawa and Ogren, 1985; Ogawa et al., 1985). This is supported by physiological and proteomic analyses showing that the NDH-1M complex involved in CO_2 uptake is essential to PS I cyclic electron transport (Mi et al., 1992, 1995; Zhang et al., 2004). Use of specific electron transport inhibitors and acceptors also suggested that CO_2 uptake

in *Synechococcus* UTEX 625 is supported by cyclic electron flow and that, in contrast, HCO_3^- transport depends on linear electron flow (Li and Canvin, 1998). In both *Synechocystis* 6803 and *Synechococcus* 7942, CO_2 uptake was severely inhibited by DCMU but the inhibition was alleviated by duroquinone (Tchernov et al., 2001). It is possible that the CO_2 uptake system is driven by PS I cyclic electron transport but also requires electron donation from PS II. Conversion of CO_2 to HCO_3^- in a light-dependent reaction, involving CA-like activity, lowers the level of CO_2 in the cytoplasm and provides the concentration gradient for inward diffusion of CO_2. A major problem here is that the HCO_3^- produced by this "converter" must leave the complex, energetically uphill, in an environment where its concentration is high. Involvement of CA would tend to push the reaction in the opposite direction (i.e., towards CO_2 formation) as it does upon darkening. It was proposed that the conversion may occur in alkaline domains associated with proton pumping from the stromal side into the thylakoid lumen, thereby pushing the conversion in the direction of HCO_3^- formation (Kaplan and Reinhold, 1999), but the mechanism involved is not clear. The possibility that the NDH-1M and NDH-1S complexes are involved in hydration of the CO_2 is yet to be established experimentally. Both cyclic and linear electron transport lead to the formation of H^+ across the thylakoid membrane, essential for the formation of ATP. This energizes the ABC-type HCO_3^- transporter directly and may also fuel the SbtA-dependent HCO_3^- transport and BicA-dependent HCO_3^- transport indirectly.

The uptake of CO_2 and HCO_3^- and leakage of these C_i species occur during the operation of the CCM (see Fig. 25.1; Tchernov et al., 1997, 2003; Kaplan et al., 2008). This carbon cycling maintains an ambient CO_2 concentration substantially above or below that expected at chemical equilibrium with the prevailing HCO_3^- concentration in the medium (Tchernov et al., 1997, 2003). Electron transport activity of the *Synechocystis* 6803 mutants lacking all or four of the five C_i uptake systems was strongly inhibited under high light intensities (Xu et al., 2008b), which supported the hypothesis that the cycling of C_i species may play an important role in protecting the cells from high light stress by dissipating excess light energy (Kaplan et al., 2008).

H. Low CO_2-Induced Expression of Genes Involved in Inorganic Carbon Acquisition Systems

The NdhD3-dependent CO_2 uptake system and HCO_3^- transporters dependent on SbtA and BCT1 are induced under LC conditions (Shibata et al., 2001, 2002; Maeda et al., 2002; Omata et al., 2001). The BicA HCO_3^- transporter is also induced under LC in *Synechococcus* 7002 (Price et al., 2004;Woodger et al., 2007). The induction occurs only in the light (McGinn et al., 2003; Mackenzie et al., 2004) and is correlated with the size of the internal C_i pool and involves oxygen (Woodger et al., 2005). Genes encoding transcriptional regulators involved in the expression of the *ndhF3-ndhD3-cupA* operon, *sbtA* and *cmpA* have been identified. The NdhR polypeptide is a LysR family protein and is encoded by *sll1594* in *Synechocystis* 6803. Inactivation of *sll1594* resulted in constitutive expression of the *ndhF3-ndhD3-cupA* operon (Figge et al., 2001) and of *sbtA* (Wang et al., 2004). Transcriptional derepression was observed on *sbtA*, *bicA* and *porB* (a homologue of *slr0042* encoding a putative porin) in *ΔndhR* of *Synechococcus* 7002 but not on the *ndhF3-ndhD3-cupA* operon in this strain (Woodger et al., 2007). However, there are many indications that other transcription factors are also involved in the regulation of these genes. Gel mobility shifts identified LexA and two AbrB-like proteins Sll0359 and Sll0822 that bind to a fragment of the *sbtA* promoter in *Synecocystis* 6803 cells maintained under HC. When a similar extract was prepared from cells grown under LC, Sll0822 did not bind to the *sbtA* promoter despite its presence in these cells. The *sbtA*, *ndhF3*, and *cmpA* genes normally expressed only under LC in wild type are expressed under both HC and LC in a Δ*sll0822* mutant. As a result, the mutant does show LC characteristics like high apparent photosynthetic affinity to external C_i when grown under HC, almost similar to those observed in LC cells (Lieman-Hurwitz et al., 2009). These data implicated Sll0822 as a repressor of LC-induced genes in this organism. It also suggested that, in *Synechocystis* 6803, raising the transcript abundance of genes involved in C_i uptake suffice to modify the photosynthetic characteristics. This may not be the case in other organisms such as *Synechococcus* 7942 where, unlike *Synechocystis* 6803, a large rise

in the abundance of *ccm* gene transcripts and of the carboxysomes is observed after exposure of HC-grown cells to LC.

Expression of the LC-induced *cmp* operon is not controlled by NdhR but is abolished by deletion of *sll0030* encoding a homologue of CbbR, designated CmpR, a LysR family transcriptional regulator (Omata et al., 2001). Recently, Nishimura et al. (2008) demonstrated that 2-phosphoglycolate, which is produced by oxygenation of ribulose 1,5-bisphosphate by Rubisco under CO_2-limited conditions, acts as the co-inducer in the activation of the *cmp* operon by CmpR. Expression of the *cmp* operon is also induced by high-light conditions and *cmpR* is required for this induction. Thus a common mechanism is involved in high-light-responsive and LC-responsive regulation. Expression of high-light-inducible *psbAII* and *psbAIII* is also induced by LC conditions and *cmpR* served as a trans-acting factor that enhances the transcription of these PS II genes under high-light conditions (Takahashi et al., 2004). However, in contrast to *psbAII*, which is induced even under HC /high light and is under redox control (Sippola and Aro, 1999), the LC-inducible CCM genes are not induced under these conditions and are apparently independent of redox control (Woodger et al., 2003). A recent study by Zhang et al. (2007) indicated the requirement of FtsH2 (Slr0228) protease in LC induction. The inducible CCM and the transcripts of their genes and regulator *ndhR* failed to accumulate upon shift of the FtsH2-deletion mutant cells from HC to LC, indicating that the regulation by the FtsH2 protease is upstream of NdhR. There appears to be a complex network of gene regulation in cyanobacteria but the master genes and the transcription regulation cascades are poorly understood.

IV. The CO_2-Concentrating Mechanism in *Chlamydomonas reinhardtii*

A model eukaryotic alga, *Chlamydomonas reinhardtii*, has been used extensively for the study of physiological and molecular aspects of the CCM. In fact, the light-dependent formation of the C_i pool caused by CCM was first discovered in this organism (Badger et al., 1980). Although the $K_m(CO_2)$ value of *C. reinhardtii* Rubisco was approximately 20 μM, apparent $K_{0.5}(CO_2)$ of the acclimated cells against CO_2-limiting stress was 2–3 μM (Jordan and Ogren, 1981). Recent studies have focused on the identification and regulation of genes expressed in response to LC conditions. Isolation of HCR mutants and LC-inducible genes has revealed several components of CCMs in this organism. The most powerful tool was comparison of gene expression profiles of HC-grown cells with those in cells grown under LC conditions. Use of a cDNA array containing 10,368 ESTs identified 51 LC-inducible genes and 32 genes repressed by LC including various classes of genes possibly encoding proteins involved in CO_2 uptake (Miura et al., 2004). Induction of the CCM is not solely dependent on absolute environmental C_i concentrations but is also affected by light intensity. The induction of several CCM-associated genes was strongly affected by high light as well as CO_2 concentrations. Novel candidates for C_i transporters and CO_2-responsive regulatory factors whose expression levels were significantly increased during the induction of the CCM were collected using cDNA array expression analyses (Yamano et al., 2008). Using the genome sequence data of this model organism (Merchant et al., 2007), several new genes related with CCM have been nominated. The identification of the isoenzmes and functions of CAs in CCM have been reviewed elsewhere (Moroney and Ynalvez, 2007).

A. Genes Responsible for C_i Uptake

Among the 51 LC-inducible genes, a number of genes have been recognized as candidates responsible for C_i uptake. Among them, *Ccp1* and *Ccp2* encode the chloroplast envelope protein LIP-36 (Chen et al., 1997) that has six transmembrane domains and shows sequence similarity with mitochondrial-carrier proteins. Knocking down the expression of both *Ccp* genes leads to HCR phenotypes, suggesting the significance of the LIP-36 proteins (Pollock et al., 2004). Other candidates include *Mcp* and *Lci21* (148a10) that encode proteins homologous to mitochondrial carrier proteins and the chromate-resistance protein, respectively. Both *LciA* and *LciB* are strongly expressed under LC. *LciA* encodes a polypeptide of 336 amino acid residues with significant similarities with the chloroplast nitrite transporter NAR1 in *C. reinhardtii* (CrNAR1;1) (Rexach et al., 1999; Miura et al., 2004) and the formate transporter, *FdhC*, from *Methanothermobacter*

thermautotrophicus. In contrast to *Nar1*, the expression of which was not affected by the change in CO_2 levels nor by a mutation in *Ccm1* (see below), the expression of *LciA* was induced under LC conditions and was completely abolished in the *ccm1* mutant. Motif analysis predicted that the mature LCIA is localized in the thylakoid or chloroplast envelope membranes and has six transmembrane domains. These results strongly suggest that LCIA functions under LC-stress conditions but not for nitrite transport. Expression of LCIA protein in the *Xenopus* oocyte revealed that LCIA facilitates HCO_3^- uptake as well as nitrate (Mariscal et al., 2006). Indeed, simultaneous knockdown of *LciA* expression along with *Hla3* (see below) by RNAi exhibited dramatically decreased cell growth, photosynthesis, and C_i uptake at alkaline conditions (Duanmu et al., 2009a). The functional importance of the LCIA in CCM is to be evaluated by determining its substrate specificity for transport.

The HCR mutant, *pmp1*, carrying a defect in the *LciB* gene corresponding to a nonsense stop codon in its coding region, and its allelic mutant *ad1,* containing a long deletion of the genomic region including the *LciB* gene, were defective in growth and C_i-accumulation (Spalding et al., 1983; Wang and Spalding, 2006). LCIB is localized in the vicinity of the pyrenoid under LC conditions in the presence of light (Yamano et al., 2010). LCIB plays some role to trap CO_2 leaking from the pyrenoid matrix and to recycle CO_2 in the chloroplast (Duanmu et al. 2009b; Yamano et al., 2010). The *Mrp1* (=*Hla3*) gene encoding a 30 kDa subunit of an ABC transporter on the chloroplast membrane, the expression of which is induced by lowering CO_2 (Im and Grossman, 2002) and is dependent on *Ccm1*, was not expressed in this mutant even under LC conditions. The substrate of the Mrp1 is yet to be identified by biochemical analysis. Additionally, *Cah3* encoding a CA isozyme located in the thylakoid lumen is essential to maintain the level of carbon fixation by Rubisco (Karlsson et al., 1998). Moreover, at least two other genes, *Cah1* and *Mca*, encoding periplasmic and mitochondrial CAs, respectively, are specifically induced under LC conditions. Although several models were proposed (Eriksson et al., 1996; Giordano et al., 2003), it is not known how these CA isozymes function. For example, a deletion mutant lacking *Cah1* was hardly impaired in C_i uptake or acclimation to LC (Van and Spalding, 1999).

Lci1 encoding a plasma membrane protein is strongly induced under LC conditions (Burow et al., 1996; Yoshioka et al., 2004, Ohnishi et al., 2010). When the *Lci1* is artificially over-expressed under HC conditions, the cells show an increase in light-dependent CO_2 exchange activity, internal C_i accumulation, and photosynthetic affinity for C_i, suggesting that LCI1 contributes to the CCM as a component of the C_i transport machinery in the plasma membrane (Ohnishi et al., 2010)

B. Photorespiration Related Genes

Seven of the LC-inducible genes are involved in photorespiration. This includes *Aat1* encoding an alanine-α-ketoglutarate aminotransferase (Chen et al., 1996), *Pgp1* for a phosphoglycolate phosphatase (Suzuki et al., 1990; Mamedov et al., 2001), *Sgat* encoding serine-glyoxylate aminotransferase, *Fhs* encoding formyltetrahydrofolate synthetase, *Gdh1* encoding glycolate dehydrogenase (Nakamura et al., 2005), *Shmt* encoding serine hydroxymethyltransferase and *GscP* encoding a glycine cleavage system P-protein (Miura et al., 2004). While it is expected that lowering the level of CO_2 will enhance photorespiration, the role, if any, of this process and of these genes in the acclimation to low levels of CO_2 is yet to be established.

C. Genes Involved in Starch Biosynthesis

Two genes, *Sta2* and *Sta3* involved in starch biosynthesis, were induced under LC conditions. The *Sta2* gene, encoding a granule-bound starch synthase I, and the *Sta3* gene, which codes for a soluble starch synthase (ADP-glucose: α-1, 4-D-glucan-4-α-D-glucosyltransferase), play an important role in starch elongation (Ball, 1998). Another LC-inducible gene, *Lci8*, encodes a protein containing the starch-binding domain and is assumed to be involved in starch metabolism (Miura et al., 2004; Yamano et al., 2008). These starch-related genes may function in the development of the starch layer surrounding the pyrenoid particularly in cells acclimated to LC (Ramazanov et al., 1995; Fukuzawa et al., 2001). The physiological role of this starch layer surrounding the pyrenoid protein body is not known but it may help to lower the leak of CO_2 formed in close vicinity to Rubisco within these bodies (Morita et al., 2000).

D. Mutants Impaired in Acclimation to Changing Levels of CO_2

Mutants impaired in acclimation to changing levels of CO_2 are desired for the study of the physiological and molecular processes involved in this process and for the identification of the relevant genes. Three such mutants are already available including the *ccm1* mutant C16 (Fukuzawa et al., 2001), *cia5* (Xiang et al., 2001) and *lcr1* (Yoshioka et al., 2004). These mutants require high levels of CO_2 for growth but the *lcr1* mutant exhibited a less stringent phenotype than the other two mutants.

E. The Mechanisms of CO_2-Sensing and Low CO_2-Induced Gene Expression

The HCR mutant C16 shows a pleiotropic phenotype including the inability to induce pyrenoid-associated starch synthesis and the 51 LC-dependent genes. The relevant gene, *Ccm1*, tagged in the mutant C16, encodes a zinc-finger protein of 699 amino acid residues (Fukuzawa et al., 2001). The same gene, *Cia5*, was cloned independently with the aid of the *cia5* mutant (Xiang et al., 2001). The *Ccm1* gene is essential for the control of CCM induction and the expression of CO_2-responsive genes, including the LC-inducible *Cah1* (Fukuzawa et al., 1990) and HC-inducible Rh1, which is assumed to be a CO_2 gas channel (Soupene et al., 2004). The fact that the induction of almost all the LC-inducible genes was impaired in the *ccm1* mutant indicated that CCM1 is a nuclear-localized master regulator of CCM through putative LC signal transduction pathways. The CCM1 has two zinc-binding domains with several conserved Cys and His residues in its N-terminal region. Binding of zinc atoms to these domains in the CCM1 was essential for regulation of LC inducible genes. In the case of the site-directed mutant proteins, H54Y, C77V and C80V, the zinc-binding ability was lost. Size exclusion chromatography followed by immunoblot analyses indicated that CCM1 is present as a protein complex with approximately 290–580 kDa independent of C_i availability (Kohinata et al., 2008).

Since the expression of *Cah1* encoding a periplasmic CA is induced under LC conditions in light but is repressed under HC conditions, the upstream region of *Cah1*was dissected into two regulatory regions, a silencer for HC repression and an enhancer for LC induction (Kucho et al., 1999). An enhancer element consensus (EEC), GANTTNC, which is essential for CO_2-responsive transcriptional activation, was identified in the region upstream of *Cah1* (Kucho et al., 2003). A regulatory mutant named *lcr1* (low-CO_2 stress response) lacking the LC induction of *Cah1* was isolated using a promoter/reporter screening system (i.e., the arylsulfatase gene driven by the *Cah1* promoter). The corresponding gene encodes a soluble protein of 602 amino acid residues bearing a single Myb domain in its N-terminal region, which binds to the upstream region of *Cah1* (Yoshioka et al., 2004). The LCR1 polypeptide binds to the upstream region of *Cah1* and presumably functions to regulate CCM-activity and LC-inducible expression of *Cah1* (Yoshioka et al., 2004). In the *lcr1* null mutant, the expression of *Cah1* was transiently induced under LC but the level of *Cah1* accumulation was not so significant in wild-type cells. Expression of *Lcr1* was induced by lowering CO_2 levels and controlled by the regulatory factor CCM1. These results indicate that LCR1 is not involved in the initial induction under LC but in the amplification and maintenance of the level of *Cah1* mRNA in response to CO_2-limiting stress. DNA array and northern analyses showed that the mRNA levels of three genes, *Cah1*, *Lci1* and *Lci6* (023e06), were impaired in this *lcr1* mutant. Since the photosynthetic affinity for C_i exhibited by the *lcr1* mutant was lower than that of the wild-type cells, *Lci1* encoding a protein with four membrane-spanning domains (Burow et al., 1996) or *Lci6* coding for a soluble protein may encode one of the C_i transporters. Taken together, the data suggested that LCR1 transmits the LC signal from CCM1 to at least three CO_2-responsive genes, *Cah1*, *Lci1* and *Lci6*. Moreover, a LC-inducible protein LCI5, whose gene has been identified by differential hybridization (Burow et al., 1996), was specifically phosphorylated after 2 h exposure to CO_2-limiting stress (Turkina et al., 2006). The phosphorylation sites of LCI5 were at the four serine and three threonine residues. Immunoblot analyses using LCI5-specific antibodies revealed that LIC5 is localized in the chloroplast and confined to the stromal side of the thylakoid membranes. These findings suggest that CO_2-limiting stress causes not only induction of specific gene expression but also protein modification through unknown CO_2-sensing mechanisms. Further understanding of the regulatory

network that confers responsiveness to the ambient CO_2 level would have to rest on the isolation and further analyses of additional regulatory mutants.

V. The CO_2-Concentrating Mechanism of Other Microalgae: Ecological Role and Possible Biotechnological Application

Our present understanding of the CCM in aquatic photosynthetic microorganisms (Raven, 1991, 1997, 2003; Johnston et al., 1992; Kaplan and Reinhold, 1999; Badger and Spalding, 2000; Colman et al., 2002; Ogawa and Kaplan, 2003; Beardall and Raven, 2004; Giordano et al., 2005; Kaplan et al., 2008; Price et al., 2008), introduced in the earlier paragraphs, relies mostly on studies on several model laboratory organisms. There is a growing body of evidence, however, that with the exception of certain organisms (Raven et al., 2005), CCMs are widely distributed among photosynthetic microorganisms belonging to different families. The reader is referred to the reviews by Raven and colleagues (Giordano et al., 2005; Raven et al., 2008) for a more comprehensive discussion of the mode of CCM operating in various algal groups and proper citations. It is widely accepted that by raising the concentration of CO_2 in close proximity to Rubisco, the CCM activates Rubisco (Schwarz et al., 1995), stimulates the rate of CO_2 fixation and inhibits photorespiration (Kaplan and Reinhold, 1999; Giordano et al., 2005). However, recent studies showed accumulation of glycolate in *Synecocystis* 6803 even under 1% CO_2 clearly indicated that Rubisco is not CO_2 saturated (Eisenhut et al., 2006). Nevertheless, there are many open questions regarding the ecological significance of CCMs, their mode of operation in divergent aquatic organisms and on the evolution of the CCM.

A. Does CO_2 Availability Under Natural Conditions Rate-Limit Photosynthesis and Growth of Microalgae?

Due to the low affinity of Rubisco from various phytoplankton for CO_2 [$K_m(CO_2)$ = 20–170 μM] as compared with the ambient oceanic CO_2 concentrations of about 5–25 μM (strongly affected by the temperature), the diffusive flux of CO_2 is not large enough to saturate photosynthetic CO_2 fixation. It is not known whether, despite the potential to express CCMs, organisms inhabiting the water bodies are rate-limited by CO_2 availability at equilibrium with the atmospheric level. Studies on this question gained momentum from the current interest in the effect of rising atmospheric CO_2 concentrations on the composition, dynamics and productivity of phytoplankton (Falkowski et al., 2000). Physiological analyses of certain phytoplankton species, performed under laboratory conditions, suggested that CO_2 availability is likely to affect the biological carbon pumps, biological calcification and organic carbon assimilation. Thus the key concept is that changes in CO_2 availability are likely to affect global carbon cycling (Engel et al., 2005).

To examine the possibility that CO_2 concentration limits productivity, we must first clarify the nature of the C_i species taken from the medium. Most of the C_i in the marine environment (and alkaline fresh water lakes) is in the form of HCO_3^- (Falkowski and Raven, 1997) but CO_2 is the substrate for the carboxylation reaction by Rubisco, the universal CO_2 fixing enzyme. The nature of the C_i species utilized from the medium by various phytoplankton species has been extensively studied (Coleman, 1991; Johnston, 1991; Matsuda and Colman, 1995; Raven, 1997; Burkhardt et al., 2001; Colman et al., 2002; Tortell and Morel, 2002; Rost et al., 2003). The ability to utilize HCO_3^- directly from the water and adjust its transport capability to changing demand, particularly during bloom conditions where CO_2 concentration may decline, strongly affects the availability of C_i for assimilation (Cassar et al., 2004). Many of the microalgae examined to date are able to utilize various proportions of HCO_3^-/CO_2 from the medium and, in many cases, this ratio increases following a reduction of the CO_2 concentration experienced during growth. This is mainly due to a larger rise in HCO_3^- transport capability than of CO_2 uptake in cells acclimating to limiting CO_2 levels (Kaplan and Reinhold, 1999; Giordano et al., 2005; Raven et al., 2008). Here the methodologies used to assess CO_2 and HCO_3^- uptake are of immense importance; in some cases it may lead to erroneous kinetic parameters followed by wrong interpretation of the data (discussed in Kaplan and Reinhold, 1999). Higher photosynthetic rates than could be accounted for by the

theoretical rate of CO_2 supply by the uncatalyzed dehydration of HCO_3^- (Raven, 1997), and data from pH shift experiments (Maberly, 1990), have been regarded as evidence for direct HCO_3^- utilization. Where the estimated CO_2 supply appears sufficient, e.g., under low cell densities, HCO_3^- utilization may nevertheless occur. It may take the form of enhanced production of CO_2 in the periplasmic space either by acidification or by induced or activated CA, followed by CO_2 diffusion into the cells without a direct uptake of HCO_3^-. Net CO_2 efflux, which may occur during net photosynthesis in certain cyanobacteria (Salon et al., 1996; Tchernov et al., 1997) and eukaryotes (Sukenik et al., 1997; Huertas et al., 2000), provides strong evidence for direct HCO_3^- utilization from the medium. Application of compounds believed to specifically inhibit CO_2 uptake, e.g., COS, Na_2S and ethoxyzolamide (Salon et al., 1996; Tyrrell et al., 1996) and Na^+-deprivation (which minimizes HCO_3^- uptake by cyanobacteria (Reinhold et al., 1984; Salon et al., 1996)) were used to study CO_2 uptake. The distinction between CO_2 and HCO_3^- uptake is best observed in experiments where either CO_2 or HCO_3^- are supplied under disequilibrium conditions (Volokita et al., 1984; Espie and Colman, 1986; Kaplan et al., 1988; Tortell and Morel, 2002). Such a distinction has also been attempted in experiments where a membrane inlet mass spectrometer (MIMS) was applied. Here the net rate of CO_2 removal is calculated from the displacement of CO_2 concentration in the cell suspension from that expected at chemical equilibrium, taking the rate constants of CO_2 hydration and dehydration into account (Badger et al., 1994). Since the MIMS does not measure HCO_3^- directly, net HCO_3^- uptake is calculated from the difference between the rate of O_2 evolution (representing net CO_2 utilization in photosynthesis) and that of CO_2 uptake. At a saturating C_i level, the maximal rate of photosynthesis is limited by the carboxylation capacity and not by the rate of C_i uptake. Consequently use of the MIMS to assess the V_{max} of uptake, and in particular of HCO_3^-, would by definition lead to underestimation of its value and that of the K_m. Thus kinetic parameters drawn from this treatment may have a limited scientific value and must be used with caution.

Extracellular CA activity and changes therein during acclimation to various CO_2 levels plays an important role, particularly in organisms that rely on CO_2 uptake alone. In these organisms, the external CA activity stimulates the otherwise rate-limiting formation of CO_2 from the bulk HCO_3^-. In addition to the extensively studied extracellular CA activity in *C. reinhardtii* (discussed above), an inducible (by low CO_2 treatment) extracellular CA was also reported in diatoms (Mitchell and Beardall, 1996; Roberts et al., 1997; Lane and Morell, 2000; Burkhardt et al., 2001; Satoh et al., 2001; Tortell and Morel, 2002; Chen and Gao, 2004), albeit with considerable variations in the CA activity. For example it was much higher in *Thalassiosira weissflogii* than in *Phaeodactylum tricornutum*, and shown to be activated by light via a transmembrane redox transfer (Nimer et al., 1999). Analysis of the genomic sequence of *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* (Armbrust et al., 2004; Montsant et al., 2005) identified many relevant genes, including those encoding CA, and is likely to yield significant progress in our understanding of these important organisms and their contribution to the global carbon cycle.

On the basis of a theoretical model, it was suggested that CO_2 diffusion to the surface of large diatoms could rate-limit photosynthesis (Riebesell et al., 1993). Laboratory and field experiments were conducted to assess the effect of ambient CO_2 concentration on the productivity of various phytoplankton species representing the main primary producers. Below we briefly summarize the present state of the art on CCMs in the major aquatic primary producers.

B. Coccolithophorids

These organisms play an important role in the global carbon cycle due to both photosynthetic and calcification activities. As both processes utilize C_i as substrate, attention was given to the interrelations between them and the possibility that CO_2 released by the calcification process serves as the substrate for CO_2 fixation. A comprehensive discussion of calcification (Paasche, 2001; Shiraiwa, 2003; Muscatine et al., 2005; Zondervan, 2007; Iglesias-Rodriguez et al., 2008) is beyond the scope of this chapter. However, the present notion is that while photosynthesis and calcification affect one another, there is no compulsory link between the C_i fluxes serving them (Riebesell, 2004). Analyses of photosynthetic

performance, using various methodologies, indicated that the coccolithophorid, *Emiliania huxleyi*, an important bloom-forming organism in the oceans, exhibits a considerably lower apparent photosynthetic affinity to C_i as compared with other phytoplankton species (Sekino and Shiraiwa, 1994; Rost et al., 2003; Shiraiwa, 2003). Furthermore, while the apparent photosynthetic affinity of most phytoplankton organisms to C_i increased dramatically after transfer to LC (Badger and Spalding, 2000; Kaplan and Reinhold, 1999; Giordano et al., 2005), it was far less affected by lowering the CO_2 concentration experienced by *E. huxleyi* during growth (Rost et al., 2003). Carbon dioxide fertilization during laboratory experiments resulted in enhanced photosynthesis by *E. huxleyi*, suggesting that its productivity is limited by the availability of CO_2 in the oceans. However, mesocosm experiments near Bergen, Norway, (Engel et al., 2005) indicated that, as expected, the "real life" situation is far more complex. Engel and coworkers provided nitrate and phosphate to mesocosm systems and thereby initiated a phytoplankton bloom dominated by *E. huxleyi*. This organism dominated all the mesocosms regardless of the CO_2 concentration applied, ranging from 190 to 710 μL CO_2 L^{-1}. While certain physiological parameters such as calcification (which declined under elevated CO_2 conditions (Riebesell et al., 2000; Rost et al., 2003; Shiraiwa, 2003)) were strongly affected by the CO_2 level, the rate of nutrient consumption was not. Taken together, the data produced by mesocosms experiments (Riebesell, 2004; Kim et al., 2006; Benthien et al., 2007; Steinke et al., 2007; Zondervan, 2007; Engel et al., 2008; Iglesias-Rodriguez et al., 2008; Larsen et al., 2008) shed some light on the complexity of the field conditions and pointed to the likelihood that extrapolations from laboratory conditions (such as showing enhanced photosynthesis and growth of *E. huxleyi* under CO_2 fertilization (Rost et al., 2003)) may lead to incorrect expectations as to the effect of rising atmospheric CO_2 concentration on biological activities in the oceans (Benthien et al., 2007). An additional study also implicated iron availability in the response of photosynthesis and calcification and the resulting fractionation of stable carbon isotopes in *Emiliania huxleyi* (Schulz et al., 2007).

C. Diatoms

More than 20% of the global primary production is performed by diatoms, underlining their importance for the ecosystems on Earth. Diatoms evolved by secondary endocytobiosis, which resulted in the combination of two eukaryotic cells and in a massive rearrangement of cellular, genetic and metabolic structures. Many of the diatoms examined to date were shown to possess both CO_2 and HCO_3^- uptake capabilities (Colman et al., 2002; but see Chen and Gao, 2004) and the apparent photosynthetic affinity of diatoms to C_i is rather high. It is therefore most unlikely that diatom productivity is rate-limited by present-day oceanic C_i levels. Support for this notion came from the large iron fertilization experiments, in high nutrient low chlorophyll regions of the oceans, which led to a large rise in the abundance of diatoms (Behrenfeld et al., 1996; Gervais et al., 2002) suggesting that under natural conditions the oceanic diatoms were not CO_2 limited. The large drawdown of CO_2 concentration during phytoplankton blooms following iron fertilization provided additional support to the argument that initially these organisms were not CO_2-limited. Field incubation experiments showed a marked effect of the CO_2 concentration on the phytoplankton assemblage (Tortell et al., 2002). Raising the CO_2 concentration in water samples from the equatorial pacific to 750 μL L^{-1} resulted in a large rise in the relative abundance of diatoms, which decreased considerably when the samples were equilibrated with 150 μL CO_2 L^{-1}. In contrast, the abundance of *Phaeocystis* sp. increased when the samples were aerated with LC (Tortell et al., 2002). It is questionable whether these data can be regarded as evidence that, prior to the changes in CO_2 concentration, the diatoms were CO_2-limited. The observed marked rise in CA activity following exposure to 150 μL CO_2 L^{-1} (Tortell and Morel, 2002) did not lend support to the suggestion that the cells were CO_2-limited prior to the aeration with LC. In addition, since the overall productivity in the field experiments was not affected by the CO_2 level (Tortell et al., 2002), we cannot conclude whether the diatoms were initially limited by CO_2 availability. Finally, the question of CO_2 limitation in diatoms should be examined in view of reports that a biochemical (C4 like) rather than a biophysical CCM might be

operating in these organisms (Reinfelder et al., 2000, 2004). Ever since the discovery of C4 metabolism more than 40 years ago, Kranz anatomy and communication between mesophyll and bundle sheath cells were the distinguishing characteristics of C4 photosynthesis (Hatch, 1992). However, studies by Bowes and colleagues characterized the C4 metabolism operating in submerged angiosperms lacking Kranz anatomy (see Bowes et al., 2004). Studies by Edwards and colleagues (Edwards et al., 2004) demonstrated the occurrence and function of C4 metabolism in single cells of two different higher plants. Thus the claim that C4 metabolism occurs in the diatom *Thalassiosira weissflogii* is no longer against an accepted dogma. A comprehensive analysis of the potential C4 metabolism in diatoms is beyond the scope of the present chapter. However, with the complete genome sequences of two diatoms available, *Thalassiosira pseudonana* (Armbrust et al., 2004) and *Phaeodactylum tricornutum* (Bowler et al., 2008), it became possible to search for the relevant genes, predict the location of the proteins and assess the function of the biophysical vs the biochemical CCMs (Kroth et al., 2008; Roberts et al., 2007, and references therein). The sequencing revealed that various metabolic pathways have been partly relocated (as compared to green algae and land plants) (Kroth et al., 2008), which may be described as "scrambling" of the cell's metabolism. One example is the unicellular C4 metabolism where the essential genes are present in the model diatom *Phaeodactylum tricornutum* but enzymes potentially involved in the initial CO_2 fixation are located in various organelles. Furthermore, a decarboxylating enzyme needed to release CO_2 within the plastids is missing while present in other organelles. We concluded that apparently the subcellular location of the various enzymes in *P. tricornutum* might not allow efficient CO_2 fixation (Kroth et al., 2008). Nevertheless, diatoms are known to be very effective in photosynthesis and to be abundant in the aquatic environments. Is it possible that the C4 metabolism in diatoms serves mainly to dissipate excess light energy?

Two lines of evidence support the notion that a biophysical CCM operates in diatoms, as in many other aquatic photosynthetic organisms. First, three genes that encode different systems for bicarbonate uptake were identified in the genome of *P. tricornutum*, one of them, also present in *T. pseudonana*, shows homology to sodium/bicarbonate transporters in various organisms and appears to be localized to the plastid (Kroth et al., 2008). The other two are homologous to sodium-dependent anion exchangers and to Cl^-/HCO_3^- exchangers abundant in red blood cells (Kroth et al., 2008). Identification of numerous genes encoding CA in the diatom genomes also lends support to the suggestion that diatoms operate a biophysical CCM (Kroth et al., 2008). The overall sequence similarities among CAs are rather low and they are commonly identified by the presence of conserved domains. Seven CAs are predicted for *P. tricornutum*, two of them are beta type and show similarity to CAs found in both plants and prokaryotes; one of those is located in the plastid (Tanaka et al., 2005). The other five identified CAs belong to the alpha family and all possess signal peptides. One of them is probably located in the periplasmic space (Szabo and Colman, 2007) perhaps functioning to maintain the concentration of CO_2 near the cell membrane close to chemical equilibrium. The situation is less clear in *T. pseudonana*, which possesses more intracellular CAs without clear localization signal and transit peptides than *P. tricornutum*. The difference between these diatoms may indicate specialization of the enzyme depending on the different ecological niches. The role of CA was further demonstrated in the recent study of Matsuda and colleagues (Harada et al., 2005) showing that expression of β-CAs may be regulated by several factors including CO_2 and light.

The presence of bicarbonate transporters in the chloroplast envelope is consistent with the operation of a biophysical CCM but is not essential for a C4-like CCM, where the initial HCO_3^- fixation occurs outside the plastid. Furthermore, enhanced uptake of inorganic carbon, both CO_2 and HCO_3^-, would be consistent with both types of CCM, whereas raising the concentration of C_i within the cells is more consistent with the biophysical CCM. The high affinity of phosphoenolpyruvate carboxylase for HCO_3^- would be expected to alleviate the need to accumulate C_i internally. Induction of an extracellular CA at low CO_2 concentrations was also observed in *P. tricornutum* (Harada et al., 2005). Such a CA would be expected to facilitate the rate of CO_2 formation in the unstirred layer surrounding the cells and

thereby CO_2 supply for photosynthesis by either CCM type. In summary, clear evidence that supports either CCM mode as the *only* way to raise the CO_2 concentration in close proximity of Rubisco is presently awaiting biochemical or genetic evidence.

D. Dinoflagellates

Despite the global importance of dinoflagelates, both as free-living cells and in symbiotic associations with various reef organisms, little is known about the C_i species utilized and their CCM activity. The large size of their genome, the fact that certain chloroplastic genes are encoded on plasmids (Koumandou et al., 2004), the primitive (in evolutionary terms) nature of Rubisco (form II) in some (Morse et al., 1995) but not all (Jenks and Gibbs, 2000) species, the circadian localization of Rubisco in the pyrenoids and stroma (Nassoury et al., 2001) and the organization of their chloroplast envelopes all complicate physiological and genetic analysis of the CCM in these organisms.

It has been observed that alkalization of the bulk water during the progress of a *Peridinium gatunense* bloom in Lake Kinneret, Israel, particularly in the *P. gatunense* patches, caused a marked decline in the availability of dissolved CO_2 (Berman-Frank et al., 1998). Though ample HCO_3^- was still available, and despite induction of periplasmic CA (Berman-Frank et al., 1995), the cells became CO_2 limited. Consequently they accumulated reactive oxygen species and entered an apoptotic-like process, including DNA fragmentation, which led to the collapse of the entire population at the end of the bloom (Vardi et al., 1999). These data clearly indicated that *P. gatunense* is a "CO_2 user" with a limited ability to acquire HCO_3^- directly from the medium. Studies on marine dinoflagellates also suggested a limited capacity to utilize HCO_3^- from the medium (Furla et al., 2000; Leggat et al., 2002; Dason et al., 2004).

A detailed study has focused on the effect of CO_2 concentration during growth on C_i acquisition by the toxic dinoflagellate *Protoceratium reticulatum* (Ratti et al., 2007). When grown under LC the apparent photosynthetic affinity for C_i was about tenfold lower than in those grown under HC. pH drift experiments suggested that it mostly uses CO_2 from the media, the acquisition of which is affected by an internal β-type CA. The level and activity of this CA declined under HC conditions. The data indicate that *P. reticulatum* has an inducible CCM that operates in the absence of pyrenoids and with little intracellular CO_2 accumulation. Other studies indicated the effect of membrane lipid composition in symbiotic dinoflagellates on temperature-dependent coral bleaching (Tchernov et al., 2004). In contrast, the interrelationships between the symbionts and the host with respect to carbon metabolism and CCM activity within the association are poorly understood.

E. Cycling of C_i Between the Cells and Their Environment

Although it is clear that fluxes of C_i between the cells and their media may alter the energy balance and affect the maintenance and regulation of the internal pH, little is known about their magnitude in various organisms. Use of MIMS indicated massive light-dependent cycling of C_i between the medium and the cells of various phytoplankton species representing the main groups of aquatic primary producers. These included diatoms, dinoflagellates, a coccolithophorid, a green alga and filamentous and single cell cyanobacteria (see Fig. 25.1 for cyanobacteria). These organisms could maintain an ambient CO_2 concentration substantially above or below that expected at chemical equilibrium with HCO_3^- (Tchernov et al., 2003). Maintenance of such conditions during steady-state photosynthesis has been attributed to CO_2 fixation during photosynthesis. However, displacement of CO_2 concentration from equilibrium in mutants that did not display net photosynthesis and differing responses of CO_2 uptake and CO_2 fixation to changing light intensity, supported the notion that these two processes, photosynthesis and CO_2 cycling, are not necessarily inseparable.

It has been reported that during steady-state photosynthesis the dissolved CO_2 concentration in the medium surrounding *Synechococcus* sp. WH7803 (Tchernov et al., 1997) and *Nannochloropsis* sp. (Sukenik et al., 1997; Huertas et al., 2000) is substantially higher than that expected at chemical equilibrium with HCO_3^-. In *Synechococcus* sp. WH7803, the magnitude of this C_i cycling reached values sevenfold higher than the photosynthetic rate and saturated

at a light intensity twice as high as that required for maximal CO_2 fixation (Tchernov et al., 1997). Quantification of the net fluxes of CO_2, HCO_3^- and of photosynthesis, in the steady-state, revealed that substantial HCO_3^- efflux accompanied CO_2 uptake and fixation in the case of "CO_2 users". On the other hand, "HCO_3^- users" were characterized by a rate of net CO_2 uptake lower than that of CO_2 fixation. A unique case was observed in *E. huxleyi*, which shifted from net CO_2 uptake to net CO_2 efflux with rising light intensity (Tchernov et al., 2003), possibly reflecting changes in the photosynthesis/calcification ratio. It was concluded that a light-dependent massive cycling of C_i is widespread through the major groups of photosynthetic microorganisms, and that it is not compulsorily linked to CO_2 fixation and may even occur in its absence. The results supported the notion that entities associated with the CCM function not only raise the CO_2 concentration at the site of Rubisco, they may also serve as a means of diminishing photodynamic damage by dissipating excess light energy. The observed C_i cycling is likely to affect the composition of stable carbon isotopes in phytoplankton (see below). Finally, it is generally accepted that the CCMs were evolved during the glacial period when the atmospheric CO_2 level dropped (Giordano et al., 2005). However, it is tempting to speculate that the entities involved in C_i uptake may have evolved as a means to dissipate excess light energy, already when atmospheric CO_2 concentration was high, and were acquired to raise the internal CO_2 when CO_2 became limiting.

It is widely accepted that the internal C_i pool serves as the immediate source of CO_2 for carboxylation. Thus, it is interesting to note that the extent of C_i accumulation within the cells varies significantly between different organisms. While *Synechococcus* 7942 may accumulate as much as 1000-fold above the extracellular C_i level, the phycoerythrin-containing marine *Synechococcus* sp. WH7803 shows a very limited ability to accumulate C_i (Hassidim et al., 1997). Nevertheless, despite the very large difference in the C_i pool, these organisms exhibit similar apparent photosynthetic affinity for extracellular C_i. Apparently the small C_i pool in *Synechococcus* sp. WH7803 is utilized more efficiently in photosynthesis than the C_i pool in *Synechococcus* 7942. While the mechanism leading to this difference is not known, it might be related to the effectiveness of C_i cycling and formation of CO_2 gradients within the cells as a consequence of rapid conversion of HCO_3^- to CO_2. A similar case of a low internal C_i pool but high apparent photosynthetic affinity to extracellular Ci was observed in *Nannochloropsis* sp. (Sukenik et al., 1997). In *E. huxleyi*, on the other hand, there is a good agreement between the low ability to accumulate C_i and low apparent photosynthetic affinity (Sekino and Shiraiwa, 1994). Surprisingly, the internal C_i pool in diatoms has been examined in very few cases (Mitchell and Beardall, 1996).

F. CO_2-Mediated Interspecies Communication

Surprisingly, despite the rising interest in the effect of the rising atmospheric CO_2 level on the phytoplankton composition, little is known on the effect of CO_2 availability on the population dynamics, interspecies communication, and changes therein. Analysis of the phytoplankton assemblage in Lake Kinneret, a fresh water lake in Israel, indicated a strong negative correlation between the presence and abundances of the dinoflagellate *Peridinium gatunense* and that of the toxic, microcystin producing, *Microcystis* sp. (Sukenik et al., 2002; Vardi et al., 2002). Growth experiments confirmed density-dependent interspecies interactions between these two organisms (Sukenik et al., 2002; Vardi et al., 2002). The presence of *Microcystis* sp. or its spent media induced intensive accumulation of reactive oxygen species in *P. gatunense* (Vardi et al., 2002) due to severe inhibition of internal CA by an allelochemical excreted by *Microcystis* sp. (Sukenik et al., 2002). The accumulation of reactive oxygen species induced an apoptotic-like process in *P. gatunense* leading to massive collapse of the *P. gatunense* population. *Microcystis* sp. mimicked the CO_2-limiting conditions experienced by *P. gatunense* at the peak of its bloom (see above). This is, to the best of our knowledge, the first report where a biotic effect on CO_2 availability was shown to directly affect the composition of the phytoplankton assemblage.

G. Fractionation of Stable Carbon Isotopes

One of the most important subjects in current environmental research is the man-made perturbation

in the global carbon cycle caused by fossil fuel utilization and deforestation. There is growing concern that the "greenhouse effect", enhanced by rising atmospheric CO_2 levels, will lead to serious climatic changes. The observed rise in atmospheric CO_2 level is only approximately half that expected on the basis of fossil fuel utilization. The "missing" half is believed to be sequestered in the oceans by two major processes: the relatively slow geochemical processes and the relatively fast "biological pump" in which photosynthetic CO_2 fixation plays a major role (see Falkowski, 1994). Accurate prediction of future atmospheric CO_2 concentration depends not only on the validity of the models and methods used but also on correct assessment of the effect of changing atmospheric CO_2 on carbon cycling between the atmosphere and the exchangeable aquatic and terrestrial carbon pools. In spite of intensive investigations, this problem is still unresolved. One reason is that it is still not clear to what extent and under what conditions CO_2 supply is rate-limiting for productivity in the aquatic environment (see above).

Fractionation of the stable carbon isotope, ^{13}C, by living organisms has been regarded as an important index of the interplay between the environment and the cells (Farquhar et al., 1989; Raven et al., 1993, 2008; Raven, 1994; Erez et al., 1998). Changes in atmospheric ^{13}C since the industrial revolution are recorded in various natural materials such as tree rings, coral skeletons and deep-sea sediments. The ^{13}C isotope has also been used to assess whether the productivity of aquatic and terrestrial organisms is rate-limited by the availability of CO_2, to investigate variations in water use efficiency in land plants, to detect various metabolic strategies, and to establish the limits of the organism's response to environmental change. The cycling of C_i described above has relevance to the fractionation of ^{13}C by organisms that possess a CCM. The Rubisco enzyme discriminates against ^{13}C-CO_2 (Sharkey and Berry, 1985; Guy et al., 1993). The ^{13}C of the organic matter formed thus reflects this discrimination and, as well, the isotopic composition of the internal C_i pool that serves as the substrate for the carboxylation reaction. The ^{13}C of the internal C_i pool will be strongly affected, first by the Rubisco fractionation resulting in ^{13}Ci enrichment of the pool and second by the balance of the fluxes of the various C_i species, inward and outward, and the carboxylation rate. The nature of the C_i species taken up (e.g., CO_2 and HCO_3^-), and their relative proportion, will also affect the ^{13}C of the internal pool. At isotopic equilibrium, HCO_3^- is approximately 9‰ richer in ^{13}C than $CO_{2(aq)}$ and uptake of HCO_3^- will thus tend to increase the abundance of ^{13}C in the internal C_i pool. The C_i cycling relative to the photosynthetic rate thus impacts on the observed ^{13}C. Any factor that affects the balance between the C_i fluxes will significantly alter the size and level of the C_i pool and hence the rate of carboxylation, the isotopic composition of the C_i pool, and the organic matter.

A large variation in the ^{13}C values has been reported for different latitudes (Rau et al., 1989; Goericke and Fry, 1994). Owing to temperature differences at the various latitudes, it is expected that the concentration of dissolved CO_2 would differ. A correlation was observed between the expected CO_2 concentration and ^{13}C (Rau et al., 1992; Goericke and Fry, 1994). The mechanisms underlying this correlation, however, are still poorly understood. The differences in CO_2 concentration with temperature are expected to modify the expression of CCMs operating in these organisms and, consequently, the unidirectional fluxes of C_i. Interestingly, the inflection point in the curve relating ^{13}C to CO_2 concentration is close to that expected at equilibrium with the atmospheric CO_2 concentration (Rau et al., 1992; Goericke and Fry, 1994), suggesting that the natural community of photosynthetic microorganisms undergoes acclimation to the low CO_2. In addition to CO_2 concentration, the growth rate has been suggested to affect the ^{13}C levels (Laws et al., 2002). Riebesell and colleagues showed that the ^{13}C of the coccolithophorid *E. huxleyi* (Rost et al., 2002; Schulz et al., 2007) and the marine diatom *Skeletonema costatum* (Gervais and Riebesell, 2001) grown in dilute batch cultures, was strongly affected by the CO_2 concentration, the growth rate, and the light conditions. They concluded that the results severely complicate the interpretation of carbon isotope measurements in suspended and sedimentary organic matter. Clearly, the value of the ^{13}C obtained reflects a complex interaction between the various biotic parameters affecting the isotopic fractionation and their response to the changing ambient conditions.

Thus the ^{13}C level cannot be used as a simple paleobarometer of CO_2 concentration in geological periods.

H. Biotechnological Implications

With the current rise in atmospheric CO_2 level, and the surging cost of fuel and food, there is a growing interest in biotechnological approaches to produce lipids from phytoplankton as a replacement for crude oil and to enhance food production. Under many environmental conditions plant photosynthesis and growth are limited by the availability of CO_2 at the site of Rubisco. Photosynthetic organisms that possess biochemical or biophysical CCMs largely overcome this limitation and depress photorespiration. It has thus been tempting to explore the possible use of cyanobacterial CCM components to raise plant productivity. One example is the use of *ictB* as a gene involved in the ability to accumulate C_i in *Synechococcus* 7942 (Bonfil et al., 1998). This gene encodes a hydrophobic protein that is highly conserved in all the cyanobacteria examined to date. Transgenic *Arabidopsis thaliana*, *Nicotiana tabacum* (Lieman-Hurwitz et al., 2003) and wheat (unpublished) expressing *ictB* exhibited significantly faster photosynthetic rates than the corresponding wild types under limiting, but not under saturating CO_2 concentrations. Similar results were obtained in *A. thaliana* plants bearing *ictB* from *Anabaena* sp. PCC 7120. Growth of transgenic *A. thaliana* maintained under low humidity was considerably faster than that of the wild type. There was no difference in the amount of Rubisco or the activity of the enzyme, when activated in vitro, in the wild type and the transgenic plants. In contrast, the in vivo Rubisco activity in plants grown under low humidity, without prior activation of the enzyme, was considerably higher in *ictB*-expressing plants than in their wild types. Moreover, the CO_2 compensation point in the transgenic plants was lower than in the wild types. The steeper slope of the curve relating CO_2 fixation to concentration, the higher in situ Rubisco activity and lower compensation points in the transgenic plants, suggested a higher CO_2 concentration in close proximity to Rubisco. These data indicated a potential use of *ictB* and perhaps other genes involved in CCMs for the stimulation of crop yield.

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References

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou SG, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kroger N, Lau WWY, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP and Rokhsar DS (2004) The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. Science 306: 79–86

Badger MR and Price GD (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J Exp Bot 54: 609–622

Badger MR and Spalding MH (2000) CO_2 acquisition, concentration and fixation in cyanobacteria and algae. In: Leegood RC, Sharkey TD and von Caemmerer S (eds) Photosynthesis: Physiology and Metabolism, Advances in Photosynthesis, Vol 9, pp 369–397. Kluwer Academic Publishers, Dordrecht

Badger MR, Kaplan A, and Berry JA (1980) Internal Inorganic Carbon Pool of *Chlamydomonas reinhardtii*: Evidence for a carbon dioxide-concentrating mechanism. Plant Physiol 66: 407–413

Badger MR, Palmqvist K and Yu JW (1994) Measurement of CO_2 and HCO_3^- fluxes in cyanobacteria and microalgae during steady-state photosynthesis. Physiol Plant 90: 529–536

Badger MR, Price GD, Long BM and Woodger FJ (2006) The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. J Exp Bot 57: 249–265

Ball SG (1998) Regulation of starch biosynthesis. In: Rochaix JD, Goldschmidt-Clermont M and Merchant S (eds) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7, pp 549–567. Kluwer Academic Publishers, Dordrecht

Battchikova N and Aro EM (2007) Cyanobacterial NDH-1 complexes: multiplicity in function and subunit composition. Physiol Plant 131: 22–32

Battchikova N, Zhang PP, Rudd S, Ogawa T and Aro EM (2005) Identification of NdhL and Ssl1690 (NdhO) in NDH-1L, and NDH-1M complexes of *Synechocystis* sp. PCC 6803. J Biol Chem 280: 2587–2595

Beardall J and Raven JA (2004) The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43: 26–40

Behrenfeld MJ, Bale AJ, Kolber ZS, Aiken J and Falkowski PG (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. Nature 383: 508–511

Benthien A, Zondervan I, Engel A, Hefter J, Terbruggen A and Riebesell U (2007) Carbon isotopic fractionation during a mesocosm bloom experiment dominated by *Emiliania huxleyi*: Effects of CO_2 concentration and primary production. Geochim Cosmochim Acta 71: 1528–1541

Berman-Frank I, Erez J and Kaplan A (1998) Changes in inorganic carbon uptake during the progression of dinoflagellate bloom in a lake ecosystem. Can J Bot 76: 1043–1051

Berman-Frank I, Kaplan A, Zohary T and Dubinsky Z (1995) Carbonic anhydrase activity in the bloom-forming dinoflagellate *Peridinium gatunense*. J Phycol 31: 906–913

Bonfil DJ, Ronen-Tarazi M, Sültemeyer D, Lieman-Hurwitz J, Schatz D and Kaplan A (1998) A putative HCO_3^- transporter in the cyanobacterium *Synechococcus* sp. strain PCC 7942. FEBS Lett 430: 236–240

Bowes G, Rao SK, Estavillo GM and Reiskind JB (2004) C_4 mechanism in aquatic angiosperms: Comparisons with terrestrial C_4 systems. Funct Plant Biol 29: 379–392

Bowler C, Allen EA, Badger JH, Grimwood J, Jabbari K, Kuo A, Maheswari U, Martens C, Maumus F, Otillar RP, Rayko E, Salamov A, Vandepoele K, Beszteri B, Gruber A, Heijde M, Katinka M, Mock T, Valentin K, Verret F, Berges JA, Brownlee C, Cadoret J-P, Chiovitti A, Choi CJ, Coesel S, De Martino A, Detter JC, Durkin C, Falciatore A, Fournet J, Haruta M, Huysman MJJ, Jenkins BD, Jiroutova K, Jorgensen RE, Joubert Y, Kaplan A, Kröger N. Kroth PG, La Roche J, Lindquist E, Lommer M, Marten-Jèzèquel V, Lopez PJ, Lucas S, Mangogna M, McGinnis K, Medlin LK, Montsant A, Oudot-Le Secq M-P, Napoli C, Obornik M, Parker MS, Petit J-L, Porcel BM, Poulsen N, Robison M, Rychlewski L, Rynearson TA, Schmutz J, Shapiro H, Siaut M, Stanley M, Sussman MR, Taylor AR, Vardi A, von Dassow P, Vyverman W, Willis A, Wyrwicz LS, Rokhsar DS, Weissenbach J, Armbrust EV, Green BR, Van de Peer Y and Grigoriev IV (2008) The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. Nature 456: 239–244

Bryant DR (ed) (1994) The Molecular Biology of Cyanobacteria, Advances in Photosynthesis, Vol 1, Kluwer Academic Publishers, Dordrecht

Burkhardt S, Amoroso G, Riebesell U and Sültemeyer D (2001) CO_2 and HCO_3^- uptake in marine diatoms acclimated to different CO_2 concentrations. Limnol Oceanogr 46: 1378–1391

Burow MD, Chen ZY, Mouton TM and Moroney JV (1996) Isolation of cDNA clones of genes induced upon transfer of *Chlamydomonas reinhardtii* cells to low CO_2. Plant Mol Biol 31: 443–448

Cassar N, Laws EA, Bidigare RR and Popp BN (2004) Bicarbonate uptake by Southern Ocean phytoplankton. Global Biogeochem Cycles 18 Article Number: GB2003

Chen XW and Gao KS (2004) Photosynthetic utilisation of inorganic carbon and its regulation in the marine diatom *Skeletonema costatum*. Funct Plant Biol 31: 1027–1033

Chen ZY, Burow MD, Mason CB and Moroney JV (1996) A low-CO_2-inducible gene Encoding an alanine:[alpha]-ketoglutarate aminotransferase in *Chlamydomonas reinhardtii.* Plant Physiol 112: 677–684

Chen ZY, Lavigne LL, Mason CB and Moroney JV (1997) Cloning and overexpression of two cDNAs encoding the low-CO_2-inducible chloroplast envelope protein LIP-36 from *Chlamydomonas reinhardtii.* Plant Physiol 114: 265–273

Colman B, Huertas IE, Bhatti S and Dason JS (2002) The diversity of inorganic carbon acquisition mechanisms in eukaryotic microalgae. Funct Plant Biol 29: 261–270

Coleman JR (1991) The molecular and biochemical analyses of CO_2 concentrating mechanisms in cyanobacteria and microalgae. Plant Cell Environ 14: 861–867

Dason JS, Huertas IE and Colman B (2004) Source of inorganic carbon for photosynthesis in two marine dinoflagellates. J Phycol 40: 285–292

Duanmu D, Miller AR, Horken KM, Weeks DP and Spalding MH (2009a) Knockdown of limiting-CO_2-induced gene HLA3 decreases HCO_3– transport and photosynthetic C_i affinity in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 106: 5990–5995

Duanmu D, Wang Y and Spalding MH (2009b) Thylakoid lumen carbonic anhydrase (CAH3) mutation suppresses air-dier phenotype of LCIB mutant in *Chlamydomonas reinhardtii*. Plant Physiol 149: 929–937

Edwards GE, Franceschi VR and Voznesenskaya EV (2004) Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Biol 55: 173–196

Eisenhut M, Kahlon S, Hasse D, Ewald R, Lieman-Hurwitz J, Ogawa T, Ruth W, Bauwe H, Kaplan A, Hagemann M (2006) The plant-like C2 glycolate cycle and the bacterial-like

glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. Plant Physiol 142: 333–342

Eisenhut M, Ruth W, Haimovich M, Bauwe H and Kaplan A (2008) The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiotically to plants. Proc Natl Acad Sci USA 105: 17199–17204

Engel A, Schulz KG, Riebesell U, Bellerby R, Delille B and Schartau M (2008) Effects of CO_2 on particle size distribution and phytoplankton abundance during a mesocosm bloom experiment (PeECE II). Biogeosciences 5: 509–521

Engel A, Zondervan I, Aerts K, Beaufort L, Benthien A, Chou L, Delille B, Gattuso JP, Harlay J, Heemann C, Hoffmann L, Jacquet S, Nejstgaard J, Pizay MD, Rochelle-Newall E, Schneider U, Terbrueggen A and Rebesell U (2005) Testing the direct effect of CO_2 concentration on a bloom of the coccolithophorid *Emiliania huxleyi* in mesocosm experiments. Limnol Oceanogr 50: 493–507

Erez J, Bouevich A and Kaplan A (1998) Carbon isotope fractionation by the freshwater cyanobacterium *Synechococcus* PCC 7942. Can J Bot 76: 1109–1118

Eriksson M, Karlsson J, Ramazanov Z, Gardestroem P and Samuelsson G (1996) Discovery of an algal mitochondrial carbonic anhydrase: molecular cloning and characterization of a low-CO_2-induced polypeptide in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 93: 12031–12034

Espie GS and Colman B (1986) Inorganic carbon uptake during photosynthesis. I: a theoretical analysis using the isotopic disequilibrium technique. Plant Physiol 80: 863–869

Espie GS and Kandasamy RA (1994) Monensin inhibition of Na^+-dependent HCO_3^- transport distinguishes it from Na^+-independent HCO_3^- transport and provides evidence for Na^+/HCO_3^- symport in the cyanobacterium *Synechococcus* UTEX 625. Plant Physiol 104: 1419–1428

Espie GS, Miller AG and Canvin DT (1989) Selective and reversible inhibition of active CO_2 transport by hydrogen sulfide in a cyanobacterium. Plant Physiol 91: 389–394

Falkowski PG (1994) The role of phytoplankton photosynthesis in global biogeochemical cycles. Photosynth Res 39: 235–258

Falkowski PG and Raven J (1997) Aquatic Photosynthesis. Blackwell Scientific, Oxford

Falkowski PG, Scholes RJ, Boyle E, Canadell J, Canfield D, Elser J, Gruber N, Hibbard K, Hogberg P, Linder S, Mackenzie FT, Moore B, Pedersen T, Rosenthal Y, Seitzinger S, Smetacek V and Steffen W (2000) The global carbon cycle: A test of our knowledge of earth as a system. Science 290: 291–296

Farquhar GD, Ehleringer JR and Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40: 503–537

Figge RM, Cassier-Chauvat C, Chauvat F and Cerff R (2001) Characterization and analysis of an NAD(P)H dehydrogenase transcriptional regulator critical for the survival of cyanobacteria facing inorganic carbon starvation and osmotic stress. Mol Microbiol 39: 455–468

Folea M, Zhang P, Nowaczyk MM, Ogawa T, Aro E-M and Boekema EJ (2008) Single particle analysis of thylakoid proteins from *Thermosynechococcus elongatus* and *Synechocystis* 6803: Localization of the CupA subunit of NDH-1. FEBS Lett 582: 249–254

Fukuzawa H, Fujiwara S, Yamamoto Y, Dionizio-Sese ML and Miyachi S (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: regulation by environmental CO_2 concentration. Proc Natl Acad Sci USA 87: 4383–4387

Fukuzawa H, Miura K, Ishizaki K, Kucho K, Saito T, Kohinata T and Ohyama K (2001) Ccm1, a regulatory gene controlling the induction of a carbon concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. Proc Natl Acad Sci USA 98: 5347–5352

Fukuzawa H, Suzuki E, Komukai Y and Miyachi S (1992) A gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC 7942. Proc Natl Acad Sci USA 89: 4437–4441

Furla P, Galgani I, Durand I and Allemand D (2000) Sources and mechanisms of inorganic carbon transport for coral calcification and photosynthesis. J Exp Biol 203: 3445–3457

Gervais F and Riebesell U (2001) Effect of phosphorus limitation on elemental composition and stable carbon isotope fractionation in a marine diatom growing under different CO_2 concentrations. Limnol Oceanogr 46: 497–504

Gervais F, Riebesell U and Gorbunov MY (2002) Changes in primary productivity and chlorophyll *a* in response to iron fertilization in the Southern Polar Frontal Zone. Limnol Oceanogr 47: 1324–1335

Giordano M, Beardall J and Raven JA (2005) CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Ann Rev Plant Biol 56: 99–131

Giordano M, Norici A, Forssen M, Eriksson M and Raven JA (2003) An anaplerotic role for mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. Plant Physiol 132: 2126–2134

Goericke R and Fry B (1994) Variation of marine plankton delta ^{13}C with latitude, temperature, and dissolved CO_2 in the world ocean. Global Biochem Cycles 8: 85–90

Guy RD, Fogel ML and Berry JA (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. Plant Physiol 101: 37–47

Harada H, Nakatsuma D, Ishida M and Matsuda Y (2005) Regulation of the expression of intracellular β-carbonic anhydrase in response to CO_2 and light in the marine diatom *Phaeodactylum tricornutum*. Plant Physiol 139: 1041–1050

Hassidim M, Keren N, Ohad I, Reinhold L and Kaplan A (1997) Acclimation of *Synechococcus* strain WH7803 to

ambient CO_2 concentration and to elevated light intensity. J Phycol 33: 811–817

Hatch MD (1992) C_4 photosynthesis: An unlikely process full of surprises. Plant Cell Physiol 33: 333–342

Herranen M, Battchikova N, Zhang PP, Graf A, Sirpio S, Paakkarinen V and Aro EM (2004) Towards functional proteomics of membrane protein complexes in *Synechocystis* sp. PCC 6803. Plant Physiol 134: 470–481

Huertas IE, Espie GS, Colman B and Lubian LM (2000) Light-dependent bicarbonate uptake and CO_2 efflux in the marine microalga *Nannochloropsis gaditana*. Planta 211: 43–49

Iglesias-Rodriguez MD, Halloran PR, Rickaby RE, Hall IR, Colmenero-Hidalgo E, Gittins JR, Green DR, Tyrrell T, Gibbs SJ, von Dassow P, Rehm E, Armbrust EV and Boessenkool KP (2008) Phytoplankton calcification in a high-CO_2 world. Science 320: 336–340

Im CS and Grossman AR (2002) Identification and regulation of high light-induced genes in *Chlamydomonas reinhardtii*. Plant J 30: 301–313

Jenks A and Gibbs SP (2000) Immunolocalization and distribution of Form II Rubisco in the pyrenoid and chloroplast stroma of *Amphidinium carterae* and Form I Rubisco in the symbiont-derived plastids of *Peridinium foliaceum* (Dinophyceae). J Phycol 36: 127–138

Johnston AM (1991) The acquisition of inorganic carbon by marine macroalgae. Can J Bot 69: 1123–1132

Johnston AM, Maberly SC and Raven JA (1992) The acquisition of inorganic carbon by four red macroalgae from different habitats. Oecologia 91: 481–492

Jordan DB and Ogren WL (1981) Species variation in the specificity of ribulose-bisphosphate carboxylase-oxygenase. Nature 291: 513–515

Kaplan A and Reinhold L (1999) The CO_2 concentrating mechanisms in photosynthetic microorganisms. Annu Rev Plant Physiol Plant Mol Biol 50: 539–570

Kaplan A, Hagemann M, Bauwe H, Kahlon S and Ogawa T (2008) Carbon acquisition by cyanobacteria: Mechanisms, comparative genomics, and evolution. In: Herrero A and Flores E (eds) The Cyanobacteria: Molecular Biology, Genomics and Evolution, pp 305–334. Horizon Scientific Press, Norwich, UK

Kaplan A, Marcus Y and Reinhold L (1988) Inorganic carbon uptake by cyanobacteria. In: Packer L and Glazer AN (eds) Methods in Enzymology, Vol 167, pp 534–539. Academic Press, New York

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Park YI, Husic HD, Moroney JV and Samuelsson G (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. EMBO J 17: 1208–1216

Kim JM, Lee K, Shin K, Kang JH, Lee HW, Kim M, Jang PG and Jang MC (2006) The effect of seawater CO_2 concentration on growth of a natural phytoplankton assemblage in a controlled mesocosm experiment. Limnol Oceanogr 51: 1629–1636

Kohinata T, Nishino H and Fukuzawa H (2008) Significance of zinc in a regulatory protein, CCM1, which regulates the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Cell Physiol 49: 273–283

Koropatkin NM, Koppenaal DW, Pakrasi HB and Smith TJ (2007) The structure of a cyanobacterial bicarbonate transport protein, CmpA. J Biol Chem 282: 2606–2614

Koumandou VL, Nisbet RER, Barbrook AC and Howe CJ (2004) Dinoflagellate chloroplasts - where have all the genes gone? Trends Genet 20: 261–267

Kroth PG, Chiovitti A, Gruber A, Martin-Jezequel V, Mock T, Parker MS, Stanley MS, Kaplan A, Caron L, Weber T, Maheswari U, Armbrust EV and Bowler C (2008) A model for carbohydrate metabolism in the diatom *Phaeodactylum tricornutum* deduced from comparative whole genome analysis. PLoS One 3: e1426.

Kucho K, Ohyama K and Fukuzawa H (1999) CO_2-responsive transcriptional regulation of CAH1 encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. Plant Physiol 121: 1329–1337

Kucho K, Yoshioka S, Taniguchi F, Ohyama K and Fukuzawa H (2003) Cis-acting elements and DNA-binding proteins involved in CO_2-responsive transcriptional activation of Cah1 encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. Plant Physiol 133: 783–793

Lane TW and Morell FMM (2000) Regulation of carbonic anhydrase expression by zinc, cobalt and carbon dioxide in the marine diatom *Thalassiosira weissflogii*. Plant Physiol. 123: 345–352

Larsen JB, Larsen A, Thyrhaug R, Bratbak G, and Sandaa RA (2008) Response of marine viral populations to a nutrient induced phytoplankton bloom at different pCO_2 levels. Biogeosciences 5: 523–533

Laws EA, Popp BN, Cassar N and Tanimoto J (2002) ^{13}C discrimination patterns in oceanic phytoplankton: likely influence of CO_2 concentrating mechanisms, and implications for palaeoreconstructions. Funct Plant Biol 29: 323–333

Leggat W, Marendy EM, Baillie B, Whitney SM, Ludwig M, Badger MR and Yellowlees D (2002) Dinoflagellate symbioses: strategies and adaptations for the acquisition and fixation of inorganic carbon. Funct Plant Biol 29: 309–322

Li Q and Canvin DT (1998) Energy sources for HCO_3^- and CO_2 transport in air-grown cells of *Synechococcus* UTEX 625. Plant Physiol 116: 1125–1132

Lieman-Hurwitz J, Haimovich M, Shalev-Maul G, Ishii A, Hihara Y, Gaathon A, Lebendiker M and Kaplan A (2009) A cyanobacterial Abr-B like protein affects the apparent photosynthetic affinity to CO_2 by modulating low-CO_2-gene expression. Environ Microbiol 11: 927–936

Lieman-Hurwitz J, Rachmilevitch S, Mittler RYM and Kaplan A (2003) Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in

HCO_3^- accumulation in cyanobacteria. Plant Biotech J 1: 43–50

Maberly SS (1990) Exogenous sources of inorganic carbon for photosynthesis by marine macroalgae. J Phycol 26: 439–449

Mackenzie TD, Burns RA and Campbell DA (2004) Carbon status constrains light acclimation in the cyanobacterium *Synechococcus elongatus*. Plant Physiol 136: 3301–3312

McGinn PJ, Price GD, Maleszka R and Badger MR (2003) Inorganic carbon limitation and light control the expression of transcripts related to the CO_2-concentrating mechanism in the cyanobacterium S*ynechocystis* sp. strain PCC 6803. Plant Physiol 132: 218–229

Maeda S, Badger MR and Price GD (2002) Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO_2 hydration in the cyanobacterium *Synechococcus* sp. PCC 7942. Mol Microbiol 43: 425–436

Maeda S, Price GD, Badger MR, Enomoto C and Omata T (2000) Bicarbonate binding activity of the CmpA protein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in active transport of bicarbonate. J Biol Chem 275: 20551–20555

Mamedov TG, Suzuki K, Miura K, Kucho K and Fukuzawa H (2001) Characteristics and sequence of phosphoglycerate phosphatase from an eukaryotic green algae *Chlamydomonas reinhardtii*. J Biol Chem 276: 45573–45579

Marco E, Ohad N, Schwarz R, Lieman-Hurwitz J, Gabay C and Kaplan A (1993) High CO_2 concentration alleviates the block in photosynthetic electron transport in an *ndhB*-inactivated mutant of *Synechococcus* sp. PCC 7942. Plant Physiol 101: 1047–1053

Marcus Y, Schwarz R, Friedberg D and Kaplan A (1986) High CO_2 requiring mutant of *Anacystis nidulans* R2. Plant Physiol 82: 610–612

Mariscal V, Moulin P, Orsel M, Miller AJ, Fernández E and Galván A (2006) Differential regulation of the *Chlamydomonas Nar1* gene family by carbon and nitrogen. Protist 157: 421–433

Matsuda Y and Colman B (1995) Induction of CO_2 and bicarbonate transport in the green Alga *Chlorella ellipsoidea.* II. Evidence for induction in response to external CO_2 concentration. Plant Physiol 108: 253–260

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ et al. (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318: 245–250

Mi H, Endo T, Ogawa T and Asada K (1995) Thylakoid membrane-bound, NADPH-specific pyridine nucleotide dehydrogenase complex mediated cyclic electron transport in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol 36: 661–668

Mi H, Endo T, Schreiber U, Ogawa T and Asada K (1992) Electron donation from cyclic and respiratory flows to photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803. Plant Cell Physiol 33: 1233–1237

Miller AG, Espie GS and Canvin DT (1990) Physiological aspects of CO_2 and HCO_3^- transport by cyanobacteria: a review. Can J Bot 68: 1291–1302

Miller AG, Espie GE and Canvin DT (1991) The use of COS, a structural analog of CO_2, to study CO_2 transport in the cyanobacterium *Synechococcus* UTEX 625. Plant Physiol 90: 1221–1231

Miller AG, Turpin DH and Canvin DT (1984) Na^+ requirement for growth, photosynthesis, and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis*. J Bacteriol 159: 100–106

Mitchell C and Beardall J (1996) Inorganic carbon uptake by an Antarctic sea-ice diatom, *Nitzschia frigida*. Polar Biol 16: 95–99

Miura K, Yamano T, Yoshioka S, Kohinata T, Inoue Y, Taniguchi F, Asamizu E, Nakamura Y, Tabata S, Yamato KT, Ohyama K and Fukuzawa H (2004) Expression profiling-based identification of CO_2-responsive genes regulated by CCM1 controlling a carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Physiol 135: 1595–1607

Montsant A, Jabbari K, Maheswari U and Bowler C (2005) Comparative genomics of the pennate diatom *Phaeodactylum tricornutum.* Plant Physiol 137: 500–513

Morita E, Abe T, Tsuzuki M, Fujiwana S, Sato N, Hirata A, Sonoike K and Nozaki H (2000) Role of pyrenoids in the CO_2-concentrating mechanism: comparative morphology, physiology and molecular phylogenetic analysis of closely related strains of *Chlamydomonas* and *Chloromonas*. Planta 208: 365–372

Moroney JV and Ynalvez RA (2007) Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*. Eukaryot Cell 6: 1251–1259

Morse D, Salois P, Markovic P and Hastings JW (1995) A nuclear-encoded form II RuBisCo in dinoflagellates. Science 268: 1622–1624

Muscatine L, Goiran C, Land L, Jaubert J, Cuif JP and Allemand D (2005) Stable isotopes (delta C-13 and delta N-15) of organic matrix from coral skeleton. Proc Natl Acad Sci USA 102: 1525–1530

Nakamura Y, Kanakagiri S, Van, K, He W and Spalding MH (2005) Disruption of glycolate dehydrogenase gene in the high CO_2 requiring mutant HCR89 of *Chlamydomonas reinhardtii.* Can J Bot 83: 820–833

Nassoury N, Fritz L and Morse D (2001) Circadian changes in ribulose-1,5-bisphosphate carboxylase/oxygenase distribution inside individual chloroplasts can account for the rhythm in dinoflagellate carbon fixation. Plant Cell 13: 923–934

Nimer N-A, Ling MX, Brownlee C and Merret MJ (1999) Inorganic carbon limitation, exofacial carbonic anhydrase activity, and plasma membrane redox activity in marine phytoplankton species. J Phycol 35: 1200–1205

Nishimura T, Takahashi Y, Yamaguchi O, Suzuki H, Maeda S and Omata T (2008) Mechanism of low CO_2-induced activation of the *cmp* bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. Mol Microbiol. 68:98–109

Ogawa T (1990) Mutants of *Synechocystis* PCC 6803 defective in inorganic carbon transport. Plant Physiol 94: 760–765

Ogawa T (1991a) A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC 6803. Proc Natl Acad Sci USA 88: 4275–4279

Ogawa T (1991b) Cloning and inactivation of a gene essential to inorganic carbon transport of *Synechocystis* PCC 6803. Plant Physiol 96: 280–284

Ogawa T (1992) Identification and characterization of the *ictA/ndhL* gene product essential to inorganic carbon transport of *Synechocystis* PCC 6803. Plant Physiol 99: 1604–1608

Ogawa T (1993) Molecular analysis of the CO_2 concentrating mechanism in cyanobacteria. In: Yamamoto HY and Smith C (eds) Photosynthetic Responses to the Environment, pp 113–125. American Society of Plant Physiologist, Rockville

Ogawa T and Kaplan A (2003) Inorganic carbon acquisition systems in cyanobacteria. Photosynth Res 77: 105–115

Ogawa T and Mi H (2007) Cyanobacterial NADPH dehydrogenase complexes. Photosynth Res 93:69–77

Ogawa T and Ogren WL (1985) Action spectra for accumulation of inorganic carbon in the cyanobacterium, *Anabaena variabilis*. Photochem Photobiol 41: 583–587

Ogawa T, Miyano A and Inoue Y (1985) Photosystem-I driven inorganic carbon transport in the cyanobacterium, *Anacystis nidulans*. Biochim Biophys Acta 808: 77–84

Ohkawa H, Pakrasi HB and Ogawa T (2000) Two types of functionally distinct NAD(P)H dehydrogenases in *Synechocystis* sp. strain PCC6803. J Biol Chem 275: 31630–31634

Ohkawa H, Sonoda M, Katoh H and Ogawa T (1998) The use of mutants in the analysis of the CCM in cyanobacteria. Can J Bot 76: 1025–1034

Ohkawa H, Sonoda M, Shibata M and Ogawa T (2001) Localization of NAD(P)H dehydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803. J Bacteriol 183: 4938–4939

Ohnishi N, Mukherjee B, Tsujikawa T, Yanase M, Nakano H, Moroney JV and Fukuzawa H (2010) Expression of a low CO_2-inducible protein, LCI1, increases inorganic carbon uptake in the green alga *Chlamydomonas reinhardtii*. Plant Cell 22: 3105–3117

Omata T (1992) Characterization of the downstream region of *cmpA*: Identification of a gene cluster encoding a putative permease of the cyanobacterium *Synechococcus* PCC7942. In: Murata N (ed) Research in Photosynthesis, pp. 807–810. Kluwer Academic Publishers, Dordrecht

Omata T and Ogawa T (1985) Changes in the polypeptide composition of the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* during adaptation to low CO_2 conditions. Plant Cell Physiol 26: 1075–1081

Omata T and Ogawa T (1986) Biosynthesis of a 42-kD polypeptide in the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* strain R2 during adaptation to low CO_2 conditions. Plant Physiol 80: 525–530

Omata T, Carlson TJ, Ogawa T and Piece J (1990) Sequencing and modification of the gene encoding the 42-kilodalton protein in the cytoplasmic membrane of *Synechococcus* PCC 7942. Plant Physiol 93: 305–311

Omata T, Gohta S, Takahashi Y, Harano Y and Maeda S (2001) Involvement of a CbbR homolog in low CO_2-induced activation of the bicarbonate transporter operon in cyanobacteria. J Bacteriol 183: 1891–1898

Omata T, Price GD, Badger MR, Okamura M, Gohta S and Ogawa T (1999) Identification of an ATP-binding cassette transporter involved in bicarbonate uptake in the cyanobacterium *Synechococcus* sp. Strain. Proc Natl Acad Sci USA 96: 13571–13576

Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. Phycologia 40: 503–529

Pollock SV, Prout DL, Godfrey AC, Lemaire SD and Moroney JV (2004) The *Chlamydomonas reinhardtii* proteins *Ccp1* and *Ccp2* are required for long-term growth, but are not necessary for efficient photosynthesis, in a low-CO_2 environment. Plant Mol Biol 56: 125–132

Price GD, Badger MR, Woodger FJ and Long BM (2008) Advances in understanding the cyanobacterial CO_2-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. J Exp Bot 59: 1441–1461

Price GD, Howitt SM, Harrison K and Badger MR (1993) Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in carboxysome assembly and function. J Bacteriol 175:2871–2879

Price GD, Woodger FJ, Badger MR, Howitt SM and Tuker L (2004) Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. Proc Natl Acad Sci USA 101: 18228–18233

Prommeenate P, Lennon AM, Markert C, Hippler M and Nixon PJ (2004) Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803 - Identification of two new *ndh* gene products with nuclear-encoded homologues in the chloroplast Ndh complex. J Biol Chem 279: 28165–28173

Ramazanov Z, Rawat M, Henk MC, Mason CB, Matthews SW and Moroney JV (1995) The induction of the CO_2-concentrating mechanism is correlated with the formation of the starch sheath around the pyrenoid of *Chlamydomonas reinhardtii*. Planta 195: 210–216

Ratti S, Giordano M and Morse D (2007) CO_2-concentrating mechanisms of the potentially toxic dinoflagellate

Protoceratium reticulatum (Dinophyceae, Gonyaulacales). J Phycol. 43: 693–701

Rau GH, Takahashi T, and Des Marais DJ (1989) Latitudinal variations in plankton ^{13}C: implications for CO_2 and productivity in past oceans. Nature 341: 516–518

Rau GH, Takahashi T, Des Marais DJ and Martin JH (1992) The relationship between delta ^{13}C of organic matter and [CO_2 (aq)] in ocean surface water: Data from a JGOFS site in the northeast Atlantic Ocean and a model. Geochim Cosmochim Acta 56: 1413–1419

Raven JA (1991) Implications of inorganic C utilization: ecology, evolution and geochemistry. Can J Bot 69: 908–924

Raven JA (1994) Carbon fixation and carbon availability in marine phytoplankton. Photosynth Res 39: 259–273

Raven JA (1997) Inorganic carbon acquisition by marine autotrophs. Adv Bot Res 27: 85–209

Raven JA (2003) Inorganic carbon concentrating mechanisms in relation to the biology of algae. Photosynth Res 77: 155–171

Raven JA, Ball LA, Beardal J, Giordano M and Maberly SC (2005) Algae lacking carbon concentrating mechanisms. Can J Bot 83: 879–890

Raven JA, Cockell CS, De La Rocha CL (2008) The evolution of inorganic carbon concentrating mechanisms in photosynthesis. Phil T R Soc B 303: 2641–2650

Raven JA, Johnston AM and Turpin DH (1993) Influence of changes in CO_2 concentration and temperature on marine phytoplankton $^{13}C/^{12}C$ ratios: an analysis of possible mechanisms. Global Planet Change 8: 1–12

Reinfelder JR, Kraepiel AML and Morel FMM (2000) Unicellular C_4 photosynthesis in a marine diatom. Nature 407: 996–999

Reinfelder JR, Milligan AJ and Morel FMM (2004) The role of the C_4 pathway in carbon accumulation and fixation in a marine diatom. Plant Physiol 135: 2106–2111

Reinhold L, Volokita M, Zenvirth D and Kaplan A (1984) Is HCO_3^- transport in *Anabaena* a Na^+-symport? Plant Physiol 76: 1090–1092

Rexach J, Montero B, Fernandez E and Galvan A (1999) Differential regulation of the high affinity nitrite transport system III and IV in *Chlamydomonas reinhardtii*. J Biol Chem 274: 27801–27806

Riebesell U (2004) Effects of CO_2 enrichment on marine phytoplankton. J Oceanogr 60: 719–729

Riebesell U, Wolf-Gladrow D and Smetacek V (1993) Carbon dioxide limitation of marine phytoplankton growth rates. Nature 361: 249–251

Riebesell U, Zondervan I, Rost B, Tortell PD, Zeebe RE and Morel FMM (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407: 364–367

Roberts K, Granum E, Leegood RC and Raven JA (2007) C_3 and C_4 pathways of photosynthetic carbon assimilation in marine diatoms are under genetic, not environmental, control. Plant Physiol 145: 230–235

Roberts SB, Lane TW and Morel FMM (1997) Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (bacillariophyceae). J Phycol 33: 845–850

Rochaix JD, Goldschmidt-Clermont M and Merchant S (eds) (1998) The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, Advances in Photosynthesis, Vol 7, Kluwer Academic Publishers, Dordrecht

Rost B, Riebesell U, Burkhardt S and Sültemeyer D (2003) Carbon acquisition of bloom-forming marine phytoplankton. Limnol Oceanogr 48: 55–67

Rost B, Zondervan I and Riebesell U (2002) Light-dependent carbon isotope fractionation in the coccolithophorid *Emiliania huxleyi*. Limnol Oceanogr 47: 120–128

Salon C, Mir NA and Canvin DT (1996) Influx and efflux of inorganic carbon in *Synechococcus* UTEX 625. Plant Cell Environ 19: 247–259

Satoh D, Hiraoka Y, Colman B and Matsuda Y (2001) Physiological and molecular biological characterization of intracellular carbonic anhydrase from the marine diatom *Phaeodactylum tricornutum.* Plant Physiol 126: 1459–1470

Schulz KG, Rost B, Burkhardt S, Riebesell U, Thoms S and Wolf-Gladrow DA (2007) The effect of iron availability on the regulation of inorganic carbon acquisition in the coccolithophore *Emiliania huxleyi* and the significance of cellular compartmentation for stable carbon isotope fractionation. Geochim Cosmochim Acta 71: 5301–5312

Schwarz R, Lieman-Hurwitz J, Hassidim M and Kaplan A (1992) Phenotypic complementation of high-CO_2-requiring mutants of the cyanobacterium *Synechococcus* sp. PCC 7942 by inosine 5'- monophosphate. Plant Physiol 100: 1987–1993

Schwarz R, Reinhold L and Kaplan A (1995) Low activation state of ribulose 1,5-bisphosphate carboxylase/oxygenase in carboxysome-defective *Synechococcus* mutants. Plant Physiol 108: 183–190

Sekino K and Shiraiwa Y (1994) Accumulation and utilization of dissolved inorganic carbon by a marine unicellular coccolithophorid, *Emiliania huxleyi*. Plant Cell Physiol 35: 353–361

Sharkey TD and Berry JA (1985) Carbon isotope fractionation in algae as influenced by inducible CO_2 concentrating mechanism. In: Lucus WJ and Berry JA (eds) Inorganic carbon uptake by aquatic photosynthetic organisms. pp 339–401. American Society of Plant Physiologists, Rockville

Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kaplan A and Ogawa T (2002) Genes essential to sodium-dependent bicarbonate transport in cyanobacteria. Function and phylogenetic analysis. J Biol Chem 277: 18658–18664

Shibata M, Ohkawa H, Kaneko T, Fukuzawa H, Tabata S, Kaplan A and Ogawa T (2001) Distinct constitutive and low-CO_2-induced CO_2 uptake systems in cyanobacteria: Genes involved and their phylogenetic relationship with homologous genes in other organisms. Proc Nat Acad Sci USA 98: 11789–11794

Shiraiwa Y (2003) Physiological regulation of carbon fixation in the photosynthesis and calcification of coccolithophorids. Comp Biochem Physiol B-Biochem & Mol Biol 136: 775–783

Sippola K and Aro EM (1999) Thiol redox state regulates expression of *psbA* genes in *Synechococcus* sp. PCC 7942. Plant Mol Biol 41:425–433

So AKC, Kassam A and Espie GS (1998) Na^+-dependent HCO_3^- transport in the cyanobacterium *Synechocystis* PCC6803. Can J Bot 76: 1084–1091

Soupene E, Inwood W and Kustu S (2004) Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO_2. Proc Natl Acad Sci USA 101: 7787–7792

Spalding MH, Spreitzer RJ and Ogren WL (1983) Reduced inorganic carbon transport in a CO_2-requiring mutant of *Chlamydomonas reinhardtii*. Plant Physiol 73: 273–276

Steinke M, Evans C, Lee GA and Malin G (2007) Substrate kinetics of DMSP-lyases in axenic cultures and mesocosm populations of *Emiliania huxleyi*. Aquat Sci 69: 352–359

Sukenik A, Eshkol R, Livne A, Hadas O, Rom M, Tchernov D, Vardi A and Kaplan A (2002) Inhibition of growth and photosynthesis of the dinoflagellate *Peridinium gatunense* by *Microcystis* sp. (cyanobacteria): a novel allelopathic mechanism. Limnol Oceanogr 47:1656–1663

Sukenik A, Tchernov D, Huerta E, Lubian LM, Kaplan A and Livne A (1997) Uptake, efflux and photosynthetic utilization of inorganic carbon by the marine *Eustigmatophyte nannochloropsis* sp. J Phycol 33: 969–974

Suzuki K, Marek LF and Spalding MH (1990) A photorespiratory mutant of *Chlamydomonas reinhardtii*. Plant Physiol 93: 231–237

Szabo E and Colman B (2007) Isolation and characterization of carbonic anhydrases from the marine diatom *Phaeodactylum tricornutum.* Physiol Plant 129: 484–492

Takahashi Y, Yamaguchi O and Omata T (2004) Roles of CmpR, a LysR family transcriptional regulator, in acclimation of the cyanobacterium *Synechococcus* sp. strain PCC 7942 to low-CO_2 and high-light conditions. Mol Microbiol 52: 837–845

Tanaka Y, Nakatsuma D, Harada H, Ishida M and Matsuda Y (2005) Localization of soluble β-carbonic anhydrase in the marine diatom *Phaeodactylum tricornutum* sorting to the chloroplast and cluster formation on the girdle lamellae. Plant Physiol 138: 207–217

Tchernov D, Gorbunov MY, de Vargas C, Yadav SN, Milligan AJ, Haggblom M and Falkowski PG (2004) Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. Proc Natl Acad Sci USA 101: 13531–13535

Tchernov D, Hassidim M, Luz B, Sukenik A, Reinhold L and Kaplan A (1997) Sustained net CO_2 evolution during photosynthesis by marine microorganisms. Curr Biol 7: 723–728

Tchernov D, Helman Y, Keren N, Luz B, Ohad I, Reinhold L, Ogawa T and Kaplan A (2001) Passive entry of CO_2 and its energy-dependent intracellular conversion to HCO_3^- in cyanobacteria are driven by a photosystem I-generated ΔH^+. J Biol Chem 276: 23450–23455

Tchernov D, Silverman J, Luz B, Reinhold L and Kaplan A (2003) Massive light-dependent cycling of inorganic carbon between photosynthetic microorganisms and their surroundings. Photosynth Res 77: 95–103

Tortell PD and Morel FMM (2002) Sources of inorganic carbon for phytoplankton in the eastern subtropical and equatorial pacific ocean. Limnol Oceanogr 47: 1012–1022

Tortell PD, DiTullio GR, Sigman PM and Morel FMM (2002) CO_2 effects on taxonomic composition and nutrient utilization in an equatorial pacific phytoplankton assemblage. Marine Ecol Prog Ser 23: 37–43

Turkina MV, Blanco-Rivero A, Vainonen JP, Vener AV and Villarejo A (2006) CO_2 limitation induces specific redox-dependent protein phosphorylation in *Chlamydomonas reinhardtii*. Proteomics 6: 2693–2704

Tyrrell PN, Kandasamy RA, Crotty CM and Espie GS (1996) Ethoxyzolamide differentially inhibits CO_2 uptake and Na^+-independent and Na^+-dependent HCO_3^- uptake in the cyanobacterium *Synechococcus* sp. UTEX 625. Plant Physiol 112: 79–88

Van K and Spalding MH (1999) Periplasmic carbonic anhydrase structural gene (Cah1) mutant in *Chlamydomonas reinhardtii*. Plant Physiol 120: 757–64

Vardi A, Berman-Frank I, Rozenberg T, Hadas O, Kaplan A and Levine A (1999) Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO_2 limitation and oxidative stress. Curr Biol 9: 1061–1064

Vardi A, Schatz D, Beeri K, Motro U, Sukenik A, Levine A and Kaplan A (2002) Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. Curr Biol 12: 1767–1772

Volokita M, Zenvirth D, Kaplan A and Reinhold L (1984) Nature of the inorganic carbon species actively taken up by the cyanobacterium *Anabaena variabilis*. Plant Physiol 76: 599–602

Wang HL, Postier BL and Burnap RL (2004) Alterations in global patterns of gene expression in *Synechocystis* sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of *ndhR*, a LysR family regulator. J Biol Chem 279: 5739–5751

Wang Y and Spalding MH (2006) An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 103: 10110–10115

Woodger FJ, Badger MR and Price GD (2003) Inorganic carbon limitation induces transcripts encoding components of the CO_2-concentrating mechanism in *Synechococcus* sp. PCC7942 through a redox-independent pathway. Plant Physiol 133: 2069–2080

Woodger FJ, Bryant DA and Price GD (2005) Sensing of inorganic carbon limitation in *Synechococcus* PCC 7942 is correlated with the size of the internal inorganic carbon pool and involves oxygen. Plant Physiol 139:1959–1969

Woodger FJ, Bryant DA and Price GD (2007) Transcriptional regulation of the CO_2-concentrating mechanism in a euryhaline, coastal marine cyanobacterium, Synechococcus sp. Strain PCC 7002: role of NdhR/CcmR. J Bacteriol 189:3335–3347

Xiang Y, Zhang J and Weeks DP (2001) The Cia5 gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 98: 5341–5346

Xu M, Bernát G, Singh G, Mi H, Rögner M, Pakrasi HB and Ogawa T (2008a) Properties of Mutants of *Synechocystis* sp. Strain PCC 6803 Lacking Carbon Sequestration Systems. Plant Cell Physiol 49: 1672–1677

Xu M, Ogawa T, Pakrasi HP and Mi H (2008b) Identification and localization of the CupB protein involved in constitutive CO_2 uptake in the cyanobacterium, *Synechocystis* sp. PCC 6803. Plant Cell Physiol 49: 994–997

Yamano T, Miura K and Fukuzawa H (2008) Expression analysis of genes associated with the induction of the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Physiol 147: 340–354

Yamano T, Tsujikawa T, Hatano K, Ozawa SI, Takahashi Y and Fukuzawa H (2010) Light and low-CO_2-dependent LCIB-LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. Plant Cell Physiol 51: 1453–1468

Yoshioka S, Tanijuchi F, Miura K, Inoue T, Yamano T and Fukuzawa H (2004) The novel Myb transcription factor LCR1 regulates the CO_2-response gene Cah1, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. Plant Cell 16: 1466–1477

Zhang P, Battchikova N, Jansen T, Appel J, Ogawa T and Aro EM (2004) Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/NdhF3/CupA/Sll1735 in *Synechocystis* sp. PCC 6803. Plant Cell 16: 3326–3340

Zhang P, Battchikova N, Paakkarinen V, Katoh H, Iwai M, Ikeuchi M, Pakrasi HB, Ogawa T and Aro EM (2005) Isolation, subunit composition and interaction of the NDH-1 complexes from *Thermosynechococcus elongatus* BP-1. Biochem J 390: 513–520

Zhang P, Sicora CI, Vorontsova N, Allahverdiyeva Y, Battchikova N, Nixon PJ and Aro EM (2007) FtsH protease is required for induction of inorganic carbon acquisition complexes in Synechocystis sp. PCC 6803. Mol Microbiol 65: 728–740

Zondervan I (2007) The effects of light, macronutrients, trace metals and CO_2 on the production of calcium carbonate and organic carbon in coccolithophores—A review. Deep Sea Research Part II: Topical Studies in Oceanography 54: 521–525

Chapter 26

Autotrophic Carbon Dioxide Fixation

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Summary

Carbon dioxide fixation is overwhelmingly carried out by reactions of the Calvin-Benson cycle of plants, green algae, and cyanobacteria. Three other carbon dioxide reduction pathways are known to allow autotrophic growth, but these are mainly limited to anaerobic organisms. In this chapter the anaerobic

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autotrophic carbon reduction pathways are briefly described followed by a more detailed look at the Calvin-Benson cycle and its regulation. The Calvin-Benson cycle reaction sequence is similar to the non-oxidative branch of the pentose phosphate pathway although the enzyme transaldolase of the pentose phosphate pathway is not used and a novel enzymatic activity, sedoheptulose-1,7-bisphosphatase, is substituted. The carbon fixation enzyme of the Calvin-Benson cycle, Rubisco, varies in its properties and is currently the subject of much research aimed at improving the efficiency of photosynthesis. Rubisco exists in three different conformations and there is also a gene coding for a related protein that does not have Rubisco activity but could be the evolutionary progenitor. The Calvin-Benson cycle is often referred to as the dark reactions of photosynthesis but it does not proceed in darkness for several reasons. In addition to providing reducing power and ATP, photosynthetic electron transport causes increased pH and magnesium in the stroma plus reducing power for thioredoxin to activate Calvin-Benson cycle enzymes and inactivate enzymes that would lead to futile cycles when Calvin-Benson cycle enzymes are active. There is a surprising regulation of phosphoglucose isomerase that can shift the regulation of the rate of starch synthesis from ADPglucose pyrophosphorylase to phosphoglucose isomerase. This may control the amount of carbon that leaves the Calvin-Benson cycle, since excessive loss of carbon from the Calvin-Benson cycle intermediates can lead to a collapse of the cycle. Finally, a recently discovered regulation mechanism based on a small protein called CP12 is discussed.

I. Introduction

Carbon fixation is the conversion of inorganic carbon into stable products. Most often, the term is used to mean the reduction of carbon dioxide or bicarbonate to compounds needed for life, especially sugars. It is generally believed that the carbon fixation of photosynthesis supports nearly all life on Earth. Chemosynthesis using molecular hydrogen, H_2S, and other reduced compounds as energy sources, may be the dominant source of reduced carbon in some environments, especially above ~70°C, for example in hot springs (Spear et al., 2005), in the deep crust of the Earth (Gold, 1992), and deep sea vents (Jannasch and Mottl, 1985; D'Imperio et al., 2008). Photosynthetic carbon fixation by plants (the Calvin-Benson Cycle) is most important to humans. The Calvin-Benson cycle is the dominant pathway in aerobic organisms but there are several other autotrophic carbon fixation pathways, typically restricted to anaerobic conditions. In terms of evolution, other carbon fixation pathways may have evolved earlier than the Calvin-Benson cycle and may be more important in understanding the early events of evolution.

A. Importance of Carbon Fixation in Photosynthesis

Like photosynthetic electron transport, photosynthetic carbon metabolism is primarily a bacterial process. Photosynthetic carbon fixation is uncommon in archaea; archaeal carbon fixation at high temperature (>70°C) is considered to be exclusively chemosynthetic (Spear et al., 2005). In eukaryotes, photosynthetic carbon fixation is restricted to plastids, believed to be of bacterial origin.

Bacteria and plants use the products of photosynthetic electron transport for a number of processes including ion transport, reduction of nitrite and sulfate, and other cellular energy needs.

Abbreviations: 6PG – 6-phosphogluconate; AGPase – ADPglucose pyrophosphorylase; CA1P – 2-carboxyarabinitol 1-phosphate; CABP – 2-carboxyarabinitol 1, 5-bisphosphate; DHAP – Dihydroxyacetone phosphate; E4P – Erythritol 4-phosphate; F6P – Fructose 6-phosphate; FBP – Fructose 1,6-bisphosphate; FBPase – Fructose-1,6-bisphosphatase; G1,6BP – Glucose 1,6-bisphosphate; G6P – Glucose 6-phosphate; GAP – Glyceraldehyde 3-phosphate; PEP – Phosphoenolpyruvate; PG – Phosphoglycolate; PGA – 3-phosphoglycerate; PGI – Phosphoglucose isomerase; PGM – Phosphoglucomutase; PRK – Phosphoribulokinase; R5P – Ribose 5-phosphate; Ru5P – Ribulose 5-phosphate; RuBP – Ribulose 1,5-bisphosphate; S7P – Sedoheptulose 7-phosphate; SBP – Sedoheptulose 1,7-bisphosphate; SBPase – Sedoheptulose-1,7-bisphosphatase; Xu5P – Xylulose 5-phosphate

However, most of the high energy intermediates produced by photosynthetic electron transport (e.g., ATP and NADPH) are used in carbon fixation. Reductive carbon fixation requires a source of electrons, but the source does not have to be derived from light energy. The different pathways of reductive carbon fixation are not linked to specific electron sources. For example, Calvin-Benson cycle carbon fixation can occur in archaea that derive reducing power from molecular hydrogen or H_2S (D'Imperio et al., 2008).

B. Terminology

A number of terms are used to describe the central reactions of photosynthetic carbon fixation. The term dark reactions is used because it can be shown that in flashing light, carbon metabolism can continue during short periods of darkness, consuming ATP and NADPH made in the "light reactions," through photochemically driven electron transport. However, most of the electron transport reactions of photosynthesis are also dark reactions by this definition. Electron transport can continue during dark periods of flashing light by consuming electrons in the plastoquinone pool (Chow et al., 1989; Tennessen et al., 1995) and using the transthylakoid energy gradient to make ATP (Sharkey et al., 1986a). The flashes must be much shorter for electron transport reactions to demonstrate their "dark reactions," but the principle is the same as that used to define carbon metabolism as dark reactions. Another problem that arises is the dark reactions of plants cannot occur in darkness because the conditions in the stroma of the chloroplast keep critical enzymes inactive in the dark. Best known is the dithiol reduction of several carbon metabolism enzymes that depend on photosynthetic electron transport. Describing the two components of photosynthesis linked by ATP and NADPH as photosynthetic carbon metabolism and photosynthetic electron transport is a clear and simple way to describe the metabolism under consideration.

This chapter will cover four pathways by which carbon dioxide is reduced for autotrophic growth and then explore some important aspects of the reductive pentose phosphate cycle, the Calvin-Benson cycle, in detail.

II. Carbon Fixation Mechanisms

With the availability of ^{14}C and the use of paper chromatography, Andrew Benson, Melvin Calvin and colleagues (see summaries and descriptions of the early work in Benson, 2002; Bassham and Calvin, 1957; Calvin and Bassham, 1962) determined the path of carbon in algae and land plants. The cycle they proposed has been confirmed to be correct. The remarkable success of elucidating the reductive pentose phosphate pathway, and the importance of the Calvin-Benson cycle to humans, slowed the elucidation of other carbon fixation pathways (Buchanan and Arnon, 1990). In this section, reductive versus non-reductive mechanisms of carbon fixation are first distinguished, and then four reductive pathways for carbon fixation are described.

A. Non-autotrophic Carbon Fixation

Autotrophy requires carbon fixation and reduction in pathways that can supply all of an organism's carbon needs. An important distinction is made between reductive carbon fixation and carbon fixation that does not lead to reduction of the carbon. One example of non-reductive carbon fixation is the carboxylation of acetyl CoA to make malonyl CoA during fatty acid synthesis. This activates the acetate group for subsequent use, which involves decarboxylation. In essence, CO_2 is used catalytically, but the carbon does not become reduced, CO_2 is released in subsequent steps of fatty acid synthesis. Similarly, phosphoenolpyruvate can be carboxylated and the resulting carboxylic acid can be incorporated into amino acids or decarboxylated, but it need not become reduced.

There are a number of carboxylations that can lead to incorporation of reduced carbon in specific compounds in anaerobic organisms. These reactions often use ferredoxin (see Buchanan and Arnon, 1990). However, incorporation and reduction of carbon into specific compounds does not necessarily lead to autotrophy and only mechanisms that allow autotrophic growth will be considered here. In the next two sections the reductive carbon fixation pathways known to support autotrophy are described in the order of least to best known.

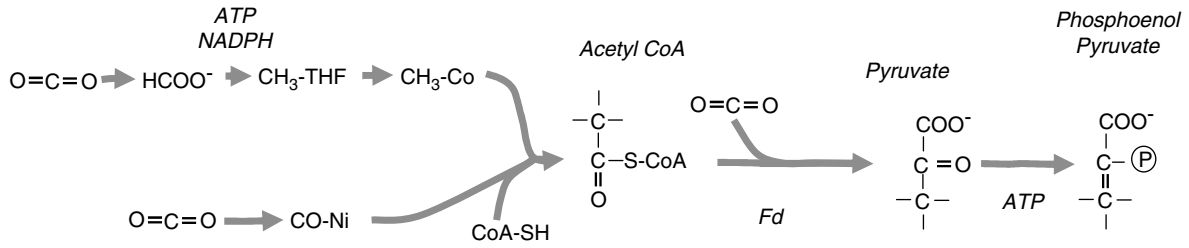

Fig. 26.1. The Wood/Ljungdahl pathway of acetate synthesis. THF is tetrahydrofolate, Co is cobalt of cobalamin. CoA-SH is Coenzyme A bound to hydrogen. Three carbon dioxide molecules are converted to pyruvate.

B. Anaerobic Reductive Carbon Fixation Mechanisms

Anaerobic organisms have several pathways that allow for autotrophic growth (Hügler et al., 2003a). The **Wood/Ljungdahl pathway** of acetate synthesis (Fig. 26.1) is known from the acetogenic bacteria such as *Clostridium sp.* (Ljungdahl, 1986; Ragsdale, 1997). This pathway is normally considered a fermentative pathway with glucose or xylulose being consumed and acetate being an electron sink and released from the bacterium. However, it has been shown that *Clostridium* can grow autotrophically using CO_2 and molecular hydrogen (Ljungdahl, 1986). The Wood/Ljungdahl pathway makes use of folic acid and cobalamin for one-carbon chemistry. The reductant is NADPH. The acetyl CoA that is produced can be carboxylated and reduced to pyruvate making use of ferredoxin. Pyruvate can undergo gluconeogenesis; the first step is conversion to phosphoenolpyruvate (PEP) using one or two ATPs. This pathway can be drawn as a linear, bifurcated pathway (Fig. 26.1) and allows chemoautotrophic growth.

The **3-hydroxyproprionate pathway** (Fig. 26.2) occurs in some archaea (Hügler et al., 2003a) and a few bacteria, notably *Chloroflexus aurantiacus* (Herter et al., 2002). The 3-hydroxyproprionate pathway makes use of NADPH for its reductant. The carboxylations of acetyl CoA and proprionyl CoA that characterize this cycle are catalyzed by a single bifunctional, biotin-containing enzyme in the archaean *Metallosphaera sedula* (Hügler et al., 2003b). All of the intermediates of this pathway are carboxylic acids. The product of the pathway can be glyoxylate but, at least in *Chloroflexus*, the glyoxylate can be combined with the intermediate proprionyl CoA to make methylmalyl CoA and eventually regenerate acetyl CoA and yield pyruvate. The 3-hydroxyproprionate pathway can be photoautotrophic, but organisms that use this pathway likely normally use molecular hydrogen for the electron source (Ward et al., 1998; Spear et al., 2005).

The **reverse Krebs cycle** (Fig. 26.3) or reductive carboxylic acid cycle (Evans et al., 1966; Buchanan and Arnon, 1990) is present in many bacteria, notably *Chlorobium limicola* forma *thiosulfatophilum* and is known to allow photoautotrophic growth. There is a report that this cycle might operate in the eukaryotic *Chlamydomonas reinhardtii* (Chen and Gibbs, 1992) but this has not been confirmed and an analysis of the genome does not support the presence of the reductive Krebs cycle in *C. reinhardtii*. A reverse Krebs cycle is known to be present in the archaean *Pyrobaculum islandicum* (Hügler et al., 2003a). Biotin is involved in the carboxylations and ferredoxin is used in some places instead of NADPH (Buchanan and Arnon, 1990). The product of the reverse Krebs cycle is acetyl CoA, which can undergo gluconeogenic reactions.

Some of the reductive steps in the reverse Krebs cycle can occur non-enzymatically in the presence of ZnS and so is of interest as a prebiotic CO_2 fixation mechanism (Zhang and Martin, 2006). The reverse Krebs cycle is only found in micro- or anaerobic situations giving rise to the speculation that the reductive Krebs cycle may have evolved before the oxidative Krebs cycle, before there was significant molecular oxygen available in the biosphere (Buchanan and Arnon, 1990). The reductive (and oxidative) Krebs cycle intermediates are all carboxylic acids.

C. Aerobic Carbon Fixation

The dominant pathway for carbon fixation in aerobic organisms is the **Calvin-Benson cycle**, also often called the Calvin Benson Bassham (CBB)

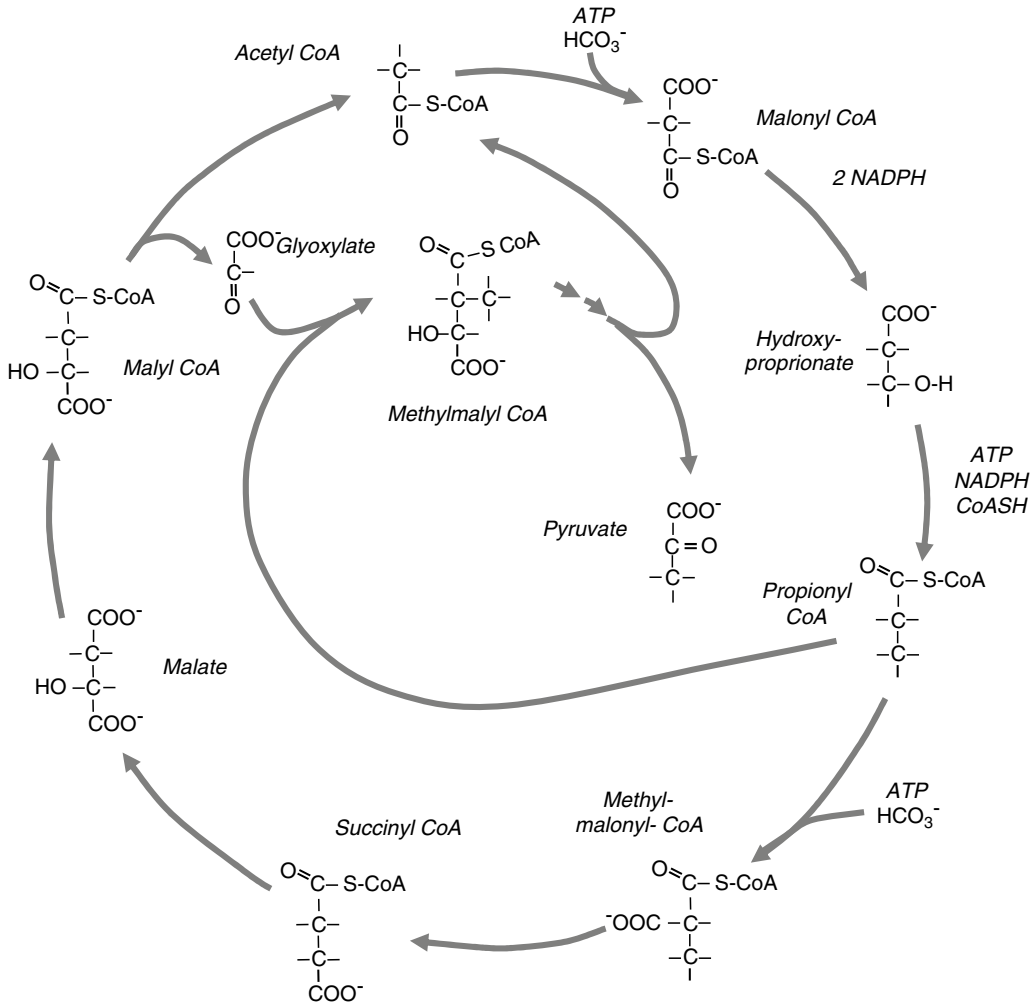

Fig. 26.2. Hydroxy proprionate pathway of *Chloroflexus*. Three bicarbonate molecules can be converted to one pyruvate in this scheme.

cycle (Fig. 26.4), or reductive pentose phosphate pathway. It is also found in many anaerobic organisms but may have been acquired secondarily (Delwiche and Palmer, 1996; Boucher et al., 2003). The Calvin-Benson cycle uses NADPH for reductant and a unique carboxylase that does not depend on either biotin or folic acid. All of the intermediates are phosphorylated and most are sugars; only one intermediate is a carboxylic acid. The reliance on sugar phosphates is a significant departure from the reactions that characterize the other autotrophic carbon fixation pathways. This pathway uses only NADPH; ferredoxin is not directly used to donate electrons to intermediates of the cycle. The reducing power for the Calvin-Benson cycle often comes from tetrapyrole-based photosynthetic electron transport, but can also come from molecular hydrogen; the Calvin-Benson cycle is not obligatorily linked to photosynthetic electron transport.

The Calvin-Benson cycle is the predominant method of autotrophy among aerobic organisms while the other pathways described are generally restricted to anaerobes or microaerobic conditions. It is reasonable to speculate that the Calvin-Benson cycle was the last of the described pathways to evolve, and there is evidence that the Calvin-Benson cycle enzymes of eukaryotes were drawn from both eukaryotic and bacterial sources (Martin et al., 2000). It must also be kept in mind that "eukaryotic" photosynthesis really represents bacterial photosynthesis because of the strong

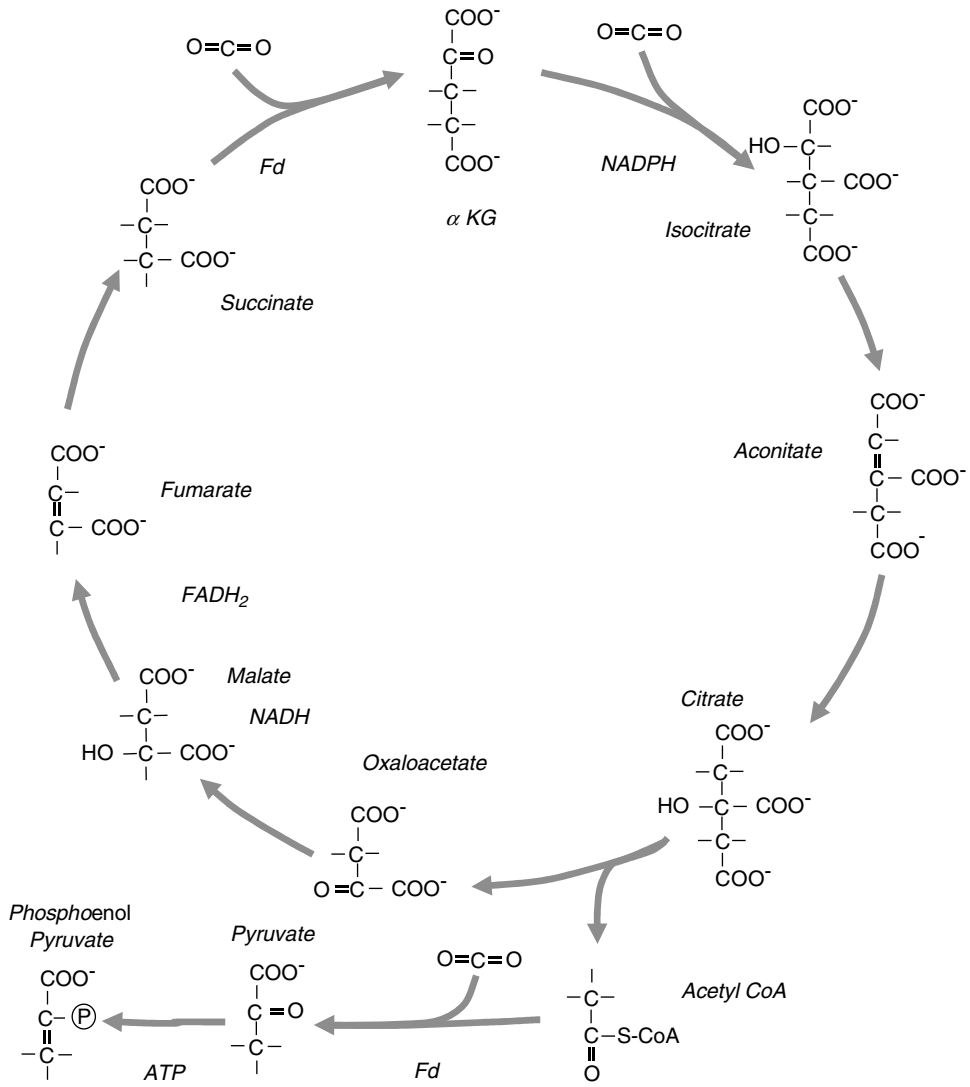

Fig. 26.3. The reverse Krebs cycle. The reverse Krebs cycle can make direct use of ferredoxin (Fd).

likelihood that all photosynthesis of eukaryotes is the result of endosymbiosis of a bacterium and some unknown progenitor more closely associated with archaea than bacteria (Margulis, 1981; McFadden, 2001).

1. The Pentose Phosphate Reaction Sequence

The pentose phosphate pathway is a phrase applied to a collection of reactions widely distributed among the domains of life that can theoretically metabolize glucose 6-phosphate (G6P) all the way to CO_2. This pathway probably supplies reducing power in the form of NADPH and carbon skeletons for anabolic pathways. The pathway is divided into an oxidative branch and non-oxidative branch. These are two very different metabolic sequences and their roles in the Calvin-Benson cycle are distinct. In the oxidative branch of the pentose phosphate pathway, G6P is converted to 6 phosphogluconate (6PG), and then to ribulose 5-phosphate (Ru5P), with two NADP reduced to NADPH. This branch of the pathway is not part of the Calvin-Benson cycle, though it is important in chloroplast metabolism (Debnam and Emes, 1999). A xylulose 5-phosphate (Xu5P)

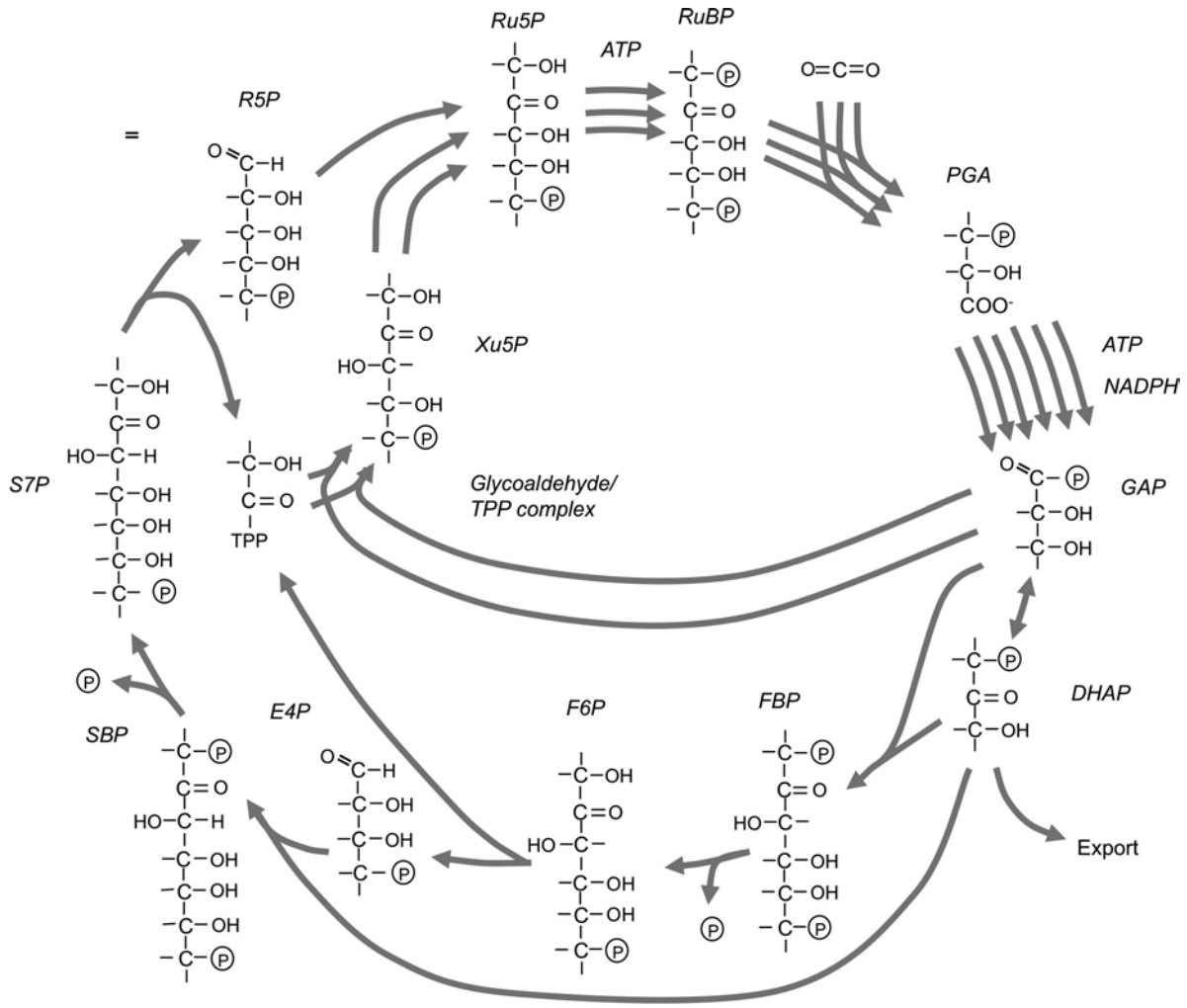

Fig. 26.4. The Calvin-Benson cycle.

translocator connects pentose metabolism on the chloroplast with the cytosol of the plant cell (Eicks et al., 2002). This pathway likely supplies reducing power at night and carbon skeletons [especially erythrose 4-phosphate (E4P)] for chloroplast metabolism such as the shikimic acid pathway.

The non-oxidative branch of the pentose phosphate pathway involves interconversions of pentose phosphates and triose phosphates, with both erythrose and sedoheptulose phosphates being involved. The pentose phosphate reactions of the Calvin-Benson cycle differ from those normally associated with the pentose phosphate pathway in that transaldolase appears not to be involved. Instead, the Calvin-Benson cycle uses a novel enzyme, sedoheptulose-1,7-bisphosphatase (SBPase), to allow a modified carbon flow. The carbon flow if transaldolase were used instead is shown in Fig. 26.5.

2. The Carboxylating Enzyme

The carboxylase of the Calvin-Benson cycle fixes carbon by combining CO_2 with the sugar phosphate ribulose 1,5-bisphosphate (RuBP), which immediately splits into two molecules of 3-phosphoglyceric acid (PGA). Plants use this mechanism of carbon fixation exclusively. Rubisco is the common name for the enzyme that carries out this reaction; the name was devised to acknowledge that ribulose is the sugar involved, that it is a

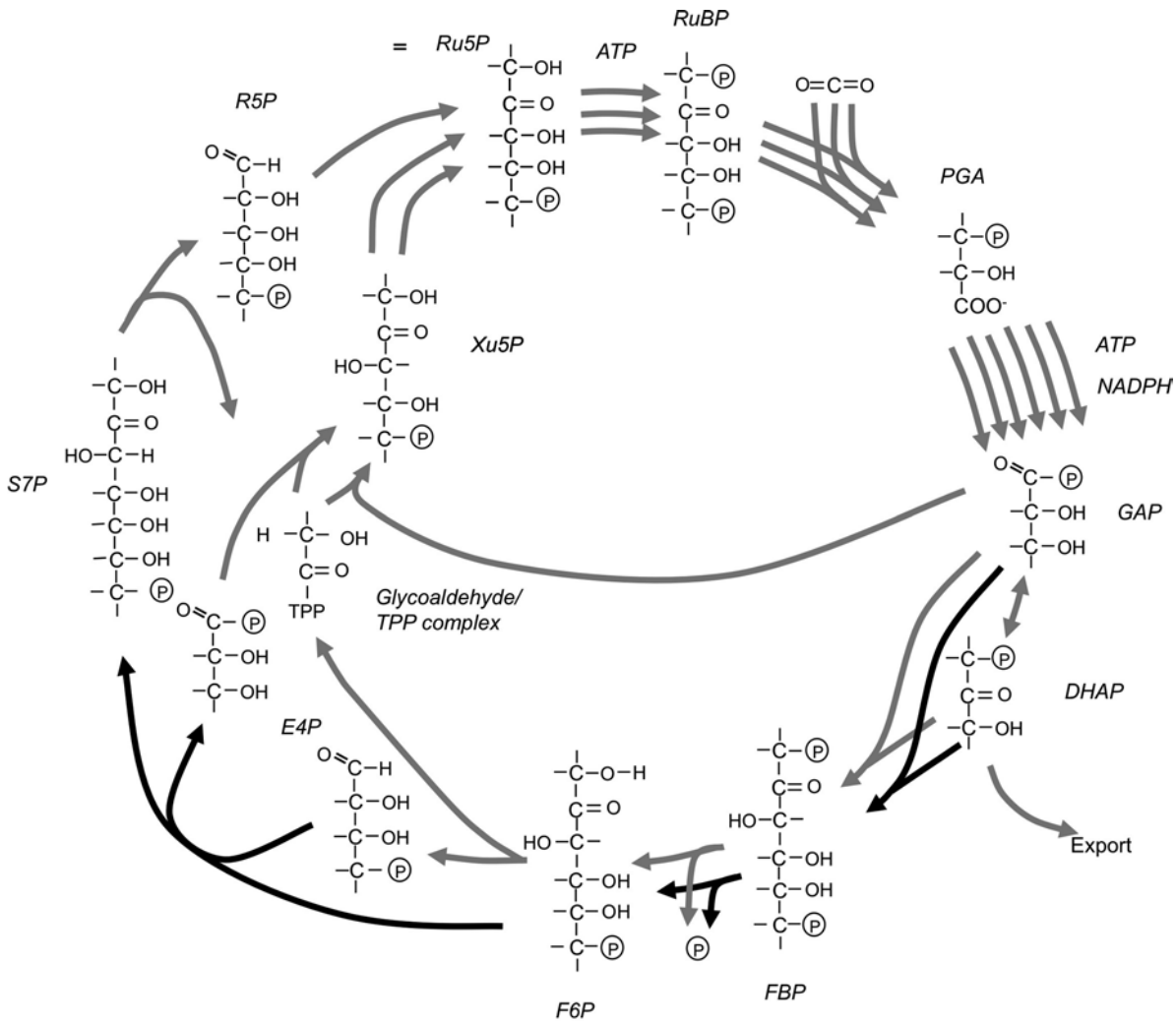

Fig. 26.5. The Calvin-Benson cycle if it were simply the reverse of the pentose phosphate pathway. The non-oxidative branch of the pentose phosphate pathway uses transaldolase to make (or breakdown) sedoheptulose 7-phosphate, which early labeling studies had shown to be important. Comparing this scheme with that in Fig. 26.4 shows how the novel enzyme sedoheptulose-1,7-bisphosphatatse can be used instead of transaldolase.

bisphosphate (two phosphates but at different parts of the molecule) and that the enzyme is bifunctional, catalyzing both carboxylation and oxygenation of the substrate.

The RuBP required by Rubisco is normally made by another enzyme unique to the Calvin-Benson cycle called phosphoribulokinase (PRK). There is a report of Rubisco activity in the archaean *Pyrodictium sp.* (Hügler et al., 2003a) but there is no evidence for PRK. At least some archaea convert phosphoribose pyrophosphate to RuBP opening up the possibility of Rubisco-mediated carbon fixation in the archaea using most of the Calvin-Benson cycle (Finn and Tabita, 2004). Even organisms that use hydrogen as an electron source sometimes use Rubisco (Chung et al., 1993). It has also been reported that seeds use Rubisco in metabolism that includes a subset of the Calvin-Benson cycle reactions (Schwender et al., 2004), not the complete Calvin-Benson cycle. This allows more efficient conversion of carbon from sugar to lipid in the seed than would be possible without the "Rubisco bypass."

Rubisco-mediated carbon fixation and the Calvin-Benson cycle as it occurs in land plant chloroplasts will be the focus of the rest of this chapter.

III. Ribulose *bis* Phosphate Carboxylase/Oxygenase (Rubisco)

A. Types of Rubisco

The minimally active form of Rubisco is a dimer. Each dimer has two active sites composed of amino acid residues from both subunits (Andersson and Taylor, 2003; Badger and Bek, 2008). In plants, four Rubisco dimers interact with eight small subunits to make an L_8S_8 enzyme. The role of the small subunits is not entirely clear but it has been suggested they might concentrate the enzyme by holding four dimers together or improve the kinetics of Rubisco, especially the selectivity for CO_2 in preference over O_2 (Spreitzer, 2003). Four small subunits sit at the top and four at the bottom of the four L2 dimers and have loops that extend into a central cavity surrounded by the four L2 dimers (Andersson and Taylor, 2003; Spreitzer, 2003). This quaternary structure of Rubisco has been described as $(L_2)_4(S_4)_2$. This form of Rubisco is called form I and has a molecular mass of ~560 kDa.

Form I (L_8S_8) Rubiscos are found in cyanobacteria and eukaryotes, but also in some halophilic archaea (Altekar and Rajagopalan, 1990; Rajagopalan and Altekar, 1994). Form I Rubiscos are further divided between those found in green algae (G type) and plants and those found in some red algae and plastids derived from them (R type) (Badger and Bek, 2008). The different Rubiscos can have widely differing kinetics, especially in the propensity for oxygenation of RuBP rather than carboxylation. Some soil bacteria have green-type form I Rubiscos but many more have red-type form I Rubiscos (Selesi et al., 2005). Rubisco appears to be especially prone to transfer among species (Delwiche and Palmer, 1996).

A number of bacteria have an L_2 form (form II) of Rubisco with a monomer molecular mass of ~55 kDa. Form II Rubiscos are found in some proteobacteria and *Gonyaulax polyedra* but not in cyanobacteria (which contain only green form I). Form II Rubiscos function as homodimers. Some bacteria, notably *Rhodobacter,* have both form I and form II Rubiscos (Delwiche and Palmer, 1996).

A third form of Rubisco was recognized from hyperthermophilic archaea (*Pyrococcus kodakaraensis*) (Ezaki et al., 1999; Watson et al., 1999). This form of Rubisco has five L2 dimers but no small subunits and this structure provides for very high temperature stability (optimum temperature for activity in excess of 90°C) (Maeda et al., 1999; Kitano et al., 2001; Maeda et al., 2002). It has not yet been proven whether this Rubisco allows autotrophic growth, but there also is no evidence against the hypothesis that this form III Rubisco provides for chemoautotrophic growth.

A fourth form (form IV) was added with the sequencing of "Rubisco-like proteins" that are related by sequence similarity to the other three forms of Rubisco but do not catalyze carboxylation (Hanson and Tabita, 2001; Ashida et al., 2003). The amino acid sequences of different Rubiscos indicate that all Rubiscos had a common origin but several of the critical amino acids conserved in the active site of Rubiscos are not present in Rubisco-like proteins (Li et al., 2005). There also is no evidence for enzyme activation through formation of a carbamate or coordination of Mg^{2+} (see Section III.B.). Form IV "Rubisco" may be an amino acid processing enzyme (Hanson and Tabita, 2001). Ashida et al. (2003) were able to demonstrate that a *Bacillus subtilus* strain lacking the methionine salvage pathway enzyme 2,3-diketo-5-methylthiopentyl-1-phosphate enolase could be rescued by expressing the *Rhodospirillum rubrum* form II Rubisco, showing that Rubisco retained the ability to function in sulfur metabolism in addition to carboxylation of RuBP.

Tabita et al. (2008) consider Form III Rubiscos of archaea to be the progenitors of all other Rubisco and Rubisco-like proteins while Ashida et al. (2008) consider the more widely distributed Rubisco-like proteins to be the source from which Rubiscos evolved. The presence of Rubiscos in all three domains of life, archaea, bacteria, and eukaryotes, might indicate an ancient evolution. However, this could also reflect the relatively common lateral gene transfer that appears to characterize photosynthetic mechanisms (Delwiche and Palmer, 1996; Boucher et al., 2003). Eukaryotic Rubisco is the result of endosymbiosis and is derived from cyanobacterial Rubisco rather than representing a common evolutionary origin of Rubisco before the domains of life diverged (McFadden, 2001).

B. Rubisco Activation

Rubisco requires post-translational modification to be catalytically competent. This modification involves formation of a carbamate by addition of

a carbon dioxide molecule to a lysine residue in the active site of Rubisco (Cleland et al., 1998). The carbon dioxide added is not the carbon dioxide that will eventually be fixed into sugars. The carbamate imparts a negative charge and Mg^{2+} is then coordinated onto the carbamylated enzyme. This process is stimulated by high pH (as high as pH 9). Only the carbamylated enzyme with Mg^{2+} is catalytically competent (Lorimer et al., 1976). Unfortunately, the uncarbamylated enzyme will bind RuBP and other sugar phosphates effectively blocking the active site and inhibiting the enzyme (Jordan and Chollet, 1983). The solution to this was discovered to be another enzyme, Rubisco activase (Somerville et al. 1982; Portis et al., 1986). This enzyme promotes Rubisco activation by carbamylation in response to light (Salvucci and Anderson, 1987) using ATP (Streusand and Portis, 1987). It is likely that Rubisco activase operates by removing bound ligands from uncarbamylated Rubisco molecules (Mate et al., 1996). A close analysis of rate constants for binding of ligands to different forms of Rubisco can help explain some of the observations about Rubisco activation by carbamylation (McNevin et al., 2006).

Is the change in activation state (carbamylation) a weakness of Rubisco or a regulatory mechanism? In cases where the potential activity of Rubisco exceeds the potential of other photosynthetic reactions to supply substrate RuBP, Rubisco often deactivates by decarbamylation leading to the paradoxical situation that when the light is low, and so should reduce the ability of the plant to regenerate RuBP, Rubisco deactivation keeps the RuBP content of leaves moderately high. That RuBP supply is the real problem can be proven by rapidly switching to lower light. At first RuBP concentrations fall, then they recover over several minutes as Rubisco deactivates (Mott et al., 1984). In the case where limited sucrose and starch synthesis restrict the ability to make ATP, again the RuBP level in a leaf at first falls, then recovers and can even be higher after the onset of feedback inhibition than before (Sharkey et al., 1986b). A similar story is possible in high temperature. When leaves are first exposed to 40°C, the RuBP level in the leaf can fall by 50% but then recover as Rubisco deactivates (Schrader et al., 2007). However, at high temperature the deactivation and RuBP build-up happen within seconds making it hard to observe the effect experimentally.

While not clear why, it is clear that when RuBP levels fall, Rubisco usually becomes decarbamylated, and thus deactivated (Portis et al., 1995) though not all treatments that lead to reduced RuBP lead to deactivation of Rubisco (Ruuska et al., 2000). It is possible that build-up of PGA, a carboxylic acid is deleterious, but it remains an enigma why Rubisco nearly always decarbamylates to match the ability of the chloroplast to provide RuBP rather than letting low RuBP concentration control the rate of Rubisco. It can be argued that the need for carbamylation of Rubisco has been exploited by land plants as a mechanism to match Rubisco activity with the other components of photosynthesis (Sharkey, 1985; Sharkey, 1990).

C. Rubisco Reaction Mechanism

The Rubisco reaction mechanism involves five distinct steps, enolization, carboxylation, hydration, carbon-carbon cleavage, and protonation (Fig. 26.6) (Roy and Andrews, 2000). Once RuBP binds to the enzyme, a loop of amino acids closes over the active site (Andersson and Taylor, 2003).

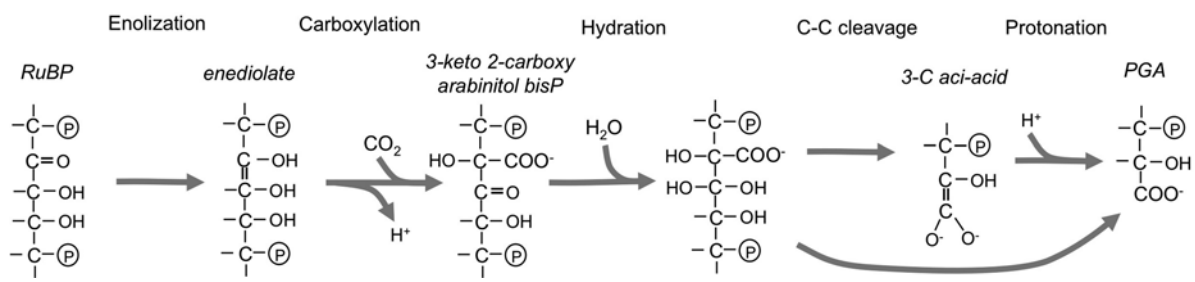

Fig. 26.6. Five steps of carboxylation catalyzed by Rubisco.

The preparation of RuBP by enolization makes an intermediate that has some similarity to the metabolite of the methionine pathway from which Rubisco could have evolved. The carboxylation step is interesting because of the possible lack of attachment of the substrate carbon dioxide to Rubisco. Instead, it is possible that RuBP is prepared for carboxylation and carbon dioxide attacks the enediolate without ever attaching to Rubisco *per se*. The carboxylation step appears to be mechanistically irreversible (Roy and Andrews, 2000).

D. Mistakes Made by Rubisco

There are several side reactions that are carried out by Rubisco that can be considered metabolic mistakes, reactions that reduce the efficiency of Rubisco (Fig. 26.7). The best known of these is oxygenation (Bowes et al., 1971; Ogren, 1984, 2003). Instead of fixing carbon dioxide, Rubisco can fix oxygen, touching off a series of reactions known as photorespiration. Another side reaction is the incorrect reversal of the enolization step.

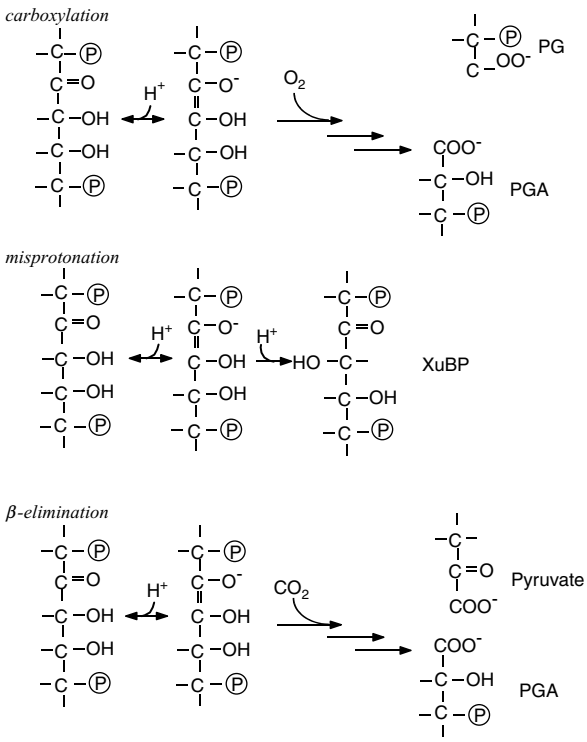

Fig. 26.7. Three catalytic "mistakes" made by Rubisco.

Enolization is readily reversible, but if the reversal proceeds with the wrong stereochemistry, xylulose 1,5-bisphosphate (XuBP) is made rather than RuBP (Edmondson et al., 1990). When Rubisco binds XuBP, loop closure occurs, but Rubisco cannot carboxylate XuBP and the loop cannot reopen. This results in XuBP being a dead-end inhibitor of Rubisco. Rubisco activase is required to remove XuBP and other sugar phosphates from Rubisco, perhaps by opening the loop that closes over the active site when it is occupied.

In the last step of the Rubisco reaction sequence, a proton is added to the three-carbon *aci*-acid to make PGA. However, about 1% of the time, β-elimination of the phosphate occurs resulting in pyruvate instead of PGA formation (Andrews and Kane, 1991). This is the only source of pyruvate inside the chloroplast and can be used for fatty acid synthesis (after conversion to acetyl CoA) or isoprenoid synthesis (in the methyl erythritol pathway) (Lichtenthaler, 2000) but it is generally considered to be relatively insignificant as a pyruvate source for chloroplast metabolism. Outside of this source, the chloroplast is dependent on the cytosol for pyruvate, which is likely supplied by import of PEP (Fischer et al., 1997). The dependence of the chloroplast on the cytosol for PEP and pyruvate is surprising in light of the biochemical flexibility of chloroplasts and universality of glycolysis among the domains of life. Nevertheless, there appears to be no phosphoglyceromutase in plastids, thus preventing the conversion of 3-PGA to 2-PGA (Stitt and ap Rees, 1979), which is needed to make PEP and pyruvate.

E. RuBP Analogs

Early on it was recognized that molecules similar to proposed reaction intermediates, especially carboxyarabinitol 1,5-bisphosphate, bind very tightly to the carbamylated enzyme. This has been an important tool for studying the carbamylation status of Rubisco. Radioactive 2-carboxyarabinitol 1,5-bisphosphate (CABP) is incubated with rapidly extracted Rubisco. Then, excess non-radioactive CABP is added and Rubisco is allowed to become fully carbamylated. The radioactive CABP will be retained on the Rubisco that was initially carbamylated but non-radioactive CABP will be bound on Rubisco originally uncarbamylated. The carbamylation state measured this way

accurately predicts the activation state as measured by activity before and after incubating with high CO_2 and Mg^{2+} (Butz and Sharkey, 1989).

It was some surprise to find that some, though not all, plants had already made use of carboxyarabinitol phosphate. In this case it was not the bisphosphate but 2-carboxyarabinitol 1-phosphate (CA1P). This compound was found to be present in the extracts of many plants, especially *Phaseolus* (Seemann et al., 1985; Servaites, 1985; Beck et al., 1989; Moore et al., 1991; Moore and Seemann, 1994). The CA1P is a nocturnal inhibitor but its role is not entirely clear and the accumulation of CA1P in the dark is not universal among plants.

IV. Other Reactions of the Calvin-Benson Cycle

A. Gluconeogenesis

Rubisco makes primarily PGA. In a reversal of glycolytic reactions, this carboxylic acid is reduced to a sugar, glyceraldehyde 3-phosphate (GAP). These reactions of gluconeogenesis consume all of the reducing power in the Calvin-Benson cycle and 2/3 of the ATP. The phosphoglycerate kinase is generally not strongly regulated, in contrast to the second enzyme, GAP dehydrogenase. GAP dehydrogenase of chloroplasts can use NADPH in addition to the NADH (McGowan and Gibbs, 1974) but because photosynthetic electron transport makes NADPH, NADPH is considered the normal cofactor for this enzyme.

The GAP produced by reduction of PGA is an aldol sugar, and it is interconverted with the corresponding ketose, dihydroxyacetone phosphate (DHAP) by triose phosphate isomerase. Triose phosphate isomerase is a very efficient enzyme and so the two triose phosphates are generally considered together, as though they were one. The ΔG^0 of the reaction of GAP↔DHAP is −7.6 kJ/mol and so the ratio of DHAP/GAP at equilibrium (25°C) is

$$K_{eq} = e^{(-7.6/(-RT))} = 21.5$$

Therefore, 95% of the triose phosphate pool is in the form of DHAP at equilibrium. In plants, the product of photosynthesis that is exported from chloroplasts is primarily DHAP (up to 1/6 of the triose phosphate made can be exported, 5/6 must be used in regeneration), which is exchanged for phosphate on the triose phosphate/phosphate antiporter (Walker and Herold, 1977; Flügge and Heldt, 1991).

B. Conversion of Triose Phosphates to Pentose Phosphates; Regeneration

The triose phosphates that result from gluconeogenic reactions of the Calvin-Benson cycle are converted to pentose phosphates using reactions of the non-oxidative branch of the pentose phosphate pathway. However, instead of reversing the transaldolase, the Calvin-Benson cycle makes use of sedoheptulose-1,7-bisphosphatase. In plants, SBPase is a novel enzyme; in cyanobacteria a bifunctional FBPase/SBPase carries out this reaction (Tamoi et al., 1996). Most reactions of the Calvin-Benson cycle have ΔG^0s near zero and so are readily reversible. However, the FBPase and SBPase reactions have substantial energy losses [$\Delta G^0 = 16.8$ kJ/mol (Robinson and Walker, 1981), $K_{eq} \sim 900$] and so are not readily reversible, providing directionality to the metabolism.

1. FBPase

The FBPase reaction is critically important for determining whether carbon will stay inside the chloroplast or be exported. The export of triose phosphates on the triose phosphate/phosphate antiporter is reversible as is the formation of FBP from triose phosphates. If FBP is dephosphorylated inside the chloroplast, that carbon will stay inside the chloroplast, especially given the lack of a hexose phosphate transporter in the membranes of autotrophic chloroplasts (other than in crassulacean acid metabolism or CAM plants) (Kammerer et al., 1998). In the cytosol, a different FBPase can act on FBP formed from triose phosphates exported from the chloroplast to commit that carbon to cytosolic reactions, especially sucrose synthesis.

2. SBPase

The SBPase represents a less-obvious branch point in Calvin-Benson cycle metabolism but regulation of SBPase affects the activity of the Calvin-Benson cycle. Starch is made inside

chloroplasts and the starting point is F6P in the Calvin-Benson cycle. If transaldolase were active inside chloroplasts, the pentose phosphates and hexose phosphates would be interconvertable. However, if transaldolase is inactive and SBPase is used instead, SBPase action commits carbon to production of pentose phosphates and that carbon can no longer be used for starch synthesis. This may explain the extremely high sensitivity of plants to genetic modification of SBPase activity (Harrison et al., 1998, 2001; Raines et al., 1999, 2000; Ölcer et al., 2001). Modeling of photosynthetic rates assuming that enzymes limit the overall rate of photosynthesis (as opposed to light availability etc.) indicates that increased investment of plant resources into SBPase activity would result in higher rates of photosynthesis (Zhu et al., 2007). This could reflect that it is not possible to simulate important aspects of the regulatory role of SBPase in current models.

Transaldolase is present in chloroplasts but may be regulated to be inactive during the day (Anderson, 1981), but the importance of the differences between the pathways shown in Figs. 26.4 and 26.5 is likely not yet fully known (Poolman et al., 2003). The original studies did not settle which pathway predominated (Bassham and Calvin, 1957; Calvin and Bassham, 1962) but later enzyme activity measurements and gene manipulation studies cited above indicate the importance of SBPase over transaldolase.

Surprisingly, transketolase and aldolase activity also have substantial effects on photosynthetic rate and plant performance (Raines, 2003). Modulating the relative activities of transketolase and aldolase (which consumes E4P) allows the concentration of E4P to be controlled. This metabolite is required for phenylpropanoid metabolism (making aromatic amino acids and many secondary compounds), which in plants is restricted to plastids.

C. Synthesis of RuBP

In plants, the pentose phosphates made by the pentose phosphate pathway are converted to the Rubisco substrate by phosphorylation of Ru5P to RuBP. This reaction appears to be unique to the Calvin-Benson cycle. Archaea appear to have arrived at a different solution by making RuBP from phosphoribose pyrophosphate, an intermediate in purine catabolism (Finn and Tabita, 2004). Phosphoribulokinase is one of the most sensitive enzymes to conditions that occur in the dark. Decreased pH, increased PGA (for lack of NADPH), and other changes in the chloroplast cause the activity of PRK to decline very quickly when darkness is imposed.

V. Regulation of the Calvin-Benson Cycle

Substantial effort has been expended studying the regulation of the Calvin-Benson cycle. The assumption is that if the weak points can be identified and then "fixed", it will be possible to improve photosynthesis. However, it is important to consider the "design" criteria, i.e., what should regulation of the Calvin-Benson cycle achieve in order to optimize plant performance, either for human purposes (e.g., crop yield) or for the plant (e.g., reproductive success of the plant and avoidance of extinction). There are fundamental conflicts between resources used for photosynthetic capacity and those used for growth and reproduction. For example, the ability of the plant to use CO_2 can be enhanced by investing more nitrogen in Rubisco making more sugar available. However, the nitrogen invested in Rubisco is unavailable for growth of roots (to get more water) or seeds (to improve harvestable yield or reproductive success). Different plants will make different tradeoffs allowing them to occupy different ecological niches. The two primary external constraints on the instantaneous rate of photosynthesis are availability of light and CO_2. Light that is not used instantly cannot be stored and is lost forever. Therefore, the Calvin-Benson cycle should be regulated to take maximum advantage of external resources (light and CO_2) with a minimum of investment of internal resources (e.g., nitrogen for proteins). The Calvin-Benson cycle regulation should be such that it has the maximal capacity for a given investment of resources. Regulation is disconnected from limitation. Photosynthesis is often limited by availability of light, and the Calvin-Benson cycle regulation should ensure that any light that does come available is used to maximum benefit. Plants adapted to environments in which much of the light is available in sunflecks have adaptations that help them use short periods of high light (Pearcy, 1990).

Plants may not be able to fully adjust for all conditions. For example, it was reported that transgenic tobacco with up to 40% less Rubisco than wild type had no reduction in growth (Quick et al., 1991; Stitt et al., 1991) which was interpreted to mean that Rubisco was generally in excess and not limiting for plant growth. However, when tobacco plants were grown in high light, **any** reduction in Rubisco content led to reduced growth (Hudson et al., 1992). Therefore, it would be wrong to conclude that Rubisco is normally present in excess, but instead, sun-adapted tobacco is limited in its ability to adjust to growth in lower light by reducing its investment in Rubisco. Regulation of the Calvin-Benson cycle does not always achieve the maximum possible use of external resources and Woodrow and Berry (1988) invoked the concept of the metabolic cost of regulation to explain this.

There are several important ways that the Calvin-Benson cycle is regulated and some of these will be covered in this section.

A. Rubisco Concentration and K_m for RuBP

Enzymes often are present in low concentration relative to their substrates, but Rubisco is an exception. Rubisco in plants is a relatively slow enzyme and so, to allow for reasonable rates of photosynthesis, is present in very high concentration, as much as 4 mM while the K_m for RuBP is in the μM range (Farquhar et al., 1980). As a result, as RuBP comes available, it will bind to Rubisco. Any increase in the concentration of RuBP will cause a linear increase in the rate of carboxylation until the concentration of RuBP exceeds the concentration of RuBP binding sites on Rubisco, then, abruptly, no further increase in the rate of Rubisco will occur, regardless of how much further the RuBP concentration increases. Farquhar et al. (1980) present methods for calculating precisely how sharp the transition would be (Fig. 26.8), but in general, the Calvin-Benson cycle can be considered as limited by the rate at which RuBP can be regenerated (e.g., when light is limiting) or by the rate at which Rubisco can catalyze carboxylation plus oxygenation assuming it is saturated with RuBP (often the limiting factor when CO_2 is low). There is, practically speaking, no intermediate state where Rubisco kinetics and RuBP regeneration kinetics co-regulate the

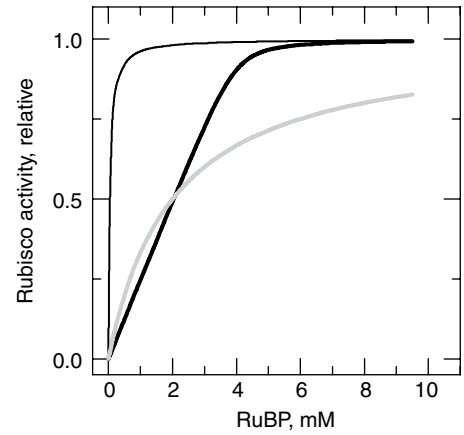

Fig. 26.8. The dependence of Rubisco activity on substrate. The *heavy black line* is the relationship given 4 mM Rubisco active sites and a K_m of Rubisco for RuBP of 40 μM (using equation A4 of Farquhar et al. (1980)). The *gray line* assumes a K_m of RuBP of 2 mM so that the curves roughly match at 50% of maximum. The *light black line* is the relationship assuming the K_m for RuBP is 40 μM but does not consider the concentration of Rubisco.

Calvin-Benson cycle (Farquhar et al., 1980). Typically somewhere between 1.5 and 2 RuBP molecules per Rubisco active site are required to cause photosynthesis to behave as predicted for RuBP-saturated conditions. The fact that this ratio must be greater than one to one before photosynthesis appears saturated with RuBP can be accounted for by binding of RuBP to Mg^{2+} and other interactions (von Caemmerer and Edmondson, 1986). The concept of single limiting factors is often traced back to Blackman (1905) but the Farquhar model of photosynthesis does not really draw on Blackman's concepts; rather, it is based on a detailed analysis of the kinetics of Rubisco (Farquhar, 1979).

B. Mg^{2+} and pH changes

Many enzymes of the Calvin-Benson cycle work best at high pH and Mg^{2+} (Werdan et al., 1975; Portis and Heldt, 1976). Photosynthetic electron transport causes protons to be pumped to the inside of the thylakoid lumen, increasing the pH of the stroma and causing some Mg^{2+} to come out of the thylakoid lumen into the stroma. This is another way that the "dark" reactions require light for activity. For example, the chloroplast FBPase is inactive below pH 7.5 and requires high [Mg^{2+}], unlike the cytosolic counterpart.

C. Redox Control of the Calvin-Benson cycle

The activity of GAP dehydrogenase was found to be sufficient if extracted from leaves harvested in sunlight but not when the leaves were harvested in low light (Ziegler and Ziegler, 1965). This led to the discovery of light activation of several Calvin-Benson cycle enzymes. This is now known to result from reduction of dithiol bridges that form in these enzymes. Closely spaced (in terms of tertiary structure) cysteine residues in the enzyme can form a dithiol bridge by oxidation. Light-driven electron transport reduces ferredoxin which reduces thioredoxin, which reduces these dithiol bridges, opening them and changing protein conformation (Buchanan, 1980, 1991; Schürmann and Jacquot, 2000; Jacquot et al., 2002). Many of the enzymes (FBPase, SBPase, PRK, ATP synthase and some forms of Rubisco activase) are known to be activated by a specific thioredoxin called thioredoxin *f* (for FBPase-type). A second thioredoxin was discovered that could activate NADP-dependent malate dehydrogenase (Scheibe, 1987) and this was called thioredoxin *m*, though now it is known that thioredoxin *f* will also reduce (and activate) NADP malate dehydrogenase. While GAP dehydogenase was the first enzyme shown to be modulated this way, it is not certain that thioredoxin is required for the light activation of this enzyme (Baalmann et al., 1995).

The redox activation of NADP-dependent GAP dehydrogenase activity, FBPase activity, and SBPase activity is substantially stimulated by the presence of substrates for these enzymes (Leegood et al., 1982; Woodrow and Walker, 1983; Baalmann et al., 1995). This provides a mechanism by which these enzyme activities can be increased substantially when needed (when there is light energy and substrates). By this mechanism, regulation can help ensure sufficient enzyme activity only when needed, reducing the metabolic cost of regulation. If these enzymes were active at night it could prove impossible to carry out some of the required chloroplast metabolism, such as the shikimic acid pathway, without causing futile cycles.

1. Redox Control of Other Enzymes of the Chloroplast

Some enzymes are inhibited by reducing conditions. Glucose-6-phosphate dehydrogenase, an enzyme in the oxidative branch of the pentose phosphate pathway, is inhibited by reduction by thioredoxin *m* (Wenderoth et al., 1997). Dithiol reduction increases the K_m of the enzyme for its substrate, G6P but does not eliminate all activity (Scheibe et al., 1989). Regulation of this enzyme is important to avoid a futile cycle (Fig. 26.9). For every molecule of G6P that enters the oxidative branch of the pentose phosphate pathway when the Calvin-Benson cycle is operating, three ATPs are lost but CO_2 and NADPH are balanced. This may be useful on occasion, especially when photosynthetic rates are changing rapidly. If Calvin-Benson cycle intermediates are depleted, the Calvin-Benson cycle will be limited by lack of substrate for Rubisco. If the carbon normally going to starch could be used to refill the Calvin-Benson cycle when it becomes depleted, this could help reduce the metabolic cost of regulation. However, the Calvin-Benson cycle can also be recharged by converting G6P to F6P so it is not clear that there is a need for G6P dehydrogenase for this purpose.

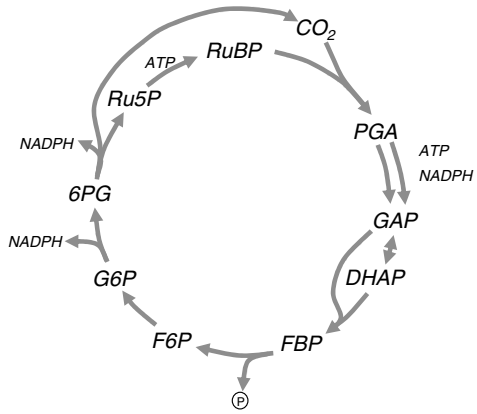

Fig. 26.9. Futile cycle that occurs if the oxidative branch of the pentose phosphate pathway operates simultaneously with the Calvin-Benson cycle.

Starch breakdown leading to carbon export for sucrose synthesis is primarily hydrolytic (Sharkey et al. 2004) perhaps phosphorolytic starch breakdown primarily supplies carbon to the chloroplast, either to recharge the Calvin-Benson cycle or for metabolism at night. Simultaneous starch synthesis and breakdown has been reported (Stitt and Heldt, 1981), and starch breakdown near the end of the photoperiod has been observed (Fondy et al., 1989). Substantial rates of starch breakdown can

be induced by holding leaves in conditions in which they are in negative carbon balance because of photorespiration (Weise et al., 2006). Interestingly, models of the Calvin-Benson cycle function better when a small capacity for recharge of the Calvin-Benson cycle from G6P dehydrogenase is allowed (Poolman et al., 2003).

D. Control of PGI/M

Starch synthesis and phosphorolytic starch degradation is a side path of the reactions in the Calvin-Benson cycle. During starch synthesis F6P is isomerized by phosphoglucose isomerase (PGI) to G6P which is transphosphorylated by phosphoglucomutase (PGM) to G1P. The G1P is converted to ADPglucose by ADPglucose pyrophosphorylase (AGPase). Finally, the glucose moiety is transferred to the growing starch chain by starch synthase. While starch degradation is not fully understood, it is thought that phosphorolytic starch degradation proceeds largely in the opposite direction as synthesis. First starch phosphorylase phosphorylates the terminal residue from the non-reducing ends of starch or maltodextrin chains, liberating G1P. G1P is then converted to G6P by PGM. The G6P can then be used by the oxidative pentose phosphate pathway or can be isomerized by PGI to F6P feeding into the Calvin-Benson cycle (Fig 26.10).

Regulation of starch synthesis by AGPase is well known. This enzyme is regulated by allosteric interactions with inorganic phosphate and 3-phosphoglycerate (PGA) and is therefore considered to control the carbon flux into starch (Preiss, 1984). However, there is a considerable body of evidence that PGI and PGM are also regulated. In the following text we discuss evidence for this regulation, how these enzymes are regulated, and propose a physiological rational for this regulation.

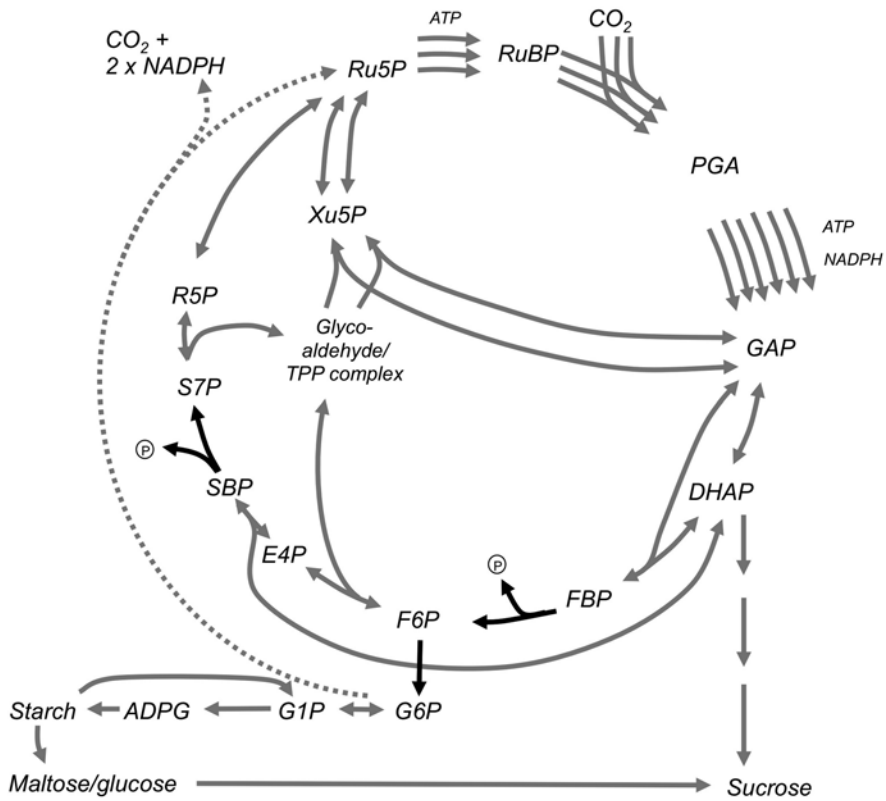

Fig. 26.10. Starch serves as a transitory storage product. During the day sucrose is made from triose phosphates that leave the Calvin-Benson cycle and starch is made from hexose phosphates that leave the Calvin-Benson cycle. Starch can be broken down phosphorolytically to resupply carbon to the Calvin-Benson cycle either as F6P or possibly as Ru5P (*dotted line*). At night starch is broken down hydrolytically and maltose (plus some glucose) is exported from the chloroplast (Weise et al., 2004) for sucrose synthesis.

There are two isoforms of PGI and PGM in plants, one set located in the plastid the other in the cytosol. Both sets of enzymes are encoded in the nuclear genome. The following discussion of regulation will be focused on the plastidic isoforms. The same regulators that are known to affect the plastidic isoform can also affect the cytosolic isoform. However, due to different stoichiometric relationships among cytosolic substrates and regulators, regulation of PGI and PGM is not believed to occur in the cytosol (Backhausen et al., 1997).

Phosphoglucose isomerase catalyzes the branch point between the Calvin-Benson cycle and starch synthesis. Evidence for regulation of PGI arises from the observation that the concentrations of F6P and G6P in illuminated leaves and chloroplasts are always displaced below equilibrium (Dietz, 1985; Sharkey and Vassey, 1989; Stitt, 1990; Schleucher et al., 1999). The equilibrium constant for the PGI reaction was determined in vitro to be K_{eq} = G6P/F6P = 3.31 (Dyson and Noltmann, 1968). The in vivo measured value in illuminated spinach chloroplasts is G6P/F6P = 1.08 (Dietz, 1985). Dietz (1985) found that PGI is competitively inhibited by PGA (K_i = 2.1 mM). Dietz also found that 6-phosphogluconate (6-PG) strongly inhibits PGI with a K_i = 80 μM. However daytime levels of 6-PG are around 50 μM in the light and 300 μM in the dark so inhibition by 6-PG is thought to mainly occur at night (Gardemann et al., 1983). Backhausen et al. (1997) found that in addition to PGA and 6-PG, E4P is also a significant competitive inhibitor of PGI with a K_i < 10 μM. The PGA concentration in the stroma of photosynthesizing spinach leaves measured using non-aqueous fractionation is 4.3 mM (Gerhardt et al., 1987). Measurements of E4P concentrations are difficult but were estimated by Backhausen et al. (1997) to be as high as 17 μM in photosynthesizing chloroplasts. Therefore both PGA and E4P inhibit PGI during the day. Calculations using hexose monophosphate concentrations from spinach measured by Dietz (1985) under standard physiological conditions (370 ppm CO_2, 25°C, and light ≈ 1,000 μmol m^{-2} s^{-1}) and subcellular volumes of spinach determined by Winter et al. (1994) demonstrate that starch synthesis can be limited by PGI not AGPase. The F6P concentration in the chloroplast is approximately 730 μM, and the G6P concentration is approximately 940 μM; this is a G6P/F6P ratio of 1.3:1. The measured G1P concentration was ≈ 40 μM; this is a G6P/G1P ratio of 23.5:1. The K_m of AGPase is 40 μM, therefore AGPase activity will be strongly controlled by G1P availability. If PGI and PGM were in equilibrium the G1P concentration would be 108 μM and AGPase would be closer to saturation and thus exert more control over the overall rate of starch synthesis.

The inhibition of PGI by PGA and E4P may be a mechanism for keeping carbon in the Calvin-Benson cycle ensuring that as photosynthetic rates increase and Calvin-Benson cycle intermediates, including F6P, rise, carbon is retained in the Calvin-Benson cycle to support the higher demand for regeneration. Without regulation it might be more difficult to keep the carbon needed to support high rates of photosynthesis in the Calvin-Benson cycle given the low K_m (40 μM) of AGPase. An excessive drainage of F6P from the Calvin-Benson cycle would result in photosynthesis being under a persistent regeneration limitation thus lowering the CO_2 fixation rate. Regulation of PGI to prevent excess carbon loss from the Calvin-Benson cycle was demonstrated in a study of transgenic plants with decreased activities of PRK. In these plants when PRK was lowered to 30% of wild type the levels of measured metabolites (Ru5P, Rib5P, F6P) that are preceding PRK increased (Paul et al., 1995). It can be assumed that levels of E4P would also increase. Even though F6P concentrations dramatically increased G6P levels remained relatively constant indicating a strong inhibition of PGI (Paul et al., 1995). Decreasing PRK by antisense RNA reflects an artificial system, however this could reflect the situation that might arise if ATP were limiting (e.g., low light) or if Rubisco were limited for CO_2 (e.g., stomatal closure).

There appears to be little regulation of PGM in photosynthesizing chloroplasts (Turner and Turner, 1980; Dietz, 1987). Sicher (1989) demonstrated that PGM activity increased two to threefold when isolated chloroplasts were incubated in the light, and that the light treated PGM was sufficient to account for measured starch synthesis rates. The activation of PGM by light could not be duplicated using dithiolthreitol (DTT) suggesting that light effects did not involve a sulfhydryl mechanism. PGA did not appear to have a large

effect on PGM activity (Sicher, 1989). PGM requires glucose 1,6-bisphosphate (G1,6BP) as a cofactor and low G1,6BP levels can greatly reduce the activity of PGM (Beitner, 1985; Sicher, 1989). However, G1,6BP concentrations in the light and dark are above levels needed to saturate PGM activity (Sicher, 1989; Sicher and Kremer, 1990). Plastidic PGM is inhibited by FBP and RuBP but this inhibition could not be explained by competition with G1,6BP (Hattenbach and Heineke, 1999; Periappuram et al., 2000). The concentrations of F1,6BP (1.5 mM) found to produce ≈ 10% inhibition are well above levels typically found in photosynthesizing tissue, 0.55 mM (Gerhardt et al., 1987). However, levels of RuBP (1.5 mM) found to produce ≈ 10% inhibition are within the physiologically relevant range found in photosynthesizing tissue (Gerhardt et al., 1987).

In summary, while PGI and PGM are not part of the Calvin-Benson cycle, their regulation may be an important control point in maintaining efficient functioning of the cycle.

E. CP12

The idea that enzymes involved in the Calvin cycle interact to give multienzyme complexes has been suggested because some of the proteins (e.g., PRK and GAP dehydrogenase) could not be easily isolated by conventional methods, such as ion exchange, gel filtration, and affinity chromatography and some enzymes co-purify with other enzymes of the Calvin-Benson cycle (Macioszek and Anderson, 1987; Malhotra et al., 1987; Nicholson et al., 1987; Macioszek et al., 1990; Clasper et al., 1991). The interaction between GAP dehydrogenase and PRK is one of the best documented examples. A specific explanation for this complex came when the chloroplast protein CP12 was discovered in pea, spinach and tobacco (Pohlmeyer et al., 1996). CP12 has since been found in *Arabidopsis thaliana*, *Ceratodon purpureus, Chlamydomonas reinhardtii*, and *Synechocystis* sp. PCC 6803 (Wedel and Soll, 1998; Marri et al., 2005). The discovery of CP12 in vascular plants, mosses, green algae, and cyanobacteria suggests that the CP12 complex and its regulation of Calvin-Benson cycle activity is conserved in all photosynthetic organisms (Wedel and Soll, 1998).

CP12 is a small, 75 amino acid, nuclear-encoded protein, which has been shown to form a stable 600-kDa complex with PRK and GAP dehydrogenase in the chloroplast (Wedel et al., 1997). The C-terminal end binds GAP dehydrogenase (normally a heterotetramer of the form A2B2) into a hexadecamer (A8B8). The GAP dehydrogenase/CP12 complex then becomes associated with PRK to form a half complex, defined as one unit. The unit then dimerizes to give the native complex composed of two tetramers of GAP dehydrogenase, two dimers of PRK, and probably two monomers of CP12 (Graciet et al., 2003). In *A. thaliana* it was found that the expression pattern of the genes for the chloroplastic GAP dehydrogenase, PRK, and CP12 all show a similar pattern. Expression is highest in the leaves and flower stalks, and is terminated during prolonged darkness or sucrose treatments (Marri et al., 2005).

The CP12 enzyme complex suggests a possible new mechanism for Calvin-Benson cycle regulation. It is known that both enzymes in the CP12 complex are regulated by thioredoxins and are active in the light (Wolosiuk and Buchanan, 1978; Brandes et al., 1996). However, these enzymes appear to be under further regulation when associated in the CP12 complex (Marri et al., 2008). Scheibe et al. (2002) found that GAP dehydrogenase and PRK are inactive when they are embedded with CP12 in the complex. The complex can be dissociated in vitro by strong reducing agents such as DTT. Further it was found that the complex dissociates in the presence of NADP and NADPH, but PRK activity is inhibited by NADP (Wedel and Soll, 1998). Therefore it is hypothesized that Calvin-Benson cycle activity would depend not only on the activation of PRK and GAP dehydrogenase by thioredoxin *f* but on the disassociation of the PRK/CP12/GAP dehydrogenase complex by NADP or NADPH and further allosteric activation of PRK by NADPH (Wedel and Soll, 1998). In cyanobacteria it appears that disassociation of the PRK/CP12/GAP dehydrogenase complex is further regulated by the NAD(H) to NADP(H) ratio. During the night when this ratio is high the enzymes remain complexed (Tamoi et al., 2005).

CP12 is uniquely poised to prevent futile cycling between the oxidative and reductive pentose phosphate pathway in the dark and light.

In the dark CP12 regulation would prevent PRK activity thus stopping carbon fixation and ATP usage by the Calvin-Benson cycle. In *Synechococcus* sp. PCC 7942 cells with lowered levels of CP12, growth was normal under a continuous photoperiod but inhibited when cells were given a light dark period (12 h/12 h). In the dark RuBP levels were 2.5-fold higher than wild type and F6P levels were 50% lower, indicating significant flow of carbon into the reductive pathway at night (Tamoi et al., 2005). Regulation of PRK by CP12 at night would be especially critical in cyanobacteria and eukaryotic algae, which do not have regulation by a ferredoxin/thioredoxin system. In the light CP12 may prevent excess activity by GAP dehydrogenase (Howard et al., 2008) reducing the likelihood of a futile cycle during the day (Fig. 26.9). While experimentation with the CP12 complex is just beginning, it presents an interesting mechanism for further redox regulation of the Calvin-Benson cycle by the direct action of NADP(H).

VI. Conclusion

Carbon dioxide can be converted to biological material by a number of mechanisms. There are four pathways that allow autotrophic carbon fixation, but three of these are mostly anaerobic. The carbon fixation pathway of aerobic organisms is the Calvin-Benson cycle, and in terms of amount of carbon fixed the Calvin-Benson cycle is probably overwhelmingly important. The path of carbon in the Calvin-Benson cycle has long been established. However, there are regulatory mechanisms that we do not fully understand. It is likely that the Calvin-Benson cycle is regulated to take maximum advantage of resources such as light and CO_2. Future work on modifying the Calvin-Benson cycle will likely focus on regulation of the pathways and branch points and improvement of Rubisco.

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References

Altekar W and Rajagopalan R (1990) Ribulose bisphosphate carboxylase activity in halophilic *Archaebacteria*. Arch Microbiol 153: 169–174

Anderson LE (1981) Light inactivation of transaldolase in pea leaf chloroplasts. Biochem Biophys Res Commun 99: 1199–1202

Andersson I and Taylor TC (2003) Structural framework for catalysis and regulation in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch Biochem Biophys 414: 130–140

Andrews TJ and Kane HJ (1991) Pyruvate is a by-product of catalysis by ribulosebisphosphate carboxylase/oxygenase. J Biol Chem 266: 9447–9452

Ashida H, Saito Y, Kojima C, Kobayashi K, Ogasawara N and Yokota, A (2003) A functional link between RuBisCO-like protein of *Bacillus* and photosynthetic RuBisCO. Science 302: 286–290

Ashida H, Saito Y, Nakano T, Tandeau de Marsac N, Sekowska A, Danchin A and Yokota A (2008) RuBisCO-like proteins as the enolase enzyme in the methionine salvage pathway: Functional and evolutionary relationships between rubisco-like proteins and photosynthetic RuBisCO. J Exp Bot 59: 1543–1554

Baalmann E, Backhausen JE, Rak C, Vetter S and Scheibe R (1995) Reductive modification and nonreductive activation of purified spinach chloroplast NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. Arch Biochem Biophys 324: 201–208

Backhausen JE, Jöstingmeyer P and Scheibe R (1997) Competitive inhibition of spinach leaf phosphoglucose isomerase isoenzymes by erythrose 4-phosphate. Plant Sci 130: 121–131

Badger MR and Bek EJ (2008) Multiple rubisco forms in proteobacteria: Their functional significance in relation to CO_2 acquisition by the CBB cycle J Exp Bot 59: 1525–1541

Bassham JA and Calvin M (1957) The Path of Carbon in Photosynthesis. 1–104

Beck E, Scheibe R and Reiner J (1989) An assessment of the rubisco inhibitor. Plant Physiol 90: 13–16

Beitner R (1985) Glucose-1,6-bisphosphate — the regulator of carbohydrate metabolism. In: Biggins, J (ed) Regulation of Carbohydrate Metabolism, pp 1–27. CRC Press, Boca Raton

Benson AA (2002) Following the path of carbon in photosynthesis: a personal story. Photosyn Res 73: 31–49

Blackman FF (1905) Optima and limiting factors. Ann Bot 19: 281–295

Boucher Y, Douady CJ, Papke RT, Walsh DA, Boudreau ME, Nesbo CL, Case RJ and Doolittle WF (2003) Lateral gene transfer and the origins of prokaryotic groups. Annu Rev Genet 37: 283–328

Bowes G, Ogren WL and Hageman RH (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochem Biophys Res Comm 45: 716–722

Brandes HK, Larimer FW and Hartman FC (1996) The molecular pathway for the regulation of phosphoribulokinase by thioredoxin *f*. J Biol Chem 271: 3333–3335

Buchanan BB (1980) Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374

Buchanan BB (1991) Regulation of CO_2 assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system: Perspective on its discovery, present status, and future development. Arch Biochem Biophys 288: 1–9

Buchanan BB and Arnon DI (1990) A reverse KREBS cycle in photosynthesis: consensus at last. Photosynth Res 24: 47–53

Butz ND and Sharkey TD (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. Plant Physiol 89: 735–739

Calvin M and Bassham JA (1962) Photosynthesis of carbon compounds. Benjamin, New York

Chen C and Gibbs M (1992) Some enzymes and properties of the reductive carboxylic acid cycle are present in the green alga *Chlamydomonas reinhardtii* F-60. Plant Physiol 98: 535–539

Chow WS, Hope AB and Anderson JM (1989) Oxygen per flash from leaf disks quantifies photosystem II. Biochim Biophys Acta 973: 105–108

Chung SY, Yaguchi T, Nishihara, H, Igarashi Y and Kodama T (1993) Purification of form L_2 RubisCO from a marine obligately autotrophic hydrogen-oxidizing bacterium, *Hydrogenovibrio marinus* strain MH-110. FEMS Microbiol Lett 109: 49–54

Clasper S, Easterby JS and Powls R (1991) Properties of two high molecular mass forms of glyceraldehyde-3-phosphate dehydrogenase from spinach leaf, one of which also possesses latent phophoribulokinase activity. Plant Physiol 202: 1239–1246

Cleland WW, Andrews TJ, Gutteridge S, Hartman FC and Lorimer GH (1998) Mechanism of Rubisco: The carbamate as general base. Chem Rev 98: 549–562

D'Imperio S, Lehr CR, Oduro H, Druschel G, Kuhl M and McDermott TR (2008) The relative importance of H_2 and H_2S as energy sources for primary production in geothermal springs. Appl Environ Microbiol, AEM.00852–00808

Debnam P and Emes M (1999) Subcellular distribution of enzymes of the oxidative pentose phosphate pathway in root and leaf tissues. J Exp Bot 50: 1653–1661

Delwiche CF and Palmer JD (1996) Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. Mol Biol Evol 13: 873–882

Dietz KJ (1985) A possible rate limiting function of chloroplast hexosemonophosphate isomerase in starch synthesis of leaves. Biochim Biophys Acta 839: 240–248

Dietz KJ (1987) Control function of hexosemonophosphate isomerase and phosphoglucomutase in starch synthesis in leaves Proc VIIth Internat Cong Photosynth, 3: 329–332

Dyson JED and Noltmann EA (1968) The effect of pH and temperature on the kinetic parameters of phosphoglucose isomerase. J Biol Chem 243: 1401–1414

Edmondson DL, Kane HJ and Andrews TJ (1990) Substrate isomerization inhibits ribulosebisphosphate carboxylase-oxygenase during catalysis. FEBS Lett 260: 62–66

Eicks M, Maurino V, Knappe S, Flugge UI and Fischer K (2002) The plastidic pentose phosphate translocator represents a link between the cytosolic and the plastidic pentose phosphate pathways in plants. Plant Physiol 128: 512–522

Evans MCW, Buchanan BB and Arnon DI (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. Proc Nat Acad Sci USA 55: 928–934

Ezaki S, Maeda N, Kishimoto T, Atomi H and Imanaka T (1999) Presence of a structurally novel type ribulosebisphosphate carboxylase/oxygenase in the hyperthermophilic archaeon, *Pyrococcus kodakaraensis* KOD1. J Biol Chem 274: 5078–5082

Farquhar GD (1979) Models describing the kinetics of ribulose bisphosphate carboxylase-oxygenase. Arch Biochem Biophys 193: 456–468

Farquhar GD, von Caemmerer S and Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149: 78–90

Finn MW and Tabita FR (2004) Modified pathway to synthesize ribulose 1,5-bisphosphate in methanogenic archaea. J Bacteriol 186: 6360–6366

Fischer K, Kammerer B, Gutensohn M, Arbinger B, Weber A, Häusler RE and Flügge U-I (1997) A new class of plastidic phosphate translocators: A putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. Plant Cell 9: 453–462

Flügge U-I and Heldt HW (1991) Metabolite translocators of the chloroplast envelope. Annu Rev Plant Physiol Plant Mol Biol 42: 129–144

Fondy BR, Geiger DR and Servaites JC (1989) Photosynthesis, carbohydrate metabolism, and export in *Beta vulgaris* L. during square and sinusoidal light regimes. Plant Physiol 89: 396–402

Gardemann A, Stitt M and Heldt HW (1983) Control of CO_2 fixation. Regulation of spinach RuBPkinase by stromal metabolite levels. Biochim Biophys Acta 722: 51–60

Gerhardt R, Stitt M and Heldt HW (1987) Subcellular metabolite levels in spinach leaves. Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol 83: 399–407

Gold T (1992) The deep, hot biosphere. Proc Nat Acad Sci USA 89: 6045–6049

Graciet E, Gans P, Wedel N, Lebreton S, Camadro JM and Gontero B (2003) The small protein CP12: A protein linker for supramolecular complex assembly. Biochemistry 42: 8163–8170

Hanson TE and Tabita FR (2001) A ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. Proc Natl Acad Sci USA 98: 4397–4402

Harrison EP, Willingham NM, Lloyd JC and Raines CA (1998) Reduced sedoheptulose-1,7-bisphosphatase levels

in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation. Planta 204: 27–36

Harrison EP, Olcer H, Lloyd JC, Long SP and Raines CA (2001) Small decreases in SBPase cause a linear decline in the apparent RuBP regeneration rate, but do not affect Rubisco carboxylation capacity. J Exp Bot 52: 1779–1784

Hattenbach A and Heineke D (1999) On the role of choroplastic phosphoglucomutase in the regulation of starch turnover. Planta 207: 527–532

Herter S, Fuchs G, Bacher A and Eisenreich W (2002) A bicyclic autotrophic CO_2 fixation pathway in *Chloroflexus aurantiacus*. J Biol Chem 277: 20277–20283

Howard TP, Metodiev M, Lloyd JC and Raines CA (2008) Thioredoxin-mediated reversible dissociation of a stromal multiprotein complex in response to changes in light availability. Proc Nat Acad Sci USA 105**:** 4056–4061

Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC and Andrews TJ (1992) Reduction of ribulose-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. Plant Physiol 98: 294–302

Hügler M, Huber H, Stetter KO and Fuchs G (2003a) Autotrophic CO_2 fixation pathways in archaea (Crenarchaeota). Arch Microbiol 179: 160–173

Hügler M, Krieger RS, Jahn M and Fuchs G (2003b) Characterization of acetyl-CoA/propionyl-CoA carboxylase in *Metallosphaera sedula*: Carboxylating enzyme in the 3-hydroxypropionate cycle for autotrophic carbon fixation. Eur J Biochem 270: 736–744

Jacquot JP, Rouhier N and Glhaye E (2002) Redox control by dithiol-disulfide exchange in plants: I. The chloroplastic systems. Ann NY Acad Sci 973: 508–519

Jannasch HW and Mottl MJ (1985) Geomicrobiology of deep-sea hydrothermal vents. Science 229: 717–725

Jordan DB and Chollet R (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J Biol Chem 258: 13752–13758

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A and Flügge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: The glucose 6-phosphate phosphate antiporter. Plant Cell 10: 105–117

Kitano K, Maeda N, Fukui T, Atomi H, Imanaka T and Miki K (2001) Crystal structure of a novel-type archaeal Rubisco with pentagonal symmetry. Struct Fold Des 9: 473–481

Leegood RC, Kobayashi Y, Neimanis S, Walker DA and Heber U (1982) Co-operative activation of chloroplast fructose-1,6-bisphosphatase by reductant, pH, and substrate. Biochim Biophys Acta 682: 168–178

Li H, Sawaya MR, Tabita FR and Eisenberg D (2005) Crystal structure of a RuBisCO-like protein from the green sulfur bacterium *Chlorobium tepidum*. Structure 13: 779–789

Lichtenthaler HK (2000) Non-mevalonate isoprenoid biosynthesis: enzymes, genes and inhibitors. Biochem Soc Trans 28: 785–789

Ljungdahl LG (1986) The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu Rev Microbiol 40: 415–450

Lorimer GH, Badger MR and Andrews TJ (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism and physiological implications. Biochemistry 15: 529–536

Macioszek J and Anderson LE (1987) Changing kinetic-properties of the 2-enzyme phosphoglycerate kinase NADP-linked glyceraldehyde-3-phosphate dehydrogenase couple from pea-chloroplasts during photosynthetic induction. Biochim Biophys Acta 892: 185–190

Macioszek J, Anderson JB and Anderson LE (1990) Isolation of chloroplastic phophoglycerate kinase - kinetics of the 2-enzyme phosphoglycerate kinase glyceraldehyde-3-phosphate dehydrogenase couple. Plant Physiol 94: 291–296

Maeda N, Kitano K, Fukui T, Ezaki S, Atomi H, Miki K and Imanaka T (1999) Ribulose bisphosphate carboxylase/oxygenase from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 is composed solely of large subunits and forms a pentagonal structure. J Mol Biol 293: 57–66

Maeda N, Kanai T, Atomi H and Imanaka T (2002) The unique pentaugonal structure of an archaeal Rubisco is essential for its high thermostability. J Biol Chem 277: 31656–31662

Malhotra OP, Kumar A and Tikoo K (1987) Isolation and quaternary structure of a complex of glyceraldehydre-3-phosphate dehydrogenase and phosphoglycerate kinase. Indian J Biochem Biophys 24: 16–20

Margulis L (1981) Symbiosis in Cell Evolution. W. H. Freeman, New York

Marri L, Sparla F, Pupillo P and Trost P (2005) Co-ordinated gene expression of photosynthetic glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, and CP12 in *Arabidopsis thaliana*. J Exp Bot 56: 73–80

Marri L, Trost P, Trivelli X, Gonnelli L, Pupillo P and Sparla F (2008) Spontaneous assembly of photosynthetic supramolecular complexes as mediated by the intrinsically unstructured protein CP12. J Biol Chem 283: 1831–1838

Martin W, Scheibe R and Schnarrenberger C (2000) The Calvin Cycle and its regulation. In: Leegood RC, Sharkey TD and von Caemmerer S (eds) Photosynthesis: Physiology and Metabolism, Advances in Photosynthesis, Vol 9, pp 9–51. Kluwer Academic Publishers, Dordrecht

Mate CJ, von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1996) The relationship between CO_2-assimilation rate, rubisco carbamylation and rubisco activase content in activase-deficient transgenic tobacco suggests a simple model of activase action. Planta 198: 604–613

McFadden GI (2001) Chloroplast origin and integration. Plant Physiol 125: 50–53

McGowan RE and Gibbs M (1974) Comparative enzymology of the glyceraldehyde 3-phosphate dehydrogenases from *Pisum sativum*. Plant Physiol 54: 312–319

McNevin D, von Caemmerer S, Farquhar G (2006) Determining RuBisCO activation kinetics and other rate and equilibrium constants by simultaneous multiple non-linear regression of a kinetic model. J Exp Bot 57: 3883–3900

Moore BD, Kobza J and Seemann JR (1991) Measurement of 2-carboxyarabinitol 1-phosphate in plant leaves by isotope dilution. Plant Physiol 96: 208–213

Moore BD and Seemann JR (1994) Evidence that 2-carboxyarabinitol 1-phosphate binds to ribulose-1,5-bisphosphate carboxylase *in vivo*. Plant Physiol 105: 731–737

Mott KA, Jensen RG, O'Leary JW and Berry JA (1984) Photosynthesis and ribulose 1,5-bisphosphate concentrations in intact leaves of *Xanthium strumarium* L. Plant Physiol 76: 968–971

Nicholson S, Easterby JS and Powls R (1987) Properties of a multimeric protein complex from chloroplasts possessing potential activities of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur J Biochem 162: 423–431

Ogren WL (1984) Photorespiration: Pathways, regulation, and modification. Annu Rev Plant Physiol 35: 415–442

Ogren WL (2003) Affixing the O to Rubisco: discovering the source of photorespiratory glycolate and its regulation. Photosynth Res 76: 53–63

Ölcer H, Lloyd JC and Raines CA (2001) Photosynthetic capacity is differentially affected by reductions in sedoheptulose-1,7-bisphosphatase activity during leaf development in transgenic tobacco plants. Plant Physiol 125: 982–989

Paul MJ, Kinght JS, Habash D, Parry MAJ, Lawlor DW, Barnes SA, Loynes A and Gray JC (1995) Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: effect on CO_2 assimilation and growth in low irradiance. Plant J 7: 535–542

Pearcy RW (1990) Sunflecks and photosynthesis in plant canopies. Annu Rev Plant Physiol Plant Mol Biol 41: 421–453

Periappuram C, Steinhauer L, Barton DL, Taylor DC, Chatson B and Zou J (2000) The plastidic phosphoglucomutase from Arabidopsis. A reversible enzyme reaction with an important role in metabolic control. Plant Physiol 122: 1193–1200

Pohlmeyer K, Paap BK, Soll J and Wedel N (1996) CP12: A small nuclear-encoded chloroplast protein provides novel insights into higher-plant GAPDH evolution. Plant Mol Biol 32: 969–978

Poolman MG, Fell DA and Raines CA (2003) Elementary modes analysis of photosynthate metabolism in the chloroplast stroma. Eur J Biochem 270: 430–439

Portis AR, Jr and Heldt HW (1976) Light-dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. Biochim Biophys Acta 449: 434–446

Portis AR Jr, Lilley RM and Andrews TJ (1995) Subsaturating ribulose-1,5-bisphosphate concentration promotes inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) - Studies using continuous substrate addition in the presence and absence of Rubisco activase. Plant Physiol 109: 1441–1451

Portis AR Jr, Salvucci ME and Ogren WL (1986) Activation of ribulose bisphosphate carboxylase/oxygenase at physiological CO_2 and ribulose bisphosphate concentrations by rubisco activase. Plant Physiol 82: 967–971

Preiss J (1984) Starch, sucrose biosynthesis and partition of carbon in plants are regulated by orthophosphate and triose-phosphates. Trends Biol Sci 9: 24–27

Quick WP, Schurr U, Scheibe R, Schulze E-D, Rodermel SR, Bogorad L and Stitt M (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. I. Impact on photosynthesis in ambient growth conditions. Planta 183: 542–554

Ragsdale SW (1997) The Eastern and Western branches of the Wood/Ljungdahl pathway: how the East and West were won. Biofactors 6: 3–11

Raines CA (2003) The Calvin cycle revisited. Photosynth Res 75: 1–10

Raines CA, Lloyd JC and Dyer TA (1999) New insights into the structure and function of sedoheptulose-1,7-bisphosphatase; an important but neglected Calvin cycle enzyme. J Exp Bot 50: 1–8

Raines CA, Harrison EP, Ölçer H and Lloyd JC (2000) Investigating the role of the thiol-regulated enzyme sedoheptulose-1,7-bisphosphatase in the control of photosynthesis. Physiol Plant 110: 303–308

Rajagopalan R and Altekar W (1994) Characterisation and purification of ribulose-bisphosphate carboxylase from heterotrophically grown halophilic archaebacterium, *Haloferax mediterranei*. Eur J Biochem 221: 863–869

Robinson SP and Walker DA (1981) Photosynthetic carbon reduction cycle. In: Hatch, MD and Boardman NK (eds) The Biochemistry of Plants. A Comprehensive Treatise, pp 193–236. Academic Press, New York

Roy H and Andrews TJ (2000) Rubisco: Assembly and mechanism. In: Leegood RC, Sharkey TD and von Caemmerer S (eds) Photosynthesis: Physiology and Metabolism, Advances in Photosynthesis, Vol 9, pp 53–83. Kluwer Academic Publishers, Dordrecht

Ruuska SA, Andrews TJ, Badger MR, Price GD and von Caemmerer S (2000) The role of chloroplast electron transport and metabolites in modulating rubisco activity in tobacco. Insights from transgenic plants with reduced amounts of cytochrome *b/f* complex or glyceraldehyde 3-phosphate dehydrogenase. Plant Physiol 122: 491–504

Salvucci ME and Anderson JC (1987) Factors affecting the activation state and the level of total activity of ribulose bisphosphate carboxylase in tobacco protoplasts. Plant Physiol 85: 66–71

Scheibe R (1987) $NADP^+$-malate dehydrogenase in C_3 plants: Regulation and role of a light-activated enzyme. Physiol Plant 71: 393–400

Scheibe R, Geissler A and Fickenscher K (1989) Chloroplast glucose-6-phosphate dehydrogenase: K_m shift upon light modulation and reduction. Arch Biochem Biophys 274: 290–297

Scheibe R, Wedel N, Vetter S, Emmerlich V and Sauermann SM (2002) Co-existence of two regulatory NADP-glyceraldehyde 3-P dehydrogenase complexes in higher plant chloroplasts. Eur J Biochem 269: 5617–5624

Schleucher J, Vanderveer P, Markley JL and Sharkey TD (1999) Intramolecular deuterium distributions reveal disequilibrium of chloroplast phosphoglucose isomerase. Plant Cell Environ 22: 525–533

Schrader SM, Kleinbeck KR, and Sharkey TD (2007) Rapid heating of intact leaves reveals initial effects of stromal oxidation on photosynthesis. Plant Cell Environ 30: 671–678

Schürmann P and Jacquot JP (2000) Plant thioredoxin systems revisited. Annu Rev Plant Physiol Plant Mol Biol 51: 371–400

Schwender J, Goffman F, Ohlrogge JB and Shachar-Hill Y (2004) Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature 432: 779–782

Seemann JR, Berry JA, Freas SM and Krump MA (1985) Regulation of ribulose bisphosphate carboxylase activity in vivo by a light-modulated inhibitor of catalysis. Proc Natl Acad Sci USA 82: 8024–8028

Selesi D, Schmid M and Hartmann A (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (cbbL) in differently managed agricultural soils. Appl Environ Microbiol 71: 175–184

Servaites JC (1985) Binding of a phosphorylated inhibitor to ribulose bisphosphate carboxylase/oxygenase during the night. Plant Physiol 78: 839–843

Sharkey TD (1985) Photosynthesis in intact leaves of C_3 plants: Physics, physiology and rate limitations. Bot Rev 51: 53–105

Sharkey TD (1990) Feedback limitation of photosynthesis and the physiological role of ribulose bisphosphate carboxylase carbamylation. Bot Mag Tokyo special issue 2: 87–105

Sharkey TD and Vassey TL (1989) Low oxygen inhibition of photosynthesis is caused by inhibition of starch synthesis. Plant Physiol 90: 385–387

Sharkey TD, Seemann JR and Pearcy RW (1986a) Contribution of metabolites of photosynthesis to postillumination CO_2 assimilation in response to lightflecks. Plant Physiol 82: 1063–1068

Sharkey TD, Stitt M, Heineke D, Gerhardt R, Raschke K and Heldt HW (1986b) Limitation of photosynthesis by carbon metabolism. II O_2 insensitive CO_2 uptake results from limitation of triose phosphate utilization. Plant Physiol 81: 1123–1129

Sharkey TD, Laporte MM, Lu Y, Weise SE and Weber APM (2004) Engineering plants for elevated CO_2: A relationship between sugar sensing and starch degradation. Plant Biol 6: 280–288

Sicher RC (1989) Evidence for a light dependent increase of phosphoglucomutase activity in isolated, intact spinach chloroplasts. Plant Physiol 89: 557–563

Sicher RC and Kremer DF (1990) Hexose and hexose-phosphate metabolism in barley leaves and roots. Role of glucose 1,6-bisphosphate. Plant Sci 67: 47–56

Somerville CR, Portis AR Jr, and Ogren WL (1982) A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase *in vivo*. Plant Physiol 70: 381–387

Spear JR, Walker JJ, McCollom TM and Pace NR (2005) Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. Proc Nat Acad Sci USA 102: 2555–2560

Spreitzer RJ (2003) Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch Biochem Biophys 414: 141–149

Stitt M (1990) Application of control analysis to photosynthetic sucrose synthesis. In: Cornish-Bowden, A and Cardenas, ML (eds) Control of Metabolic Processes, pp 363–376 Plenum Press, New York

Stitt M and ap Rees T (1979) Capacities of pea chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. Phytochemistry 18: 1905–1911

Stitt M and Heldt HW (1981) Simultaneous synthesis and degradation of starch in spinach chloroplasts in the light. Biochim Biophys Acta 638: 1–11

Stitt M, Quick WP, Schurr U, Schulze E-D, Rodermel SR and Bogorad L (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbcS*. II. Flux-control coefficients for photosynthesis in varying light, CO_2, and air humidity. Planta 183: 555–566

Streusand VJ and Portis AR Jr (1987) Rubisco activase mediates ATP-dependent RuBPCase activation. Plant Physiol 85: 152–154

Tabita FR, Satagopan S, Hanson TE, Kreel NE and Scott SS (2008). Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. J Exp Bot 59: 1515–1524

Tamoi M, Ishikawa T, Takeda T and Shigeoka S (1996) Molecular characterization and resistance to hydrogen peroxide of two fructose-1,6-bisphosphatases from *Synechococcus* PCC 7942. Arch Biochem Biophys 334: 27–36

Tamoi M, Miyazaki T, Fukamizo T and Shigeoka S (2005) The Calvin cycle in cyanobacteria is regulated by CP12 via the NAD(H)/NADP(H) ratio under light/dark conditions. Plant J 42: 504–513

Tennessen DJ, Bula RJ and Sharkey TD (1995) Efficiency of photosynthesis in continuous and pulsed light emitting diode irradiation. Photosynth Res 44: 261–269

Turner JF and Turner DH (1980) The regulation of glycolysis and the pentose phosphate pathway. In: Davies, DP

(ed) The Biochemistry of Plants, pp 279–316. Academic Press, New York

von Caemmerer S and Edmondson DL (1986) The relationship between steady-state gas exchange, in vivo RuP_2 carboxylase activity and some carbon reduction cycle intermediates in *Raphanus sativus*. Aust J Plant Physiol 13: 669–688

Walker DA and Herold A (1977) Can the chloroplast support photosynthesis unaided. Plant Cell Physiol SI: 295–310

Ward DM, Ferris MJ, Nold SC and Bateson MM (1998) A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol Mol Biol Rev 62: 1353–1370

Watson GM, Yu JP and Tabita FR (1999) Unusual ribulose 1,5-bisphosphate carboxylase/oxygenase of anoxic archaea. J Bacteriol 181: 1569–1575

Wedel N and Soll J (1998) Evolutionary conserved light regulation of Calvin cycle activity by NADPH-mediated reversible phosphoribulokinase/CP12/ glyceraldehyde-3-phosphate dehydrogenase complex dissociation. Proc Nat Acad Sci USA 95: 9699–9704

Wedel N, Soll J and Paap BK (1997) CP12 provides a new mode of light regulation of Calvin cycle activity in higher plants. Proc Nat Acad Sci USA 94: 10479–10484

Weise SE, Schrader SM, Kleinbeck KR and Sharkey TD (2006) Carbon balance and circadian regulation of hydrolytic and phosphorolytic breakdown of transitory starch. Plant Physiol 141: 879–886

Weise SE, Weber APM and Sharkey TD (2004) Maltose is the major form of carbon exported from the chloroplast at night. Planta, 218: 474–482

Wenderoth I, Scheibe R and von Schaewen A (1997) Identification of the cysteine residues involved in redox modification of plant plastidic glucose-6-phosphate dehydrogenase. J Biol Chem 272: 26985–26990

Werdan K, Heldt HW and Milovancev M (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochim Biophys Acta 396: 276–292

Winter H, Robinson DG and Heldt HW (1994) Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193: 530–535

Wolosiuk RA and Buchanan BB (1978) Activation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase by ferredoxin-thioredoxin system. Plant Physiol 61: 669–671

Woodrow IE and Berry JA (1988) Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants. Annu Rev Plant Physiol 39: 533–594

Woodrow IE and Walker DA (1983) Regulation of stromal sedoheptulose-1,7-bisphosphatase activity and its role in controlling the reductive pentose phosphate pathway of photosynthesis. Biochim Biophys Acta 722: 508–516

Zhang XV and Martin ST (2006) Driving parts of Krebs cycle in reverse through mineral photochemistry. J Amer Chem Soc 128: 16032–16033

Zhu X-G, de Sturler E and Long SP (2007) Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: A numerical simulation using an evolutionary algorithm Plant Physiol, 145: 513–526

Ziegler H and Ziegler I (1965) The influence of light on the $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase. Planta 65: 369–380

Chapter 27

Photosynthetic Sucrose Biosynthesis: An Evolutionary Perspective

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Summary

In this chapter, we explore the origins and evolutionary history of sucrose synthesis in plants. Our journey begins with the photosynthetic proteobacteria and cyanobacteria, which were probably the first organisms to synthesize this disaccharide sugar. Sucrose remained an obscure metabolite, used mainly for osmoregulation, until the endosymbiosis of a sucrose-synthesizing cyanobacterium gave rise to the chloroplasts of green plants. This unique event opened up new opportunities for sucrose, and ultimately led to its achieving global importance as one of the principal products of photosynthesis in plants. The rise of sucrose parallels the evolution of plants themselves, with the appearance of long distance vascular transport systems and the adoption of sucrose as a major transport sugar being key developments.

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During this process the two enzymes in the committed pathway of sucrose synthesis – sucrose-phosphate synthase (SPS) and sucrose-phosphatase (SPP) – evolved new structural and regulatory features, such as the phosphorylation sites involved in light-dark modulation and osmotic stress activation of SPS. We describe how these and other regulatory mechanisms act to coordinate the rate of sucrose synthesis in leaves with photosynthetic CO_2 fixation, sucrose export and storage. As our evolutionary journey nears its end, we discover how gene duplications and divergence gave rise to the four SPS gene families – A, B, C and D — that we see in angiosperms today, and how plants have recruited each of the SPS isoforms for different purposes, although these may vary from species to species. Having charted the meteoric rise of sucrose, we finish with some speculation on why this sugar of humble origin came to achieve such prominence in the life of plants, concluding that the secrets of its success remain largely a mystery.

I. Introduction

Sucrose is one of the main products of photosynthesis, and the most commonly transported carbohydrate in vascular plants (see Chapter 28). As such it provides most of the energy and carbon needed for the growth of non-photosynthetic organs and the laying down of starch, fructan and oil reserves in seeds, tubers, stems and fruits. Sucrose itself is a common storage form of carbon, and is often accumulated by plants exposed to cold or drought, acting as a compatible solute to protect cell integrity and viability. Many metabolic and developmental processes, such as storage reserve synthesis and the transition to flowering, are dependent on the availability of sucrose, and sucrose plays yet another role in plants as a signal molecule, acting at multiple levels to modulate metabolic and developmental gene expression (Lunn and MacRae, 2003). Few other compounds challenge the dominance of sucrose in plant metabolism. The raffinose-family (raffinose, stachyose and verbascose) and other galactose-containing oligosaccharides (e.g., planteose) can be quantitatively important transport and storage sugars (Avigad and Dey, 1997), especially in symplasmic phloem loading species in the Cucurbitaceae and Lamiaceae (Turgeon and Medville, 2004), but all of these molecules are essentially derivatives of sucrose. Some members of the Apiaceae and many fruit trees in the Rosaceae transport substantial amounts of sugar alcohols, especially mannitol or sorbitol, in addition to a certain amount of sucrose, but sucrose is still a significant product of photosynthesis and an important storage reserve even in these plants. The central role of sucrose in plant metabolism is clear, but the reasons why this should be so remain uncertain.

The pathway of sucrose biosynthesis in angiosperms (flowering plants) is well established, and we have a good understanding of the regulatory mechanisms that control flux through the pathway, at least in a few model species such as spinach (*Spinacia oleracea*), maize (*Zea mays*) and *Arabidopsis thaliana*. In this chapter, we shall give a brief overview of pathway regulation, but as this topic has been reviewed in detail (see MacRae and Lunn, 2005) we decided to focus on sucrose synthesis from an evolutionary perspective.

Molecular phylogenetics has revolutionized our understanding of evolutionary relationships among the green plants. Several traditional classifications, e.g., "green algae", "bryophytes" and "dicots", are now thought to be paraphyletic rather than monophyletic and some other traditional groupings have doubtful validity. This new understanding of the plant branch of the "tree of life" will set the context for our discussion on the origins and evolution of sucrose synthesis in plants. We shall concentrate on the two enzymes in the committed pathway of

Abbreviations: aa – Amino acid; CAM – Crassulacean acid metabolism; DHAP – Dihydroxyacetone phosphate; F1,6BPase – Fructose-1,6-bisphosphatase; F2KP – Fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; $Fru1,6P_2$ – Fructose 1,6-bisphosphate; $Fru2,6P_2$ – Fructose 2,6-bisphosphate; Fru6P – Fructose 6-phosphate; G3P – Glyceraldehyde 3-phosphate; Glc6P – Glucose 6-phosphate; Gly3P – Glycerol 3-phosphate; PEP – Phosphoenolpyruvate; PGA – 3-phosphoglycerate; ORF – Open reading frame; SPS – Sucrose-phosphate synthase; SPP – Sucrose-phosphatase; Suc6P – sucrose 6^F-phosphate; SuSy – Sucrose synthase; UDPGlc – UDPglucose

sucrose biosynthesis: (i) sucrose-phosphate synthase (SPS; EC 2.3.1.14), which catalyzes the synthesis of sucrose 6^F-phosphate (Suc6P) from a nucleoside-diphosphoglucose, usually UDPglucose (UDPGlc), and fructose 6-phosphate (Fru6P), and (ii) sucrose-phosphatase (SPP; EC 3.1.3.24), which hydrolyzes Suc6P to release free sucrose and orthophosphate (P_i). Both of these enzymes probably originated in the bacteria, and were inherited by plants from the cyanobacterial ancestor of chloroplasts. In attempting to retrace their history, we discuss how the appearance of new structural and regulatory features in SPS might be linked to sucrose taking on new roles during the evolution of vascular plants from simple eukaryotic green algae, and we identify where gaps still remain in our knowledge of this evolutionary history. We conclude by re-examining the question of why, out of all the possible options, sucrose came to be so important to plants.

II. Sucrose Synthesis in Bacteria

A. The Origins of Sucrose Synthesis

Apart from green plants (Viridiplantae) and some single-celled photosynthetic eukaryotes (e.g., *Euglena gracilis*), only a few groups of bacteria (Eubacteria) are known to synthesize sucrose. Sucrose has been found in both freshwater and marine species of cyanobacteria (Reed et al., 1984; Salerno et al., 1995; Page-Sharp et al., 1999), including unicellular (e.g., strains belonging to *Synechocystis*, *Synechococcus* and *Scytonema*) and filamentous types (e.g., strains of *Nostoc* and *Anabaena*). Sucrose is also present in two species of halo/alkali-tolerant, methanotrophic γ-proteobacteria – *Methylobacter alcaliphilus* 20Z and *Methylobacter modestohalophilus* (Khmelenina et al., 1999, 2000). To our knowledge, there is no experimental evidence of sucrose synthesis in any other groups of bacteria. Nevertheless, the ability to synthesize sucrose can be inferred in at least one species from two other groups of bacteria – the planctomycetes and firmicutes. The genome of the marine planctomycete, *Rhodopirellula baltica*, has an open reading frame (ORF) that encodes a protein (GenBank accession No. NP_866562) with 42% identity (60% similarity) to a cyanobacterial SPS from *Synechocystis* sp. PCC 6803. For reasons that will be discussed in more detail later, the putative *Rhodopirellula baltica* SPS is predicted to be a bifunctional enzyme, with both SPS and SPP activities. An ORF encoding another SPS-like protein has been found in the firmicute bacterium *Halothermothrix orenii*. Although the *Halothermothrix orenii* sequence shows only moderate similarity to the *Synechocystis* sp. PCC 6803 SPS, it has been reported that the protein does have SPS activity (Huynh, 2004).

From similar analyses of genomic sequences, putative *sps* genes have been identified in β-proteobacteria (*Nitrosomonas europea*, *Methylobacillus flagellatus*), γ-proteobacteria (*Acidithiobacillus ferrooxidans*) (Mijts and Patel, 2001) and δ/ε-proteobacteria (*Desulfuromonas acetoxidans*), and in several species of cyanobacteria (Cumino et al., 2002; Lunn, 2002). No putative *sps* genes have so far been identified in any other groups of bacteria, or in the archaea, even though many genomic sequences from representative species of these groups are now available. Therefore, our current view is that sucrose synthesis in prokaryotes is restricted to just four groups of bacteria: cyanobacteria, proteobacteria, planctomycetes and firmicutes, and is absent from the archaea.

The presence of sucrose in many cyanobacterial species from different lineages (Reed et al., 1984) suggests that the capacity to synthesize sucrose existed in an early ancestral cyanobacterium, before the various cyanobacterial lineages diverged about 2.0–3.5 billion years ago. But were cyanobacteria the first organisms to make sucrose? The answer to this question is shrouded in the mists of time, and efforts to trace the evolutionary history of the bacteria themselves, let alone the history of sucrose metabolism in these organisms, are hampered by horizontal gene transfer between unrelated species. Nevertheless, the relatively high incidence of *sps*-and *spp*-related genes in the proteobacteria suggests that sucrose metabolism might also have been present in this group from an early date. Phylogenetic analysis of genes encoding photosystem (PS) I and II reaction center proteins led to the proposal that the oxygenic photosynthetic apparatus of the cyanobacteria evolved from heterologous fusion of ancestral types related to those in the heliobacteria/green

sulfur bacteria (PS I) and proteobacteria/green non-sulfur bacteria (PS II) (Xiong et al., 1998). It therefore seems reasonable to propose that cyanobacteria could have inherited the enzymes of sucrose metabolism, along with PS II, from a common ancestor with the proteobacteria (Lunn, 2002).

The occurrence of sucrose-related genes in other bacterial groups appears to be much less common, and there could be several reasons for this patchy distribution. One possible explanation is that there has been extensive loss of sucrose synthetic enzymes from most lineages of the bacteria. Alternatively, and perhaps more likely, sporadic horizontal gene transfer from cyanobacteria or proteobacteria could account for isolated examples of sucrose synthesis in other groups of bacteria. Cyanobacteria share the same type of habitats where the marine planctomycete *Rhodopirellula baltica* and the firmicute *Halothermothrix orenii* (isolated from a desert salt pan in Tunisia) live, so it seems almost certain that there has been the opportunity for these two species to acquire their *sps* genes by horizontal gene transfer from cyanobacteria at some time in their past. Presumably such gene transfer has occurred many times into other types of bacteria too, but unless the ability to synthesize sucrose conferred some competitive advantage in a particular habitat there would be no selective pressure for its retention.

B. The Functions of Sucrose in Bacteria

Many aquatic species of cyanobacteria use sucrose as a compatible solute to help balance their intracellular water potential with that of the external environment (Reed et al., 1984; 1986; Reed and Stewart, 1985), and it seems likely that non-cyanobacterial species also use sucrose as a compatible solute. Some cyanobacteria use other compounds (e.g., α,α-trehalose or glucosylglycerol) instead of, or in addition to, sucrose, and glycine betaine or glutamate betaine are often the preferred compatible solutes in species exposed to the most hostile conditions (Page-Sharp et al., 1999; Hagemann and Marin, 1999). Cyanobacteria living in dry or hot environments, e.g., salt pans and thermal springs, use these compounds to protect themselves from the effects of desiccation and high temperature. Sucrose and α,α-trehalose can displace water from the hydration shell of the polar head groups of membrane lipids, and this can prevent membrane fusions as well as lateral phase separations in lipid bilayers, which could otherwise lead to increased membrane permeability and loss of solutes from the cell (Caffrey et al., 1988; Elbein et al., 2003; Hincha and Hagemann, 2004). This property appears to be an important factor in the ability of these sugars to confer stress tolerance to cells living in hot or dry habitats. Expression of the *Synechocystis* sp. PCC 6803 SPS in *Escherichia coli* led to the accumulation of sucrose in this species, which does not normally synthesize this sugar, and a 10,000-fold increase in desiccation tolerance (Billi et al., 2000), demonstrating the effectiveness of sucrose as a protective agent in vivo.

Curiously, sucrose appears to have less importance as a compatible solute in *Synechocystis* sp. PCC 6803 itself, as this species accumulates glucosylglycerol rather than sucrose under osmotic stress conditions (Hagemann and Marin, 1999). Deletion of the *sps* gene had no effect on the growth of *Synechocystis* sp. PCC 6803 cells under normal conditions, showing that sucrose is not essential for survival in this strain (Hagemann and Marin, 1999). However, partial deletion of the ADPglucose pyrophosphorylase gene prevented the synthesis of glucosylglycerol and glycogen, and the cells were able to adapt to salt stress by accumulating sucrose instead (Miao et al., 2003). Expression of the *Synechocystsis* sp. PCC 6803 *sps* gene is transiently upregulated within the first hour of exposure to salt stress, probably due to a salt-induced increase in transcription, and this leads to a transitory accumulation of sucrose over the first 5 h (Marin et al., 2004; Desplats et al., 2005), but after 24 h of exposure to salt stress the amount of SPS protein was found to be the same as in unstressed cells (Lunn et al., 1999). It has been suggested that the transitory accumulation of sucrose acts to trigger a signal transduction cascade leading to longer-term adaptation, with only a minor role in direct protection against osmotic stress (Desplats et al., 2005).

In addition to being a compatible solute, sucrose could also have a transport function in filamentous, N_2-fixing cyanobacteria. The nitrogenase enzyme responsible for N_2 fixation is extremely sensitive to oxygen, and to protect it from the oxygenic reactions of photosynthesis it is sequestered in specialized, non-photosynthetic

cells called heterocysts. These cells contain high alkaline invertase activity, and it has been suggested that sucrose is synthesized in the photosynthetic cells and then transported to the heterocysts, where it is hydrolyzed by the invertase to provide substrates for respiration (Schilling and Ehrnsperger, 1985). A more recent study showed that sucrose synthase (SuSy) also contributes to sucrose cleavage in heterocysts (Curatti et al., 2002). Some single-celled cyanobacteria also fix N_2, and these overcome the incompatibility of oxygenic photosynthesis and N_2 fixation by a temporal separation of the two processes, by means of circadian control of gene expression. It has been suggested that these species might accumulate sucrose as a temporary storage reserve during the day, and then use it to support respiration and N_2 fixation at night (Lunn, 2002).

C. The Pathway of Sucrose Synthesis in Bacteria

As we have described, bacteria use sucrose for several purposes, and the source of substrates for sucrose synthesis is likely to vary depending on the species and the conditions. Cyanobacteria fix CO_2 via the Calvin-Benson cycle (White, 1995), and one of the phosphorylated intermediates in this pathway, Fru6P, can be used for sucrose synthesis. The enzymes phosphoglucose isomerase and phosphoglucomutase convert Fru6P to glucose 1-phosphate (Glc1P), via glucose 6-phosphate (Glc6P), providing the substrate for synthesis of UDPglucose or ADPglucose by UDPGlc or ADPGlc pyrophosphorylases, respectively (Fig. 27.1). In plants, only UDPGlc can serve as the glucosyl donor in the SPS reaction, but cyanobacterial SPSs are more promiscuous and can often use other types of nucleoside-diphosphoglucose molecule as well. For example, the *Synechocystis* sp. PCC 6803 SPS can use UDPGlc, ADPGlc, GDPGlc or CDPGlc as the glucosyl donor in vitro, with K_m values of 2.9, 2.5, 1.8 and 7.2 mM, respectively (Lunn et al., 1999), although UDPGlc and ADPGlc are probably the most common substrates for sucrose synthesis in vivo. In cyanobacteria, the enzymes of CO_2 fixation often occur within a protein shell, called the carboxysome, but it is not known if the enzymes necessary for sucrose synthesis are located inside or outside this microcompartment, or whether a transport step is needed somewhere along the pathway. In non-photosynthetic bacteria, the precursors for sucrose synthesis may be supplied by gluconeogenesis, breakdown of sugar polymers (e.g., glycogen), or by uptake of external sugars.

The synthesis of two other common compatible solutes in cyanobacteria, α,α-trehalose and glucosylglycerol, occurs via phosphorylated intermediates (trehalose 6-phosphate and glucosylglycerol 3-phosphate) in reactions analogous to those catalyzed by SPS and SPP (Cabib and Leloir, 1958; Marin et al., 1998). Nucleoside-diphosphoglucose molecules also serve as the glucosyl donor in these reactions, with the acceptor molecules being Glc6P and glycerol 3-phosphate, respectively (Fig. 27.1). With at least one substrate in common, sucrose synthesis must compete with these other pathways, if present, and this competition will influence which is the dominant solute accumulated. Synthesis of polyols (e.g., mannitol and sorbitol) from hexose-phosphate precursors, which occurs in a few species of bacteria, could also compete for substrates with sucrose synthesis.

Bacteria contain at least three different types of SPS enzyme (Lunn, 2002; Cumino et al., 2002). The simplest form (type 1), found in some cyanobacteria (e.g., *Prochlorococcus marinus* subsp. *pastoris* strain CCMP1986), has a molecular mass of about 53 kDa, and contains a single domain. This domain shows similarity with other glucosyltransferase enzymes, such as sucrose synthase (SuSy), as well as the catalytic domain of the plant SPS (Fig. 27.2). Several filamentous species, e.g., *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102, possess one or more type-1 SPSs, which show less similarity with the plant enzyme and are slightly smaller (45–47 kDa) (Cumino et al., 2002). The *Anabaena* sp. PCC 7120 SPS1 is dependent on a divalent metal cation (Mg^{2+} or Mn^{2+}) for activity, but the *Anabaena* sp. PCC 7120 SPS2 is not (Porchia and Salerno, 1996). The native *Anabaena* sp. PCC 7120 enzymes are both monomeric. It appears that the 59-kDa SPS from *Halothermothrix orenii*, which has a specific activity of 6 μmol min^{-1} mg^{-1} protein using UDPGlc and Fru6P as substrates, also belongs to this group (Mijts and Patel, 2001; Huynh, 2004). There is usually a separate SPP enzyme in species with a type-1 SPS (Lunn, 2002).

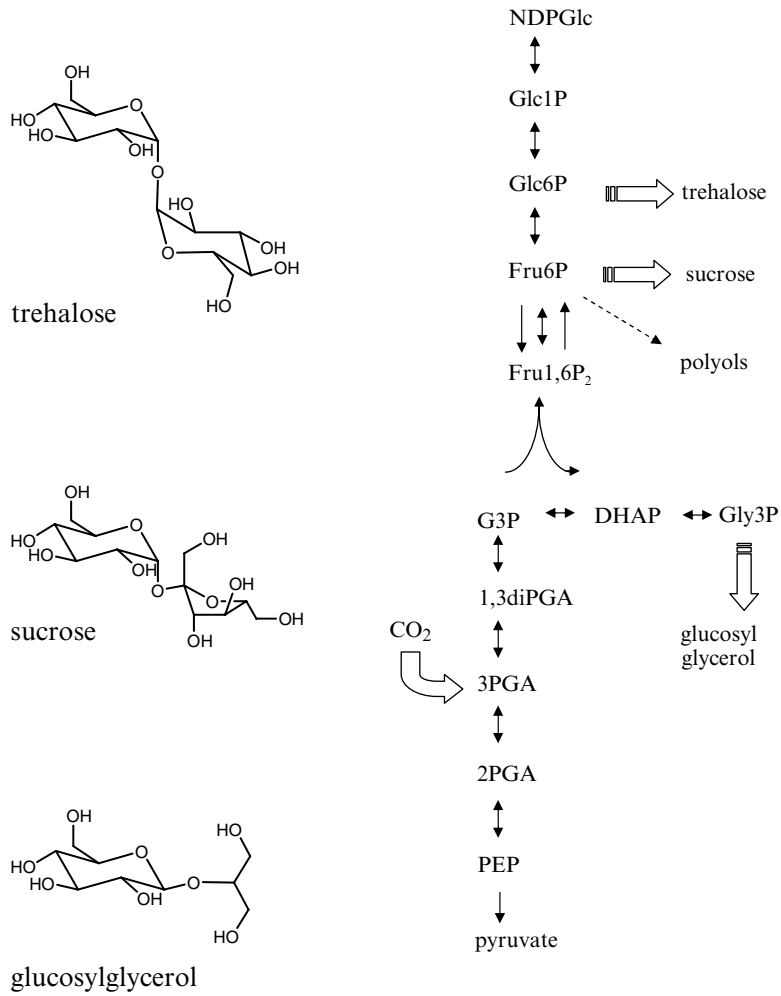

Fig. 27.1. Structures of key disaccharide and polyol sugars and the basic pathway of sugar synthesis in prokaryotes.

The type-2 SPSs are larger than the type-1 enzyme, with a molecular mass of 79–81 kDa, and contain a 260–270 amino acid (aa) domain at the C-terminus in addition to the catalytic glucosyltransferase domain. This C-terminal domain has significant similarity to cyanobacterial SPPs, and includes most of the conserved residues found in the active site of the *Synechocystis* sp. PCC 6803 SPP, suggesting that the type-2 SPS is a bifunctional enzyme with both SPS and SPP activities (Lunn, 2002). In further support of this proposal, species with only a type-2 SPS do not appear to have a separate SPP enzyme. Although this proposal has yet to be tested experimentally, it has been shown that an artificial, chimeric form of the *Synechocystis* sp. PCC 6803 enzymes can have both SPS and SPP activities (Lunn, 2002). The type-2 SPS is found in several cyanobacteria (e.g., *Synechococcus elongatus* PCC 6301, *Prochlorococcus marinus* strain MIT 9319 and *Thermosynechococcus elongatus* BP-1), some proteobacteria (e.g., *Nitrosomonas europea* and *Acidithiobacillus ferooxidans*) and the planctomycete, *Rhodopirellula baltica*. The cyanobacterium *Gloeobacter violacea* appears to have both an *Anabaena*-like type-1 SPS and a type-2 SPS, as well as a separate SPP.

The *Synechocystis* sp. PCC 6803 SPS (81 kDa) belongs to the third group of SPSs found in bacteria. These also have a C-terminal, SPP-like domain, but this lacks several of the active site residues that are critical for catalytic activity in SPP (Lunn et al., 2000; Lunn, 2002; Fieulaine et al., 2005), which is consistent with the

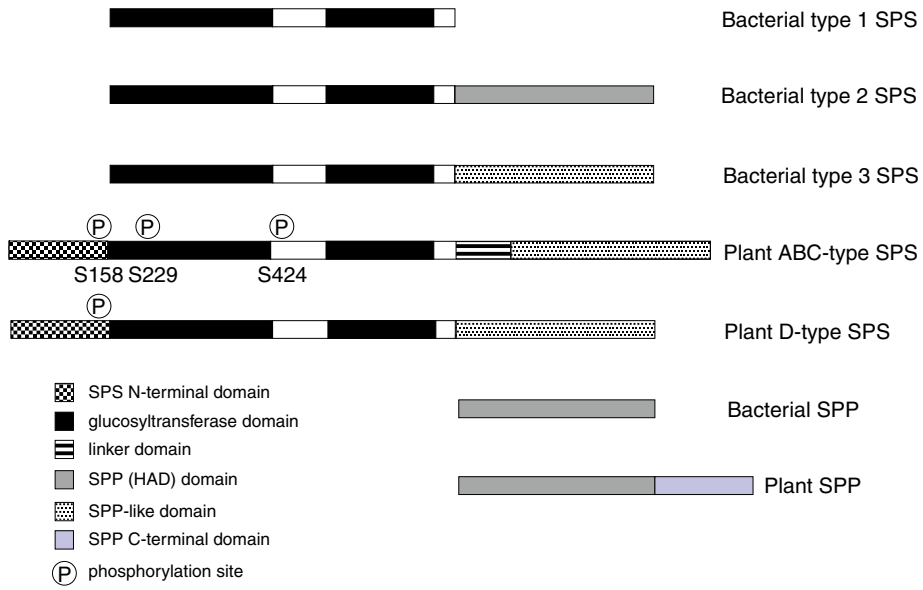

Fig. 27.2. Domain architecture of SPS and SPP proteins from bacteria and plants. Schematic alignment of SPS and SPP proteins from bacteria and plants showing the various catalytic and non-catalytic domains and known phosphorylation sites. In spinach leaf SPS (A-family), Ser158 is involved in light-dark regulation, phospho-Ser229 is the binding site for 14-3-3 proteins, and osmotic stress-induced phosphorylation of Ser424 activates the enzyme.

Synechocystis sp. PCC 6803 SPS's lack of SPP activity (Curatti et al., 1998) and the presence of a separate SPP enzyme in this species (Lunn et al., 2000). Modelling of the SPP-like domain of the *Synechocystis* sp. PCC 6803 SPS indicated that the residues involved in binding Suc6P are mostly conserved, so even though this domain has no SPP activity it might still bind Suc6P (Fieulaine et al., 2005). Based on previous reports that SPS and SPP can form a complex (Echeverria et al., 1997), it was proposed that the SPP-like domain of SPS could act to channel Suc6P between the active sites of these two enzymes (Fieulaine et al., 2005). The *Synechocystis* sp. PCC 6803 SPS is not Mg^{2+}-dependent and shows no obvious regulatory properties, and the native enzyme is monomeric (Curatti et al., 1998; Lunn et al., 1999; 2003).

The *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 SPPs have a molecular mass of 26–28 kDa, and the native enzymes are monomeric (Lunn et al., 2000; Cumino et al., 2001; Lunn, 2002). The activities of both enzymes are strictly dependent on the presence of a divalent metal cation, with Mg^{2+} being preferred to Mn^{2+}. They are highly specific for Suc6P, showing little or no hydrolytic activity with other sugar phosphates (e.g., Fru6P, trehalose-6-phosphate), and sucrose is a weak competitive inhibitor (K_i = 80 to > 200 mM). The enzymes show similarity to members of the haloacid dehalogenase (HAD) superfamily of proteins, which includes many other phosphatases and hydrolases (Lunn et al., 2000) that have three characteristic motifs associated with the active site.

Crystal structures of the *Synechocystis* sp. PCC 6803 SPP, alone and in combination with various ligands, have been determined at a resolution of 1.4 Å (Fieulaine et al., 2005). The protein consists of two domains: a core domain containing the catalytic site (including the HAD motif residues) and a smaller cap domain, joined by two flexible hinge loops allowing movement of the two domains. The enzyme was crystalized in two conformations, open and closed, and the latter was found to be catalytically active. The cap domain contains a glucose-binding site, which is largely responsible for the enzyme's remarkable substrate specificity and its inhibition by sucrose. Based on crystal structures of the enzyme with Suc6P bound in the active site, it was postulated that the catalytic reaction is initiated by nucleophilic attack on the substrate by Asp9, leading to formation of a covalent

phospho-Asp9-enzyme intermediate and free sucrose. In the second step of the reaction, a water molecule hydrolyzes the phosphoacyl bond to liberate P_i and regenerate the free enzyme. The Mg^{2+} ion cofactor plays a central role in substrate binding and catalysis, and appears to become displaced within the active site during the catalytic cycle, possibly to allow post-catalytic release of the P_i (Fieulaine et al., 2005). Apart from weak competitive inhibition by sucrose, the cyanobacterial SPPs, like the SPS counterparts, show no obvious regulatory properties. Therefore, it seems likely that the rate of sucrose synthesis is controlled mainly at the level of *sps* and *spp* gene transcription and by the availability of substrates.

III. Eukaryotic Sucrose Synthesis

A. The Cyanobacterial Origin of Sucrose Synthesis in Eukaryotes

The mitochondria of eukaryotic cells are thought to be derived from an ancient endosymbiosis of an α-proteobacterium, probably closely related to present day *Rickettsia* sp., whilst the chloroplasts of eukaryotic green algae and green plants (Viridiplantae) are believed to have a cyanobacterial ancestor (Cavalier-Smith, 2000; Keeling, 2004). As the enzymes of sucrose synthesis are present in both of these groups of bacteria, it is conceivable that eukaryotes could have inherited them from either of these organellar progenitors. However, the occurrence of sucrose synthesis only in the green plants and green algae, and not in any other group of eukaryotes, argues strongly in favor of a cyanobacterial origin for these enzymes in eukaryotes (Lunn, 2002; Salerno and Curatti, 2003). It is unclear which of the three types of cyanobacterial SPS was inherited by the first green eukaryotes, but the type-3 SPS (e.g., *Synechocystis* sp. PCC 6803) would seem to be the most likely type as it most closely resembles the enzyme found in plants.

B. Eukaryotic Algae

There are two major lineages of green plants, the first of which includes three classes of green algae (Chlorophyceae, Trebouxiophyceae and Ulvophyceae) and a basal, paraphyletic group,

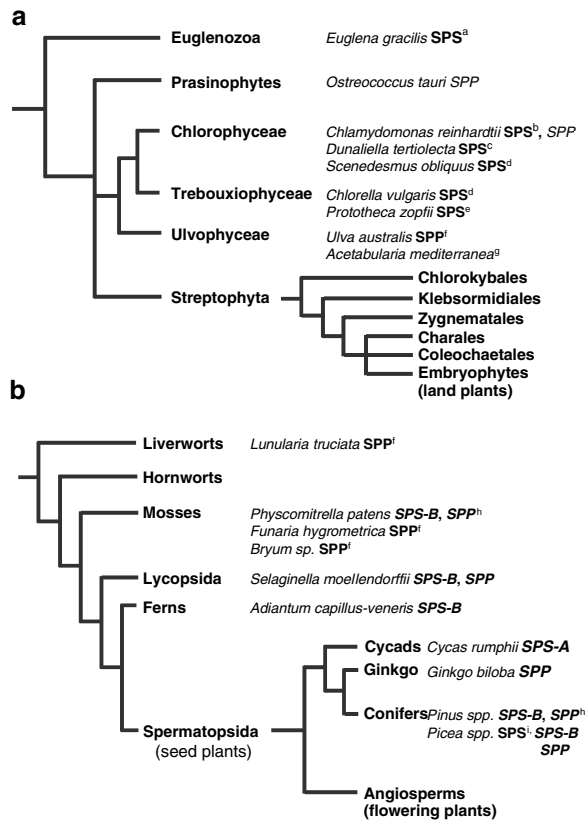

Fig. 27.3. SPS and SPP in green algae, lower plants and gymnosperms. (**a**) Phylogram (Adapted from the Tree of Life Web Project – http://tolweb.org) showing species of green algae in which SPS and/or SPP activities have been reported. Putative *SPP* genes (shown in *italics*) have also been identified from genomic sequences of two species. (**b**) Phylogram (Adapted from the Tree of Life Web Project and Palmer et al., 2004) showing non-Angiosperm species of Embryophytes (land plants) in which SPS and/or SPP activities have been reported. The presence of putative *SPS-A* (A-family), *SPS-B* (B-family) or *SPP* genes (shown in *italics*) is also indicated ([a]Porchia et al. (1999); [b]Salerno et al. (1995); [c]Müller and Wegmann (1978b); [d]Duran and Pontis (1977); [e]Salerno (1985a,b); [f]Hawker and Smith (1984); [g]Winkenbach et al. (1972); [h]Lunn (2003); [i]Loewe et al. (1996). Other data from unpublished analysis of sequences from the GenBank Expressed Sequence Tag (EST) (http://www.ncbi.nlm.nih.gov/) and Joint Genome Institute (JGI) Eukaryotic Genomes (http://genome.jgi-psf.org/euk_home.html) databases).

the prasinophytes, collectively known as the Chlorophyta (Lewis and McCourt, 2004) (Fig. 27.3a). Most of these chlorophyte green algae are microscopic freshwater organisms or large seaweeds. The second lineage of green plants, the Streptophyta (Fig. 27.3a), includes the land plants together with several groups of "green algae" (e.g., the Charales) and we shall return to

these plants in Section III.C. Sucrose is known to be present in several species from the Chlorophyceae (e.g., *Chlamydomonas reinhardtii, Dunaliella tertiolecta* and *Scenedesmus obliquus*), Ulvophyceae (e.g., *Acetabularia mediterranea*) and Trebouxiophyceae (Winkenbach et al., 1972; Salerno, 1985a, b). The latter class includes *Chlorella vulgaris*, which was used for many of the early $^{14}CO_2$-labeling experiments that led to the discovery of the Calvin-Benson cycle. These experiments revealed that sucrose is synthesized via a phosphorylated intermediate, which was initially thought to be sucrose 1^F-phosphate using Glc6P or UDPGlc and fructose 1-phosphate as substrates (Buchanan, 1953). However, the discovery of SPS in wheatgerm extracts established that UDPGlc and Fru6P are the substrates for sucrose synthesis, and that sucrose 6^F-phosphate is the true intermediate (Leloir and Cardini, 1955). In addition to *Chlorella vulgaris*, sucrose has also been reported to be a major product of photosynthetic CO_2 fixation in *Acetabularia mediterranea* (Ulvophyceae) (Winkenbach et al., 1972). SPS and/or SPP activities have been detected in at least one species from each of the three main classes of green algae (Fig. 27.3a).

The occurrence of sucrose synthesis in the Prasinophytes is uncertain. The fully sequenced genome of one species from this group, *Ostreococcus tauri*, contains an ORF that encodes a protein with significant similarity to cyanobacterial and plant SPPs, but no SPS-like ORF has been found. The only study of carbohydrate metabolism in this species found that it is able to synthesize starch, but there was no report on the presence or absence of sucrose (Ral et al., 2004). The genome of *Chlamydomonas reinhardtii*, which does have the capacity to synthesize sucrose (Salerno et al., 1995), also has a good candidate for an *SPP* gene, but no obvious SPS-encoding sequence. The closest match to known *SPS* genes is an ORF encoding a 170-kDa protein that contains two small (80–100 aa) regions with similarity to the glucosyltransferase domain of plant and cyanobacterial SPS proteins (JGI Eukaryotic Genomes). If this gene does encode the *C. reinhardtii* SPS, then the enzyme is very different to both the bacterial and plant SPSs. In addition to the chlorophyte green algae, the eukaryotic green protist *Euglena gracilis* can also synthesize sucrose (Porchia and Salerno, 1999). This species belongs to a separate lineage of organisms, the Euglenozoa, whose plastids were derived from the chloroplasts of green algae during a secondary endosymbiotic event (Keeling, 2004). It seems likely that *E. gracilis* acquired the enzymes of sucrose synthesis from green algae too. A few species of eukaryotic green algae from the genera *Trebouxia* (Trebouxiophyceae) and *Trentepohlia* (Ulvophyceae), as well as cyanobacteria from the genus *Nostoc*, can exist as the photobiont partners of lichen fungi, and these probably account for the presence of sucrose in these organisms, as reported for several Antarctic species (Roser et al., 1992).

The red algae (Rhodophyta) also contain plastids derived from the same cyanobacterial ancestor as chloroplasts, diverging from the green algal lineage after this single endosymbiotic event (Keeling, 2004). Although sucrose has never been found in these organisms (Avigad, 1982; Dancer and ap Rees, 1989; Karsten et al., 1993), the genomes of two red algae, *Galdieria sulphuraria* and *Cyanidioschyzon merolae* do contain ORFs that encode proteins with similarity to SPP and SPS, respectively (Barbier et al., 2005). A possible explanation for this apparent discrepancy is that the enzymes encoded by these genes are involved in synthesis of floridoside (α-D-galactopyranosyl-(1,2)-glycerol), or its isomers D- and L-isofloridoside (α-D/L-galactopyranosyl-(1,1)-glycerol), which are commonly found in red algae and even regarded as a diagnostic feature of these organisms (Avigad, 1982; Barbier et al., 2005). There are several groups of photosynthetic organisms, e.g., brown algae, diatoms and dinoflagellates, that derived their plastids from red algae in separate secondary endosymbiotic events (Palmer et al., 2004). As none of these groups appears to make sucrose either, it seems likely that the capacity to synthesize sucrose was lost from the red algal lineage at an early date, before these secondary endosymbioses.

Very little is known about the function of sucrose in green algae. In marine species (e.g., *Dunaliella tertiolecta*) one obvious role would be in osmoregulation, but sucrose synthesis also occurs is several freshwater green algae under normal growth conditions, so this might not be its only function. The rate of sucrose synthesis in *Dunaliella tertiolecta* was increased by raising the growth temperature above 25°C, and this response was attenuated by salt (NaCl) stress (Müller and Wegmann, 1978a). This suggests that

sucrose is involved in adaptation to high temperature, rather than osmoregulation, in this species. In *Acetabularia mediterranea*, sucrose synthesis was inhibited when nitrite, but not nitrate or ammonium, was supplied to the cells (Winkenbach et al., 1972). However, the rate of photosynthetic CO_2 fixation was also strongly decreased by nitrite, indicating a general inhibition of metabolism by this anion.

Our knowledge of the enzymes and control of sucrose synthesis in green algae is also scanty. SPS activity has been reported in the Chlorophyceae (*Chlamydomonas reinhardtii*, *Dunaliella tertiolecta* and *Scenedesmus obliquus*), Trebouxiophyceae (*Chlorella vulgaris* and *Prototheca zopfii*) and Euglenozoa (*Euglena gracilis*) (Duran and Pontis, 1977; Müller and Wegmann, 1978b; Salerno, 1985a, b; Salerno et al., 1995) and SPP activity has been detected in *Ulva australis* (Ulvophyceae) (Hawker and Smith, 1984). The *Chlorella vulgaris* and *Scenedesmus obliquus* SPSs showed similar kinetic properties to those of the higher plant enzyme (Duran and Pontis, 1977). Activity of the *Chlorella vulgaris* enzyme was found to be inhibited by P_i and activated by Glc6P (Salerno, 1985a), but insensitive to sucrose, whereas the *Dunaliella tertiolecta* SPS was reported to be activated by P_i (Müller and Wegmann, 1978b). The *Chlorella vulgaris* SPS has a native molecular mass of 400 kDa, suggesting that it is likely to be a multimeric enzyme, as it is in plants (Duran and Pontis, 1977). The *Ulva australis* SPP activity was Mg^{2+}-dependent and 100 mM sucrose inhibited activity by 51% (Hawker and Smith, 1984). The putative *Ostreococcus tauri* and *Chlamydomonas reinhardtii SPP* genes encode 33-kDa and 32-kDa proteins, respectively. Like the cyanobacterial SPPs from *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, the *C. reinhardtii* protein contains only the catalytic HAD domain, whereas the *O. tauri* protein has a small (approx. 26 aa) N-terminal extension.

One of the most interesting unanswered questions concerning sucrose synthesis in chlorophyte green algae is its intracellular location. Presumably in the earliest eukaryotic green algae sucrose would have been synthesized in the chloroplasts, but in vascular plants it is synthesized exclusively in the cytosol (Nishimura and Beevers, 1979; Robinson and Walker, 1979). Therefore, at some point during evolution the pathway must have been transferred from the chloroplast to the cytosol, perhaps via an intermediate stage where sucrose was synthesized in both compartments. Intriguingly, sucrose was reported to be one of the major products of photosynthetic CO_2 fixation by isolated chloroplasts of *Acetabularia mediterranea* (Winkenbach et al., 1972). Although there was significant cytosolic contamination of these chloroplast preparations, we should keep an open mind towards the possibility that some sucrose synthesis might occur within the chloroplasts in some species of green algae. The transfer of sucrose synthesis to the cytosol probably occurred in parallel with transfer of the genes encoding SPS and SPP from the primitive chloroplast genome to the nucleus. In plants these enzymes are encoded only by nuclear genes, and no trace of *sps* or *spp* genes is to be found in the chloroplast genomes of green algae or higher plants.

We can only speculate on the reasons for this shift in the site of sucrose synthesis. One possible explanation is that if sucrose were a major product of photosynthesis, high rates of sucrose synthesis in the chloroplasts would pose too big a challenge for maintaining osmotic balance between these organelles and the cytosol. Rapid export of sucrose into the cytosol would alleviate this problem, but this would require some kind of sucrose transporter in the chloroplast envelope, and no such transporter has been found in plants. The main sucrose transporters in plants are sucrose:H^+ symporters, and the high stromal pH in the light would be unfavorable for such transporters to export sucrose from the chloroplast. Conversely, although transport by a sucrose:H^+ antiporter would be feasible, this would lower the stromal pH, and thus the proton gradient across the thylakoid membranes, and inhibit photophosphorylation.

C. Charales, Bryophytes and Lower Vascular Plants

In recent years, molecular phylogenetic studies have shown that the traditional classification of "green algae" as a single group is phylogenetically untenable, with this paraphyletic grouping containing both the true chlorophyte green algae and other species that belong to a separate, monophyletic lineage (Streptophyta) that also includes the land

plants (Embryophyta) (Lewis and McCourt, 2004). Within this streptophyte lineage, the characean algae (Charales), e.g., *Chara*, are the most closely related to land plants, forming a sister clade to the Embryophyta (Fig. 27.3b). Sucrose is known to contribute to osmoregulation and maintenance of turgor pressure in several species of characean plants including: *Chara vulgaris*, *Tolypella nidifica*, *Tolypella glomerata* and *Nitellopsis obtusa* (Kirst et al., 1988; Winter et al., 1996, 1999), and sucrose is the main transported carbohydrate in *Chara vulgaris* and *Chara corallina* (Ding et al., 1992; Schulte et al., 1994). Essentially nothing is known about the enzymology and control of sucrose synthesis in these plants.

The land plants have traditionally been classified into two groups – bryophytes (liverworts, hornworts and mosses) and vascular plants (Tracheophyta), but molecular phylogenetic studies have shown that the "bryophyte" grouping, like the traditional "green algae", is paraphyletic. The true phylogenetic relationship within these primitive plants is still controversial, with dispute over whether the liverworts or the hornworts were the first to diverge from the lineage leading to vascular plants. For a proper understanding of the evolution of sucrose metabolism, this is an important question to be resolved, for it was the development of long distance transport systems, and the adoption of sucrose as the main transported carbohydrate, that probably elevated sucrose to its central position in plant metabolism.

Among these groups of "bryophyte" plants, sucrose was found to be the major soluble carbohydrate in the Antarctic liverwort *Cephaloziella exiliflora* (Roser et al., 1992), whereas in another liverwort, *Plagiochila asplenioides*, sucrosyl-fructans were quantitatively more important than sucrose itself (Suleiman et al., 1979). Nevertheless, labeling studies in the latter species showed sucrose to be one of the major products of photosynthetic CO_2 fixation (Suleiman and Lewis, 1980). Extracts from another liverwort species, *Lunularia truciata*, were reported to contain SPP activity that was Mg^{2+}-dependent and weakly inhibited by sucrose (Hawker and Smith, 1984). The same study found SPP activities with similar properties in extracts from two species of moss, *Bryum* sp. and *Funaria hygrometrica*. Sucrose was found to be the most common sugar in almost all of the species examined in two surveys of Antarctic and European mosses, and their high sucrose content was suggested to contribute to the desiccation tolerance of these plants (Roser et al., 1992; Smirnoff, 1992).

The moss *Physcomitrella patens* has become an important model species for investigation of gene function in plants, due to the possibility of targeting genes for precise modification or knockout by homologous recombination. Consequently, a large number of expressed sequence tags (ESTs) have been generated from this species, and its genome has been sequenced using the whole genome shotgun sequencing approach. Two sets of ESTs with significant similarity to higher plant SPS sequences were identified from the *Physcomitrella patens* EST collection, and two representative cDNA clones were fully sequenced. *PpSPS1* (GenBank accession No. DQ157858) and *PpSPS2* (DQ157859) both encode 120-kDa proteins, which are 90% identical (92% similar) to each other and show 62% identity (71% similarity) with the maize leaf SPS (J. Lunn and R. Zrenner, unpublished). The features of these genes and the proteins they encode will be discussed in more detail in Section IV. Searches of the unassembled genome sequences failed to find any other recognizable SPS-like sequences. Two putative *SPP* genes have also been identified from *Physcomitrella patens* (Lunn, 2003, and unpublished data). In addition to the catalytic HAD domain, these also have an extra domain at the C-terminus, and therefore closely resemble the enzyme from vascular plants in size and domain architecture (Fig. 27.2). The function of this C-terminal domain is unknown. The higher plant SPP differs from the cyanobacterial form in being dimeric, suggesting that the C-terminal domain could be involved in dimerization of the subunits (Lunn et al., 2000; Lunn, 2003).

Moving on to the vascular plants (Tracheaophyta), these can be divided into the lycophytes (Lycopsida) and euphyllophytes. The fossil record shows that the lycophytes diverged soon after the appearance of vascular plants, becoming one of the most diverse and abundant groups of plants during the Carboniferous era; in fact much of the organic material from which coal deposits were formed came from extinct, tree-like forms of these plants. There are three

extant groups of lycophytes: club mosses (e.g., *Lycopodium* spp.), spike mosses (genus *Selaginella*) and quillworts (genus *Isoetes*). Some species from the genus *Selaginella* are known as resurrection plants because of their ability to withstand extreme desiccation. Physiological studies have linked this extreme desiccation tolerance to the accumulation of large amounts of α,α-trehalose, which is present only in vanishingly small amounts in most other vascular plants (Holligan and Drew, 1971; Avigad, 1982). Although α,α-trehalose was the most abundant sugar found in hydrated as well as dehydrated *Selaginella lepidophylla* plants, sucrose was also present in substantial amounts (Adams et al., 1990; Vásquez-Ortíz and Valenzuela-Soto, 2004). Because of the desiccation tolerance of several plants in this genus and their key position in the evolutionary tree, the genome of a representative species, *Selaginella moellendorffii*, has been sequenced by the whole genome shotgun sequencing method (JGI Eukaryotic Genomes). Preliminary analysis of the unassembled sequence revealed that the genome contains six putative *SPS* genes, which probably represent allelic pairs from three loci (J.E. Lunn, unpublished). There are also at least two putative *SPP* genes that encode typical plant SPPs with a catalytic HAD domain and a C-terminal extension. Five other *SPP*-like genes were also found, however, comparison with *SPP* genes from other species suggested that none of these is likely to encode a functional SPP enzyme, due to the presence of multiple stop codons and loss of intron splice sites. Again, we shall return to discussion of the putative *SPS* genes and the proteins they encode in Section IV.

The euphyllophytes are divided into two sister clades – the Spermatophyta (seed plants) and the Monilioformopses (ferns and their allies). The latter clade includes several lineages of ferns (Polypodiales, Marratiales and Ophioglossaceae), the whisk ferns (Psilotaceae) and horsetails (Equisetales) (Pryer et al., 2001). Apart from a report of sucrose uptake and transport by intact shoots of the fern *Davallia trichomanoides* (Croxdale, 1977), there appears to be little experimental evidence concerning the physiological role of sucrose in these plants. Sucrose was detected in salt-stressed gametophytes of the mangrove forest fern *Acrostichum aureum*, but D-pinitol (a polyol) was the major soluble carbohydrate (Sun et al., 1999). We were unable to find any information on SPS activity in ferns, but SPP activity has been reported in leaf extracts from *Nephrolepsis cordifolia* (Hawker and Smith, 1984), and an acid invertase has been partially purified from *Pteris deflexa* (Sayago et al., 2001). Many ferns appear to be palaeopolyploids and have very large genomes, so it is unlikely that these will be the target of whole genome sequencing efforts in the near future. However, some EST projects are now underway, and the GenBank EST database contains an EST (accession No. BP916605) from *Adiantum capillus-veneris* (maidenhair fern) that shows considerable similarity to SPS sequences from seed plants.

D. Seed Plants – Gymnosperms and Angiosperms

There are five extant lineages of seed plants (Spermatophyta): cycads (Cycadophyta), ginkgos (Ginkgophyta), conifers (Coniferophyta), gnetophytes (Gnetophyta) and angiosperms (Magnoliophyta – flowering plants). The first four have traditionally been grouped together as the gymnosperms, but the phylogenetic relationship within this category is uncertain, and some studies have even suggested that it may be paraphyletic, with the gnetophytes belonging to a separate lineage more closely allied to the angiosperms (Palmer et al., 2004). One obstacle to resolving these relationships is that the cycads and gnetophytes are represented by only a handful of extant species, and *Ginkgo biloba* is the only living species of ginkgophyte. A further complication, particularly for resolving the relationship of the gymnosperms with the angiosperms, is that the true ancestors of the flowering plants probably belonged to either the seed ferns (glossopterids) or the Bennettitales, two extinct groups known only from the fossil record. However, at present, the weight of opinion seems to be in favor of the gymnosperms being a monophyletic sister clade to the angiosperms (Palmer et al., 2004).

Very little is known about sucrose metabolism in any of the gymnosperms except the conifers. There have been several studies of seasonal effects on carbohydrate metabolism in the conifer Norway spruce (*Picea abies*). In the spring,

before bud break, the mature needles show high levels of SPS protein indicating a high capacity for sucrose synthesis. Sucrose produced in the needles is exported to sink organs, including the bark of woody stems and leaf buds, where it is partly converted to starch (Egger et al., 1996). After bud break, three distinct phases in needle development were observed. During the first 45 days, the young needles had high acid invertase activity and showed a transient accumulation of starch. The second phase (45–70 days) of needle development was characterized by high SuSy activity, and increases in the dry weight/fresh weight ratio and soluble sugar content of the needles. During these two phases the developing needles are supplied with carbohydrate by export of photoassimilates from mature needles and remobilization of storage reserves in the stems. The final phase (70 days after bud break) saw the transition of the needles from sink to source organs, marked by a large increase in the activity of SPS relative to SuSy (Hampp et al., 1994). The activity of SPS in mature needles in the winter was high, and this could help to maintain photosynthetic CO_2 fixation in the overwintering needles (Loewe et al., 1996). Another study found that SPS activity increased in the needles of Norway spruce seedlings after formation of an ectomycorrhizal association with the roots led to increased sink demand (Hampp et al., 1995). The SPS from Norway spruce needles is activated by Glc6P and this activation is inhibited by Pi (Loewe et al., 1996). The enzyme has a subunit molecular mass of around 139 kDa, and evidence was found for inhibition by protein phosphorylation (Loewe et al., 1996).

The GenBank EST database contains an EST (accession No. CB091348) from the cycad *Cycas rumphii* that shows strong similarity to SPS sequences from angiosperms. There are also several SPS-like ESTs from various species of spruce (*Picea* sp.) and pine (*Pinus* sp.) in the database. Analysis of these sequences showed that species from both genera have at least two *SPS* genes, and these probably come from two pairs of orthologous genes (J. Lunn, unpublished). There are two SPP-like ESTs (accession Nos DR064611 and CB075279) from *Ginkgo biloba*, and one (CK751237) from the gnetophyte, *Welwitschia mirabilis*. The deduced protein sequences of these ESTs show strong similarity to a full-length SPP sequence from loblolly pine (*Pinus taeda*) (Lunn, 2003). There are also several ESTs from white spruce (*Picea glauca*) and Sitka spruce (*Picea sitchensis*) that are highly similar (89–93% nucleotide identity) to the loblolly pine *SPP* cDNA.

The last stage in our evolutionary journey brings us to the angiosperms (flowering plants). In the introduction to this chapter, we outlined the diverse functions of sucrose in these plants – photoassimilate, transport sugar, storage reserve and signal compound. The pathway of photosynthetic sucrose synthesis has been studied extensively in angiosperms, and in the next section we shall present an overview of pathway control in angiosperm leaves, with emphasis on the committed steps catalyzed by SPS and SPP. For a more detailed coverage of the whole pathway from photosynthetic CO_2 fixation to sucrose, the reader is referred to our earlier review on this topic (MacRae and Lunn, 2005).

E. The Control of Sucrose Synthesis in Leaves

In plants, sucrose synthesis is localized in the cytosol, but in illuminated leaves the precursors for the pathway are supplied by photosynthetic CO_2 fixation in the chloroplasts, in the form of triose-phosphates. These are exported from the chloroplast in exchange for P_i via the triose-phosphate/phosphate translocator. In the cytosol, triose-phosphates are used to synthesize fructose 1,6-bisphosphate (Fru1,6P_2), which is hydrolyzed by the cytosolic fructose-1,6-bisphosphatase (F1,6BPase) to produce Fru6P – the acceptor molecule for the SPS reaction. Isomerisation of Fru6P by phosphoglucose isomerase generates Glc6P, and phosphoglucomutase converts this to Glc1P, which is the substrate for UDPGlc pyrophosphorylase and synthesis of UDPGlc – the glucosyl donor in the SPS reaction (Fig. 27.1). The sucrose synthesized by SPS and SPP is mostly exported from the leaves to supply non-photosynthetic tissues, although some is also retained in the leaf to support respiration and export at night. Many species of angiosperms (e.g., *Arabidopsis thaliana*, tobacco and pea) store starch rather than sucrose in their leaves, breaking it down at night and exporting the products, maltose and a smaller amount of glucose, from the chloroplast to the cytosol, where they can be used for respiration or sucrose synthesis

(Niittylä et al., 2004). Other species store hexoses or fructans in their vacuoles, and these can also be remobilized at night for sucrose synthesis (MacRae and Lunn, 2005). It should be noted that these night-time pathways of sucrose synthesis do not require any cytosolic F1,6BPase activity.

A model describing the coordination of sucrose synthesis in the cytosol with photosynthetic CO_2 fixation and starch synthesis in the chloroplasts was developed in the late 1980s, based primarily on research with spinach (Stitt et al., 1987). This model invoked changes in the stromal and cytosolic concentrations of various metabolites, e.g., triose-phosphates, hexose-phosphates and P_i, to explain changes in the activities of two key enzymes, the cytosolic F1,6BPase and SPS, which lead to altered flux of photoassimilates into sucrose. The cytosolic F1,6BPase is strongly inhibited by the regulatory metabolite fructose 2, 6-bisphosphate ($Fru2,6P_2$), whilst SPS is allosterically activated by Glc6P and inhibited by P_i (Huber and Huber, 1996). $Fru2,6P_2$ is synthesized and degraded by a bifunctional enzyme (F2KP) that has both fructose-6-phosphate,2-kinase and fructose-2,6-bisphosphatase activities. The two activities of the enzyme are regulated allosterically by several metabolites, including triose-phosphates, Fru6P and P_i, and by phosphorylation of the F2KP protein (Nielsen et al., 2004). $Fru2,6P_2$ and the F2KP protein appear to be specific to eukaryotes. Their presence in both plants and non-photosynthetic eukaryotes (e.g., animals and fungi) argues for an early origin in the eukaryotic lineage, before the divergence of these groups. In non-photosynthetic eukaryotes, $Fru2,6P_2$ plays an important role in control of glycolysis as well as gluconeogenesis, and it may also contribute to control of glycolysis in plants, through its activation of the pyrophosphate-dependent phosphofructokinase (Nielsen et al., 2004).

The original model of control of sucrose synthesis described two basic processes: (i) feed-forward activation and (ii) feedback inhibition. It was proposed that the onset of photosynthetic CO_2 fixation and export of triose-phosphates from the chloroplast leads to a decrease in the concentration of $Fru2,6P_2$ in the cytosol, due to differential changes in the activities of F2KP. The fall in $Fru2,6P_2$ relieves inhibition of the cytosolic F1,6BPase, leading to increased hexose-phosphate synthesis. In turn, this provides more substrates for SPS, and higher Glc6P levels will also allosterically activate the enzyme. By these mechanisms, supply of photoassimilates from the chloroplast could activate both the cytosolic F1,6BPase and SPS, leading to increased sucrose synthesis. If the rate of synthesis exceeds the capacity of the leaf to export or store sucrose, it was proposed that feedback inhibition of SPS and/or SPP by sucrose would lead to accumulation of hexose-phosphates, and restrict the release of P_i in the cytosol. The higher hexose-phosphate/P_i ratio in the cytosol would affect the two activities of the F2KP enzyme, bringing about an increase in $Fru2,6P_2$ levels and inhibition of the cytosolic F1,6BPase. Lower cytosolic P_i would also limit exchange for photosynthetic intermediates across the chloroplast envelope, and the resulting build-up of 3-phosphoglycerate in the chloroplasts and drop in stromal P_i levels would activate ADPglucose pyrophosphorylase, diverting surplus photoassimilate into starch synthesis.

This model has been tested experimentally using transgenic plants and mutants with altered enzyme activities. Such studies have largely supported the importance of the cytosolic F1,6BPase and SPS in control of flux through the pathway, and the key role of $Fru2,6P_2$ in coordinating chloroplast and cytosolic metabolism, but also brought to light other important regulatory mechanisms. Consequently, the basic model has evolved considerably over time as new discoveries have been incorporated, with a greater emphasis on the importance of changes in the amount and activation state of key enzymes, including regulation of SPS by protein phosphorylation and redox-modulation of ADPGlc pyrophosphorylase (Hendriks et al., 2003; Huber and Huber, 1996; MacRae and Lunn, 2005).

Three regulatory phosphorylation sites have been identified from studies of the spinach leaf SPS: Ser158, Ser229 and Ser424, which are involved in light-dark regulation, 14-3-3 protein binding and osmotic stress activation, respectively (Table 27.1) (Huber and Huber, 1996). For ease of comparison, the spinach residue numbers, e.g., Ser158, will be used to describe the equivalent residues in SPS sequences from other species in the following discussion. Phosphorylation of Ser158 by SNF1-related or calcium-dependent protein kinases leads to inactivation of the enzyme in the dark, and dephosphorylation by a type 2A

Table 27.1. Properties of different families of SPS proteins from plants and *Synechocystis* sp. PCC 6803. Comparison of molecular mass and phosphorylation site motifs in representatives from each of the main families of SPS proteins in angiosperms and two primitive plants, the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* with the cyanobacterial SPS from *Synechocystis* sp. PCC 6803

			Phosphorylation site motifs		
Species	Gene family	Molecular mass kDa	**Ser158**	**Ser229**	**Ser424**
Consensus			**BH**X**B**XX**S**XXX**H**	**H**X**R**XX**S**X**P**	**BH**X**B**XX**S**
Synechocystis	bacterial	81	(NS)	**LTRLIKDP**	(NS)
*Physcomitrella*1	B	120	SSLALA**S**SNVG	**LTRQI**S**SP**	**R**ARRGVN
*Physcomitrella*2	B	120	SALTLA**S**SNFG	**LTRQI**S**SP**	**R**ARRGV**S**
*Selaginella*1	B	122	S**MKRI**AS**D**LEE	**LTRQI**S**AP**	**R**LK**S**GV**S**
*Selaginella*2	B	115	R**LSR**NS**S**ETD**V**	**LTRQI**L**AP**	**RIKRGVS**
*Selaginella*3	B	119	**CMLR**NL**S**VLHS	**LTRQI**CAT	**RAKKGL**N
*Arabidopsis*1	B	119	QL**QR**NL**S**NLE**I**	**FTRQI**CSS	**R**ARRGVN
*Arabidopsis*4	C	119	**HMPRI**R**S**EMQ**I**	**LTRQI**S**SP**	**RRRRGVS**
*Arabidopsis*5a	A2	117	**RMSR**VS**S**VDA**M**	**LTRQV**S**S**A	**R**MK**R**GV**S**
*Arabidopsis*5b	A1	117	**RLPRI**N**S**AES**M**	**LTRQV**S**SP**	**RIKR**NV**S**
Rice1	B	119	**KFQR**NF**S**ELT**V**	**FTRQV**S**SP**	**RARRGVS**
Rice2	D1	108	SSP**K**TS**S**IDK**L**	**LTRQI**L**AP**	**RVKR**GAN
Rice6	D2	109	NTP**RI**S**S**VDK**L**	**FTRQI**L**AP**	**RVKR**GAN
Rice8	A	119	**RMPRI**G**S**TDA**I**	**VTRQI**S**AP**	**RIKRGVS**
Rice11	C	119	**RFARI**N**S**DPR**I**	**LTRQI**S**CP**	**RRRRGVS**

The amino acid sequences that align with the phosphorylation site motifs in the spinach leaf SPS are shown, with conserved residues from the consensus sequences highlighted in bold
NS no region of similarity

protein phosphatase activates it in the light (Huber and Huber, 1996). The function of 14-3-3 protein binding is uncertain, but it might be involved in regulation of protein turnover (Cotelle et al., 2000). Unlike phosphorylation of Ser158, osmotic-stress induced phosphorylation of Ser424 leads to activation of SPS (Toroser and Huber, 1997). Each of the phosphorylation sites lies within a recognition motif for the respective protein kinases, and the corresponding residues in some representative examples are shown in Table 27.1. The allosteric site in SPS is unknown, therefore, we cannot predict the presence or absence of allosteric regulation from the sequences of SPSs from species where this has not been experimentally tested.

Accumulation of sucrose in spinach leaves reduces the activation state of SPS, and this, rather than direct inhibition of the enzyme by sucrose, appears to be the main mechanism of short-term feedback inhibition in this species. Interestingly, no evidence of such feedback mechanisms was found in maize or wheat leaves (Lunn and Hatch, 1997; Trevanion et al., 2004), both of which accumulate high levels of sucrose during the day. At the other end of the spectrum, starch-storing plants (e.g., *Arabidopsis thaliana*) show relatively small changes in the activation state of SPS during the diurnal cycle, consistent with substantial sucrose synthesis occurring at night as well as during the day. It has also been reported that SPS is directly inhibited by sucrose in some starch-storing species (Huber, 1981). In the seagrasses, which are submerged marine angiosperms from the monocot family Posidoniaceae, sucrose appears to play a major role in osmoregulation, and SPS activity responds to changes in salinity and temperature rather than light (Touchette and Burkholder, 2000, and references therein).

As noted previously, much of our knowledge of the control of sucrose synthesis comes from work on spinach, which is a C_3 plant, and there may be important differences in the regulatory mechanisms of sucrose synthesis in plants with

other photosynthetic pathways. In maize, which is an NADP-malic enzyme type C_4 plant, the cytosolic F1,6BPase, which is located in the mesophyll cells where sucrose synthesis is localized, has a lower affinity for Fru1,6P_2 than in spinach. This adaptation helps to maintain high concentrations of triose-phosphates in the mesophyll cells, which are needed to drive diffusive transport of triose-phosphates, produced by reduction of 3-phosphoglycerate in the mesophyll cells, back into the bundle sheath cells for re-entry into the Calvin-Benson cycle (Leegood, 1985). This exchange of 3-phosphoglycerate and triose-phosphates between mesophyll and bundle sheath cells is necessary because the bundle sheath cell chloroplasts have very little PS II activity, and their main source of NADPH is from decarboxylation of malate, which is sufficient to reduce only about half of the 3-PGA produced by Rubisco. The situation may be different in other C_4 species belonging to the different decarboxylation types (NAD-malic enzyme and PEP carboxykinase) or in other NADP-malic enzyme species where sucrose is also synthesized in the bundle sheath cells (Lunn and Furbank, 1997), but little is known about the properties of the cytosolic F1,6BPase from C_4 species other than maize.

The maize leaf SPS shows a very strong diurnal rhythm of activation, which closely tracks irradiance (Lunn and Furbank, 1999). Photosynthesis in C_4 plants is often limited by light, so this response of SPS activity should help to keep the rate of sucrose synthesis in balance with the rate of CO_2 fixation. Light-dark regulation of the maize leaf SPS is brought about by reversible phosphorylation of Ser162 (equivalent to Ser158 in spinach), in a similar manner to that in spinach leaves (Huber and Huber, 1996). However, there is evidence that light activation of SPS in other C_4 species, especially those with substantial SPS in the bundle sheath cells, may be significantly different, with the phosphorylated enzyme being the activated form (Lunn et al., 1997). It is not known whether this indicates phosphorylation of a different site in the enzyme or an altered response to phosphorylation of the Ser158 site. From these observations, we conclude that most plants probably use fairly similar regulatory mechanisms for controlling the flux through the pathway of sucrose synthesis, but there is a considerable degree of flexibility in the basic mechanisms that allows adaptation to meet the specialized needs of each individual species.

In the last few years new information about the enzymes in the pathway of sucrose synthesis has come from genome and EST sequencing efforts, and from genome-wide transcript profiling studies. It is now clear that many of the enzymes in the pathway of sucrose synthesis are encoded by multiple genes with different expression patterns, and one of the main aims of current research on sucrose synthesis is to understand the physiological role of the various enzyme isoforms. In the next section, we shall take a more detailed look at the diversity of genes encoding one of the key enzymes of sucrose synthesis, SPS, in angiosperms and other plants, and discuss what we know about their evolutionary history and function.

IV. Sucrose Phosphate Synthase (SPS) Gene Families – Evolution, Structure and Function

Genes encoding SPS were first identified from maize (Worrell et al., 1991) and spinach (Klein et al., 1993; Sonnewald et al., 1993), and we now have SPS sequences from over 30 other plants (Lunn and MacRae, 2003). Initially, as *SPS* genes from more species were discovered, a trend emerged for the sequences to cluster into two groups. One group contained sequences mostly from eudicots and was more similar to the spinach SPS than the maize SPS, whilst the other group was mostly from monocots and was more similar to the maize SPS, which led to the idea that there was a simple monocot-dicot divide among *SPS* genes. Based on Southern hybridization analysis, it was also thought that each species contained only a single *SPS* gene (Worrell et al., 1991; Valdez-Alarcón et al., 1996). However, both of these ideas were later found to be incorrect. Three different *SPS* genes were discovered in the Satsuma orange (*Citrus unshiu*) (Komatsu et al., 1996), and two different *SPS* cDNAs were cloned from sugarcane (*Saccharum officinarum*), one of which closely resembles the maize gene, whereas the other encodes a smaller protein with some similarity to the eudicot SPSs known at that time (Sugiharto et al., 1997). Two *SPS* cDNAs were also cloned from a eudicot species of resurrection plant, *Craterostigma plantaginea*, with

one falling into the eudicot group and the other into the monocot group (Ingram et al., 1997). These results showed that plants have multiple *SPS* genes, and there is no simple monocot-dicot divide in the angiosperm *SPS* gene family.

Sequencing of the *Arabidopsis thaliana* genome showed that this species has four *SPS* genes. A phylogenetic analysis of these and all the other known SPS sequences, mostly from eudicots, revealed that they cluster into three distinct families, which were designated A, B and C (Langenkämper et al., 2002). Of the four *Arabidopsis thaliana* genes, *AtSPS5a* (At5g11110) and *AtSPS5b* (At5g20280) both belong to the A family, which included most of the known sequences from eudicots, whilst *AtSPS1* (At1g04920) belongs to the B family, which was previously thought to be the monocot-specific family. The fourth gene, *AtSPS4* (At4g10120), belongs to the C family.

To investigate *SPS* gene diversity in monocots, which were poorly represented among the SPS sequences available for previous analyses, Castleden et al. (2004) sequenced several *SPS* cDNA clones from wheat, and carried out a survey of the extensive collections of EST sequences from cereals and other grasses (rice, maize, barley, sugarcane and sorghum). This survey, together with analyses of the rice and maize genomic sequences, showed that all six species have at least one *SPS* gene from each of the A, B and C families, although these cluster somewhat separately from the eudicot sequences in the same family (Fig. 27.4). It was also discovered that each of these species has at least two closely related genes that belong to a separate lineage – the D family (Castleden et al., 2004). The D family contains two distinct subfamilies of genes, although these encode similar isoforms of SPS that have several distinctive features. These unusual isoforms (108–109 kDa) are smaller than the A, B or C type SPSs (114–119 kDa), lacking a linker region of about 85 residues that is located between the glucosyltransferase and SPP-like domains in the other three types (Table 27.1; Fig. 27.2). Except for the Ser424 site, which is absent from the B-family SPSs in eudicots, the three phosphorylation sites are present in almost all other A, B and C family SPSs in angiosperms (Table 27.1). In contrast, the D-type SPSs lack both the Ser229 and Ser424 phosphorylation sites, and some of the residues critical for protein kinase recognition are also missing from the Ser158 phosphorylation site motif (Table 27.1).

Present evidence suggests that the D-family is restricted to the grasses (Poaceae), as searches of the publicly available ESTs from other monocot families, or from dicots and non-angiosperms, failed to discover D-type sequences from any other plants (Castleden et al., 2004). It should be noted though that sequences from non-grass monocots are still poorly represented in the publicly available EST collections, so it is too early to draw any firm conclusions on this point. The genome of a species of banana (*Musa acuminata*), is currently being sequenced (http://www.musagenomics.org/), and as this species belongs to the order Zingiberales, which is closely related to the grasses, this should help to answer the question of whether the D-type SPS is restricted to the grasses or is also present in other monocot families.

The genome of the black cottonwood tree (*Populus trichocarpa*) has now been sequenced (http://genome.jgi-psf.org/), and this contains six *SPS* genes: three from the A family, two from the B family and one from the C family. This species is thought to be an ancient tetraploid, and this could account for the presence of multiple genes in some families. Similarly, maize, which is also an ancient tetraploid, has duplicated genes in the B family and one of the subfamilies within the D family (Castleden et al., 2004). In hexaploid bread wheat (*Triticum aestivum*), evidence was found of three homologous genes in each of the A, B and C families, and in both of the D subfamilies, and most of these homologs are expressed (Castleden et al., 2004), as are the multiple A-family homologs in polyploid and palaeopolyploid kiwifruit species (*Actinidia deliciosa* and *Actinidia chinensis*) (Fung et al., 2003).

A closer look at the eudicot sequences within the A family tree shows that these form two separate clusters (Fig. 27.4). Interestingly, the fully sequenced genomes of *Arabidopsis thaliana* and black cottonwood both contain representatives in each cluster, and there are cDNA sequences from sugar beet (*Beta vulgaris*) and tomato that follow the same pattern. In addition to these four species, sequences from both of the A-family clusters have now been identified from apple (E. MacRae, unpublished). From these findings, we suggest that the eudicot A family of *SPS* genes has undergone a relatively recent duplication

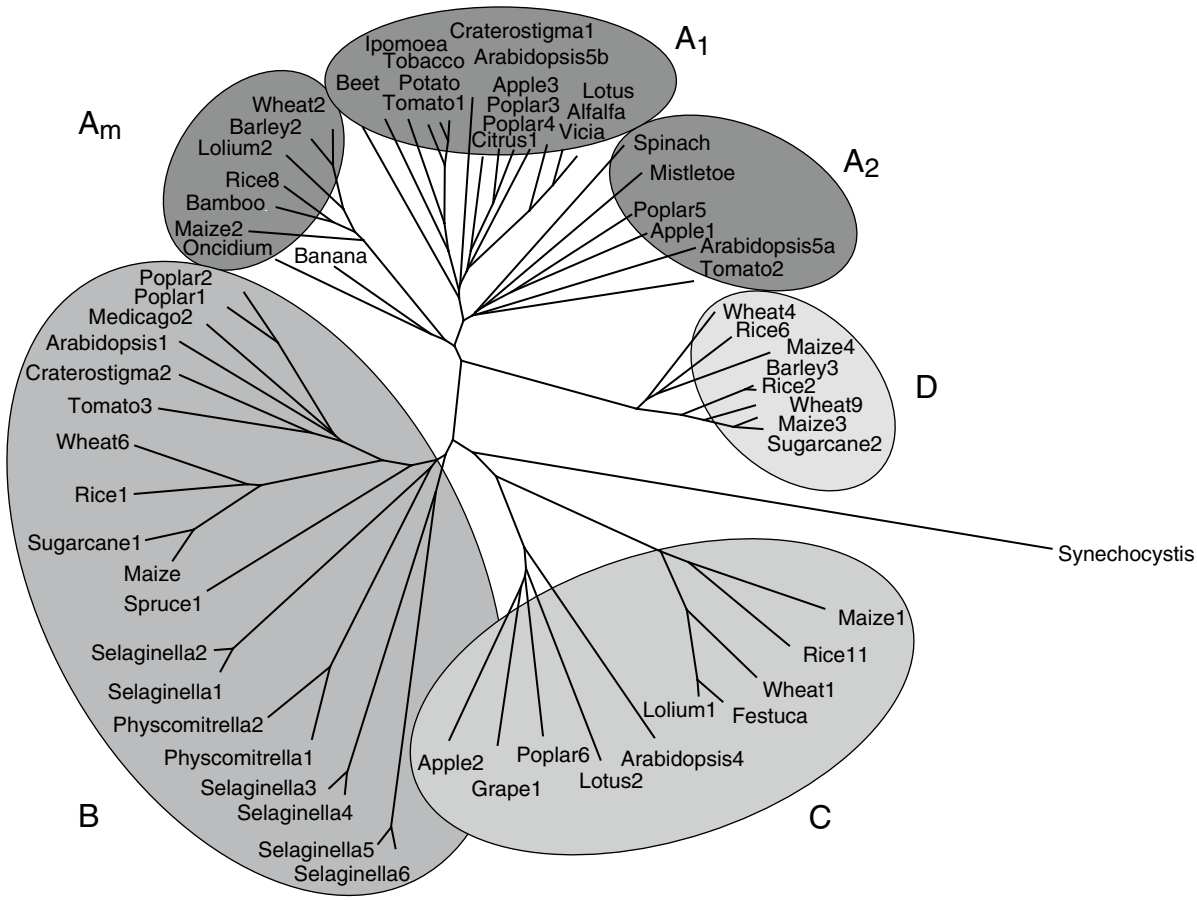

Fig. 27.4. SPS families in plants. This unrooted phylogenetic tree shows the four main families of SPS proteins in plants: A, B, C and D. The A-family can be further divided into the A_1 and A_2 subfamilies, both from dicotyledonous species, and the A_m subfamily from monocotyledonous species. The B- and C-families contain mono- and dicotyledonous branches. The B-family also contains several SPSs from lower plants. The D-family appears to be restricted to the Poaceae and is divided into two subfamilies. A prokaryotic SPS from the cyanobacterium *Synechocystis* sp. PCC 6803 is also shown. See Lunn and MacRae (2003) and Castleden et al. (2004) for the GenBank accession Nos of the sequences except: *Physcomitrella patens* SPS1 – DQ157858; *Physcomitrella patens* SPS2 – DQ157859 and grape SPS1 (*Vitis vinifera*) – AY899999. Other sequences derived from unpublished analyses of the JGI Eukaryotic Genomes and GenBank EST and HTGS databases.

event, with divergence of the duplicated genes to form two subfamilies, which we shall refer to as A_1 and A_2 (Fig. 27.4). Presumably this split predates the divergence of the rosid, asterid and Caryophyllales lineages within the eudicots (Fig. 27.5), as both A_1 and A_2 sequences are present in at least one species from each of these groups. It is worth noting that the spinach SPS, on which most of our detailed knowledge of the enzyme's regulation is based, appears to belong to the A_2 group (Fig. 27.4).

A survey of genomic and cDNA (EST) sequences identified A, B and C family SPS sequences from grapevine (*Vitis vinifera*), apple (*Malus X domestica*) and tomato (*Lycopersicon esculentum*), A and B sequences from onion (*Allium cepa*) and *Medicago truncatula*, and A and C sequences from *Lotus corniculatus* var. *japonicus* (J. Lunn, unpublished). The occurrence of A, B and C family genes in these, and the other species mentioned above, make it clear that the A, B and C families arose before the divergence of the eudicots and monocots, which occurred at least 125 million years ago (Soltis and Soltis, 2004a) (Fig. 27.5). Beyond this it is difficult to make firm conclusions due to the paucity of information from other angiosperm groups. Nevertheless, we do know from ESTs

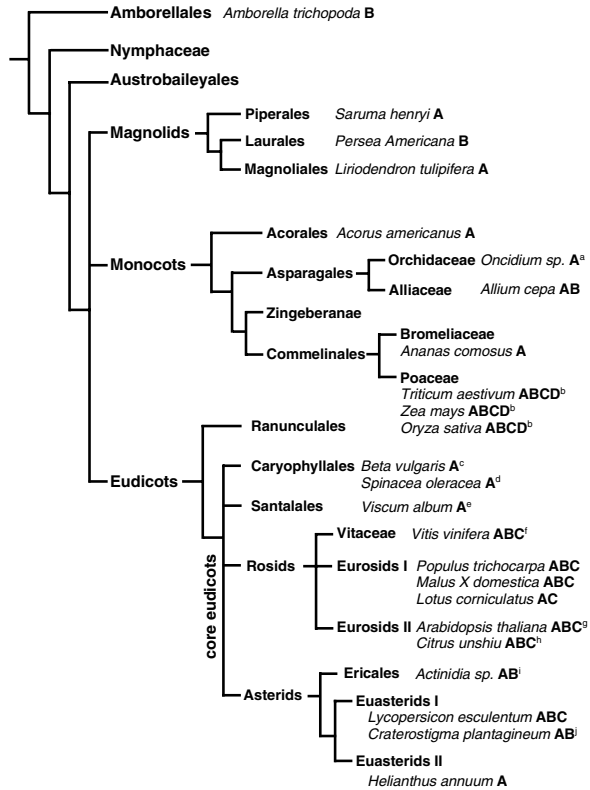

Fig. 27.5. Distribution of SPS gene families in angiosperms. Phylogram (Adapted from the Angiosperm Phylogeny Group II, 2003) showing the distribution of *SPS* gene families in representative species from the major groups of angiosperms (flowering plants) ([a]Li et al. (2003); [b]Castleden et al. (2004); [c]Hesse et al. (1995); [d]Sonnewald et al. (1993), Klein et al. (1993); [e]GenBank accession No. AY331261; [f]GenBank accession No. AY899999; [g]Langenkämper et al. (2002); [h]Komatsu et al. (1996); [i]Fung et al. (2003); [j]Ingram et al. (1997). Other data from unpublished analysis of sequences from the GenBank EST, GenBank High Throughput Genomic Sequence (HTGS), GenBank Whole Genome Shotgun sequence (WGS) and JGI Eukaryotic Genomes databases).

that the A and B families are present in species among the magnolids, a sister clade of the eudicots and monocots, and there is a single B-family EST from *Amborella trichocarpa* (accession No. CD482209) (Fig. 27.5). This species is the sole surviving representative from what is widely thought to be the most ancient lineage of angiosperms, although the position of *Amborella trichocarpa* as the basal angiosperm is still controversial (Stefanovic et al., 2004; Soltis and Soltis (2004b); Lockhart and Penny, 2005). The deduced protein sequence fragment from the *Amborella trichocarpa* SPS EST includes both the Ser158 and Ser229 phosphorylation site motifs, whereas the B-family SPSs from the eudicots lack Ser229.

Comparison of cDNA and genomic sequences from *Arabidopsis thaliana*, rice, maize and black cottonwood shows that the A, B, C and D-family *SPS* genes all have a similar exon-intron structure, suggesting that they have a common evolutionary origin (Langenkämper et al., 2002; Castleden et al., 2004; J. Lunn, unpublished). When the cyanobacterial SPS from *Synechocystis* sp. PCC 6803 was included as an outgroup in the phylogenetic trees, it invariably joined the plant sequences on the branch leading to the C-family sequences (Castleden et al., 2004). This was interpreted as showing that the C family was the first to diverge from the rest, with the B family next to diverge, with a split in the A family in the grasses giving rise to the D family (Fig. 27.4). Use of other bacterial SPSs as an outgroup does not appear to alter this tree topology, but the SPS sequences from non-angiosperm plants appear to tell a slightly different story. Within the gymnosperms, all of the

publicly available SPS ESTs from the conifers (*Picea* and *Pinus* spp.) belong to the B family, and only the cycad (*Cycas rumphii*) EST aligns more closely with the A family, although it lies close to the branch point between the A and B families. The only known SPS EST from a fern (*Adiantum capillus-veneris*) also belongs to the B family. Although these ESTs show that the A and B families existed before the divergence of the gymnosperms and angiosperms, and that the B family existed even earlier, they may not represent the full range of *SPS* genes in these groups of plants. In contrast, the availability of whole genome sequences from the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* should give us a more complete picture of the *SPS* gene diversity in these more primitive plants. Phylogenetic analysis indicates that all of the *Physcomitrella patens* and *Selaginella moellendorffii* SPSs belong to the B family (Fig. 27.4). The putative *SPS* genes from these two primitive plants have essentially the same exon-intron structure as those from angiosperms, and the deduced protein sequences all have a typical angiosperm SPS (ABC-type) architecture, with N-terminal, glucosyltransferase, linker and SPP-like domains (Fig. 27.2). All of the *Physcomitrella patens* and *Selaginella moellendorffii* SPSs have a Ser158 residue, but only those from *Selaginella moellendorffii* have any of the other conserved residues in the putative phosphorylation site motif. The Ser229 site is present in both *Physcomitrella patens* SPSs, but the Ser424 site is found only in SPS1 (Table 27.1). Among the *Selaginella moellendorffii* SPSs, SPS1 has both the Ser229 and Ser424 sites, whereas SPS2 has only the Ser424 site and SPS3 has neither (Table 27.1).

The earliest steps in the evolutionary path of SPS before the emergence of multicellular plants are uncertain, in particular, the transition from the presumed cyanobacterial-type SPS in the first green eukaryotes to that in higher plants remains obscure as we have no examples of intermediate types. The chlorophyte green algal SPSs may have diverged so greatly from those in the streptophyte lineage, that they are unrecognizable using current search algorithms, and are unlikely to shed much light on the early evolution of SPS in plants. There are some conflicting data on which is the most ancient family of SPS in streptophyte plants. The most primitive types of plant from which we have SPS sequences are the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* and these only have B type SPSs. The earliest evidence we have of the A family is from the gymnosperms (cycad), and *SPS* genes from the C and D families have only been found in angiosperms so far. As the genomes of ferns and gymnosperms are generally very large it is unlikely we shall have full genome sequences in the near future, therefore, we shall depend on larger scale EST projects, or targeted searches for the missing families, to establish when the A and C families arose. Although the presence of only B-family genes in primitive plants points to this family being the ancestral type, this disagrees with some topologies obtained for the phylogenetic tree, which appear to indicate the C family was the first to diverge. However, the robustness of these trees with the newly available sequences has not yet been fully evaluated, and may not be strongly supported. In any event, it is likely that more sequences from other primitive plant species will be needed to settle this issue. Sequences from the Charales or the other "algal" groups from the streptophyte lineage, could be particularly interesting and we suggest that this should be a priority for future investigations.

Whatever the evolutionary relationship between the SPS families, we see that most of the new features that distinguish the plant and cyanobacterial enzymes – the N-terminal and linker domains, and the Ser229 and Ser424 regulatory phosphorylation sites – had been acquired by the time the mosses diverged within the streptophyte lineage (estimated to be >400 million years ago). The Ser158 phosphorylation site motif, however, is clearly recognizable only in the *Selaginella moellendorffii* SPSs. It is tempting to speculate that acquisition of the Ser158 phosphorylation site, allowing light-dark regulation of SPS, was linked to sucrose becoming a transport sugar and major product of photosynthesis, whereas the Ser424 site linked to osmotic stress regulation had already appeared in the mosses, in which sucrose functions mainly as a compatible solute. Curiously, the D-family SPSs in grasses appear to show secondary loss of several of these "advanced" features, including the linker domain and the Ser229 and Ser424 phosphorylation sites, and appear to have less regulatory potential than the other types (Castleden et al., 2004).

The significance of this is unclear as we do not know what their function is in grasses.

As mentioned above, even primitive plants such as the mosses have multiple isoforms of SPS, and the functional significance of the diversity of SPS isoforms in plants is only partly understood. The first clues have come from analysis of gene expression patterns, both experimentally and in silico from the EST databases. Northern hybridization analysis of all five *SPS* gene families in wheat showed that each has distinct, but often overlapping, spatial and temporal expression patterns (Castleden et al., 2004). However, comparison with other species of grasses did not reveal any obviously conserved patterns of expression across species. For example, the B-family genes are highly expressed in maize and rice leaves, whereas the wheat and barley B-family genes are only expressed in anthers and germinating seeds. It has been pointed out though, that thousands of years of breeding and selection for higher yields and other agronomic traits may have greatly modified the original expression patterns of the *SPS* gene families in these domesticated cereal species (Castleden et al., 2004).

Genome-wide transcript profiling in *Arabidopsis thaliana* using microarrays has shown that the *SPS* genes in this wild species also have distinct spatial and temporal expression patterns (http://jsp.weigelworld.org/atgenDev/atgen.jsp). The B-family gene, *AtSPS1*, appears to be expressed mainly in reproductive organs (stamens and developing seeds), whereas the C-family gene, *AtSPS4*, is highly expressed in leaves and shows a marked diurnal rhythm, with transcript levels increasing during most of the night and declining during the day (Harmer et al., 2000; Gibon et al., 2004a). The A_1-family gene, *AtSPS5b*, is also highly expressed in leaves, and reduced expression of this gene in transgenic antisense plants was reported to decrease the rate of sucrose synthesis (Strand et al., 2000), showing that this isoform makes a significant contribution to photosynthetic sucrose synthesis. Transcript levels of the *AtSPS5b* gene also show a diurnal rhythm, but in the opposite phase to *AtSPS4*, rising during the day and falling again at night (Gibon et al., 2004a). This has led to speculation that there might be a "day shift" and a "night shift", where different isoforms, perhaps with different kinetic properties, are used for photosynthetic sucrose synthesis during the day and sucrose synthesis from remobilization of starch at night, respectively (MacRae and Lunn, 2005). In this context, it is worth remembering that night-time sucrose synthesis from the products of starch breakdown bypasses the cytosolic F1,6BPase, and so SPS might play a greater role in control of flux than during the day.

Some plants that are adapted to very dry environments use Crassulacean acid metabolism (CAM) to temporarily fix CO_2 into organic acids at night, using the enzyme phosphoenolpyruvate carboxylase. During the day, the organic acids (mostly malate) are released from the vacuole where they are stored at night, and decarboxylated to provide a source of CO_2 for fixation by the conventional Calvin-Benson (C_3) cycle in the light (see Chapter 26). This biochemical adaptation allows the plants to keep their stomata closed during the day, opening them only at night when it is cooler to minimize water loss by transpiration. Many of these plants accumulate starch or fructans during the day, and these are remobilized at night mostly for synthesis of phosphoenolpyruvate, which is the acceptor molecule for initial CO_2 fixation, rather than for sucrose synthesis and export as in C_3 plants. Therefore, we can speculate that there is less need for a "night shift" of sucrose synthesis in CAM plants, and the expression patterns of the *SPS* genes may differ accordingly. Some facultative CAM plants (e.g., *Mesembryanthemum crystallinum*) are able to switch between CAM photosynthesis, when conditions are hot and dry, and conventional C_3 photosynthesis when conditions are more favourable. In such species we might predict changes in the expression of the "night-time" SPS isoform as the plant switches from C_3 to CAM, and vice versa.

Returning to *Arabidopsis thaliana*, expression of the A_2-family gene, *AtSPS5a*, in leaves, is strongly up-regulated by cold, osmotic and salt stresses, as well as by treatment with abscisic acid (ABA), suggesting that one role of this isoform is to increase sucrose synthesis as part of the plant's adaptation to abiotic stress. Although these transcript data provide a starting point for understanding the function of the various isoforms, changes in transcript abundance may not necessarily lead to changes in the amount of enzyme. Measurements of protein levels, using either specific antibodies for each isoform or proteomic approaches, will

be required to test this. Detailed kinetic analyses of the individual isoforms, and analyses of transgenic plants or mutants with specifically altered activity of one isoform, are also needed to understand the full physiological significance of this diverse gene family.

V. Conclusions and Perspectives

In this chapter, we have outlined the current state of knowledge concerning the origins and evolutionary history of sucrose synthesis in plants. Biochemical and molecular evidence strongly supports a bacterial origin for the key enzymes of sucrose synthesis, SPS and SPP, and their inheritance by plants from the cyanobacterial ancestor of chloroplasts. Many important changes have occurred in sucrose metabolism during the evolution of plants; the genes encoding SPS and SPP were transferred from the ancestral chloroplast genome to the cell nucleus, and sucrose synthesis moved from the chloroplast to the cytosol. To coordinate sucrose synthesis in the cytosol with photosynthetic CO_2 fixation in the chloroplast, new regulatory mechanisms appeared, e.g., regulation of the cytosolic F1,6BPase by Fru2,6P_2. The enzymes in the committed pathway of sucrose synthesis also acquired new regulatory properties, including modulation by reversible protein phosphorylation, and allosteric control by Glc6P and P_i. The phosphorylation site motifs associated with osmotic stress activation of SPS (Ser424) and 14-3-3 protein binding (Ser229) are already present in the mosses, but the light-dark regulation by phosphorylation of Ser158 appears to have arisen at a later date, after the divergence of the mosses within the streptophyte lineage. We can speculate that the appearance of this type of regulation could be linked to the evolution of vascular plants that use sucrose as a long-distance transport sugar, in which sucrose became a major product of photosynthesis.

The genes encoding SPS appear to have been duplicated several times in the evolution of plants, and then diverged to form several distinct gene families, of which the B family may be the most ancient. This process appears to be ongoing, with evidence for the D family splitting from the A family around the time of divergence of the grasses from other monocots (approx. 77 million years ago). A subsequent duplication event, before the major clades within the grass lineage diverged (approx. 50 million years ago), gave rise to the two subfamilies within the D family (Castleden et al., 2004). Similarly, a newly identified division among the eudicot sequences in the A family points to a relatively recent split here to form the A_1 and A_2 subfamilies. These events probably allowed specialization of the resulting isoforms, which are known to be expressed in different parts of the plant or at different times. It is only in the last few years that the multiplicity of SPS isoforms has been recognized and we still know very little about the specific functions of each isoform. On present evidence, there appears to be little obvious conservation of function of the SPS isoforms across species, suggesting that different isoforms have been recruited for the same task in different species, and vice versa.

Finally, we return to the puzzle, or riddle, of why sucrose became so central to plant metabolism (Edelman, 1971; Pontis, 1977; Avigad, 1982). Other options were certainly available to plants, with another disaccharide sugar, α,α-trehalose, probably being one of the strongest contenders. Unlike sucrose, α,α-trehalose is almost universal in living organisms, occurring in all major types of bacteria and archaea, and in most groups of eukaryotes, including plants (Elbein et al., 2003). Many different organisms use trehalose as a protectant against osmotic or oxidative stress, but it also functions as a transport and storage carbohydrate in fungi and insects (Wera et al., 1999; Benaroudj et al., 2001; Crowe et al., 2001). The enzymes of trehalose synthesis are widespread in plants, from chlorophyte green algae to angiosperms, but only a few species are known to synthesize large amounts of trehalose. These are mostly resurrection plants that can withstand extreme desiccation, and include species from diverse groups, including lycophytes (e.g., *Selaginella lepidophylla*), ferns (e.g., *Pellaea ovata*), grasses (e.g., *Sporobolus atrovirens*) and eudicots (e.g., *Myrothamnus flabellifolius*) (Iturriaga et al., 2000). In other plants the main purpose of trehalose metabolism appears to be in sugar signaling, with the intermediate of trehalose synthesis – trehalose 6-phosphate – rather than trehalose itself, playing the main role (Schluepmann et al., 2003).

Apart from sucrose and α,α-trehalose, organisms use several other carbohydrates as compatible solutes, including: glucosylglycerol, floridoside, glycerol, sorbitol and mannitol. Glucosylglycerol is common in cyanobacteria, but there have been a only few reports of this compound in plants, including the resurrection plant *Myrothamnus flebellifolia* and some *Lilium* species (Bianchi et al., 1993). Floridoside and isofloridoside synthesis appears to be a characteristic feature of the red algae, but we know of no reports of these heterosides in green plants. It is unclear whether green plants never possessed the ability to synthesize these compounds, or if this ability was lost at some time during their evolution, therefore, we do not know if they were ever potential alternatives to sucrose. Glycerol is obviously used by plants for both membrane and storage lipid synthesis, but does not appear to have any importance as a compatible solute, storage reserve or transport compound in plants. In contrast, quite a few plants use various polyols, of which the most common are sorbitol and mannitol, for both transport and storage. The fact that, even in these plants, polyols only supplement sucrose and do not entirely replace it, suggests that sucrose is essential and has some unique property.

Sucrose has several properties that make it suitable for its various roles – it is highly soluble, uncharged and non-reducing – but the same is also true for α,α-trehalose, polyols and the other compounds mentioned above. The non-reducing nature of sucrose means that it is less chemically reactive than hexose sugars, such as glucose, which can react directly with the amino groups of proteins and other cell components (Crowe et al., 2001). Such glycation reactions are thought to be responsible for damage to the optic nerve, leading to blindness, in diabetic patients whose blood glucose levels are poorly regulated. Clearly free hexoses would not be a good alternative to sucrose in plants, which accumulate high levels of carbohydrates, and hexoses are usually sequestered away in the vacuoles if present in large amounts in plant cells.

The β-D-fructofuranoside nature of sucrose is one feature that is unique among these compounds. The free energy of hydrolysis of the glycosidic bond in sucrose is higher than in α,α-trehalose, partly due to the extra free energy released as the liberated furanose form of D-fructose partly isomerizes to the pyranose form (Neufeld and Hassid, 1963). This extra free energy released by the liberation of fructose is thought to explain why sucrose can be cleaved not only hydrolytically by invertase, but also by sucrose synthase to produce UDPGlc and fructose. The latter reaction, when coupled to pyrophosphorolysis of UDPGlc, allows conversion of sucrose to hexose-phosphates with the consumption of just one pyrophosphate molecule, whereas the hydrolytic route of sucrose catabolism requires two molecules of ATP (Edwards et al., 1984). The energetic saving is relatively small when sucrose is respired via glycolysis and the Krebs cycle, but could become more significant under oxygen-limiting conditions, e.g., in flooded roots, when the Krebs cycle is not fully operational. Until recently, no comparable reaction involving cleavage of trehalose was known (Salerno and Curatti, 2003), but there have now been reports of such an enzyme in at least two species of hyperthermophilic archaea, *Thermococcus litoralis* and *Pyrococcus horikoshii* (Qu et al., 2004; Ryu et al., 2005). Both catalyze the synthesis of trehalose from a nucleoside-diphosphoglucose (UDPGlc, ADPGlc or GDPGlc) and glucose, and the reaction catalyzed by the *Thermococcus litoralis* enzyme was shown to be reversible, although the trehalose-synthesizing reaction is strongly favored. The optimal temperature for activity of the *Thermococcus litoralis* enzyme was 90°C, with less than 5% of this activity at 30°C (Qu et al., 2004). Although these reports demonstrate the existence of a "trehalose-synthase" enzyme, it appears that the reverse reaction would be very slow under the conditions in which most plants live, unlike the sucrose synthase reaction, which is freely reversible in vivo.

Another potential explanation for why α,α-trehalose did not become the major carbohydrate in plants comes from studies of heat-shock recovery in yeast. Heat-shock induces yeast cells to accumulate high levels of α,α-trehalose, which protects them during stasis, but this is rapidly degraded after heat-shock when the cells resume normal metabolic activity and growth. High levels of trehalose were reported to inhibit the recovery phase by interfering with the chaperone-assisted renaturation of partially unfolded proteins (Singer and Linquist, 1998), and yeast mutants with

decreased trehalase activity showed impaired recovery from heat-shock (Wera et al., 1999). Interestingly, resurrection plants that accumulate high levels of α,α-trehalose as they become dehydrated, and enter stasis, degrade most of this sugar when rehydrated (Adams et al., 1990). These results suggest that if α,α-trehalose were a major product of photosynthesis instead of sucrose, then it might interfere with other metabolic processes within the plant cell.

In conclusion, there appear to be several plausible arguments why α,α-trehalose did not become the dominant carbohydrate in plants, and it is the polyols (e.g., sorbitol and mannitol) that have come closest to displacing sucrose from its position as the main transport and storage compound in plants. The reasons why they are only found in some plants and have not completely replaced sucrose even in those species are not immediately obvious. It seems that the riddle of sucrose remains as puzzling, yet as fascinating, as ever.

Editorial Note

The original manuscript for this chapter was submitted in September 2005. Since submission, further bacterial genome sequences have become available, which suggest that sucrose synthesis may be present in the Flavobacteria, in addition to the other bacterial groups described. The crystal structure of the *Halothermothrix orenii* SPS has also been determined, revealing details of the substrate binding and catalytic mechanism of the enzyme [Chua TK, Bujnicki JM, Tan T–C, Huynh F, Patel BK, Sivaraman J (2008) The structure of sucrose phosphate synthase from *Halothermothrix orenii* reveals its mechanism of action and binding mode. Plant Cell 20: 1059–1072]. The grapevine (*Vitis vinifera*), papaya (*Carica papaya*), sorghum (*Sorghum bicolor*) and soybean (*Glycine max*) genomes have also been sequenced, and these contain similar *SPS* and *SPP* gene families to other eudicot and monocot species, confirming the general pattern described.

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References

Adams RP, Kendall E and Kartha KK (1990) Comparison of free sugars in growing and dessicated plants of *Selaginella lepidophylla*. Biochem Syst Ecol 18: 107–110

Angiosperm Phylogeny Group II (2003) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Bot J Linn Soc 141: 399–436

Avigad G (1982) Sucrose and other disaccharides. In: Loewus FA and Tanner W (eds) Encyclopedia of Plant Physiology, New Series, Vol 13A. Plant Carbohydrates I. Intracellular Carbohydrates, pp 217–347. Springer Verlag, Berlin

Avigad G and Dey PM (1997) Carbohydrate metabolism: storage carbohydrates. In: Dey PM and Harborne JB (eds) Plant Biochemistry, pp 143–204. Academic Press, San Diego

Barbier G, Oesterhelt C, Larson MD, Halgren RG, Wilkerson C, Garavito RM, Benning C and Weber APM (2005) Comparative genomics of two closely related unicellular thermo-acidophilic red algae, *Galdieria sulphuraria* and *cyanidioschyzon merolae*, reveals the molecular basis of the metabolic flexibility of *Galdieria sulphuraria* and significant differences in carbohydrate metabolism of both algae. Plant Physiol 137: 460–474

Benaroudj N, Lee DH and Goldberg AL (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. J Biol Chem 276: 24261–24267

Bianchi G, Gamba A, Limiroli R, Pozzi N, Elster R, Salamini F and Bartels D (1993) The unusual composition in leaves of the resurrection plant *Myrothamnus flebellifolia*. Physiol Plant 87: 223–226

Billi D, Wright DJ, Helm RF, Prickett T, Potts M and Crowe JH (2000) Engineering desiccation tolerance in *Escherichia coli*. Appl Environ Microbiol 66: 1680–1684

Buchanan JG (1953) The path of carbon in photosynthesis. XIX. The identification of sucrose phosphate in sugar beet leaves. Arch Biochem Biophys 44: 140–149

Cabib E and Leloir LF (1958) The biosynthesis of trehalose phosphate. J Biol Chem 231: 259–275

Caffrey M, Fonseca V and Leopold AC (1988) Lipid-sugar interactions: relevance to anhydrous biology. Plant Physiol 86: 754–758

Castleden CK, Aoki N, Gillespie VJ, MacRae EA, Quick WP, Buchner P, Foyer CH, Furbank RT and Lunn JE (2004) Evolution and function of the sucrose-phosphate synthase gene families in wheat and other grasses. Plant Physiol 135: 1753–1764

Cavalier-Smith T (2000) Membrane heredity and early chloroplast evolution. Trends Plant Sci 5: 174–182

Cotelle V, Meek SE, Provan F, Milne FC, Morrice N and MacKintosh C (2000) 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. EMBO J 15: 2869–2876

Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W and Tablin F (2001) The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. Cryobiol 43: 89–105

Croxdale JG (1977) Accumulation of ^{32}P and [^{14}C] sucrose in decapitated and intact shoots of the fern *Davallia trichomanoides* Blume. Planta 133: 111–115

Cumino A, Ekeroth C and Salerno GL (2001) Sucrose-phosphate phosphatase from *Anabaena* sp. strain PCC 7120: isolation of the protein and gene revealed significant structural differences from the higher-plant enzyme. Planta 214: 250–256

Cumino A, Curatti L, Giarrocco L, Salerno GL (2002) Sucrose metabolism: *Anabaena* sucrose-phosphate synthase and sucrose-phosphate phosphatase define minimal functional domains shuffled during evolution. FEBS Lett 517:19–23

Curatti L, Folco E, Desplats P, Abratti G, Limones V, Herrera-Estrella L and Salerno G (1998) Sucrose-phosphate synthase from *Synechocystis* sp. PCC 6803: identification of the *spsA* gene and characterization of the enzyme expressed in *Escherichia coli*. J Bacteriol 180: 6776–6779

Curatti L, Flores E and Salerno G (2002) Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp. FEBS Lett 513: 175–178

Dancer JE and ap Rees T (1989) Relationship between pyrophosphate : fructose-6-phosphate 1-phosphotransferase, sucrose breakdown, and respiration. J Plant Physiol 135: 197–206

Desplats P, Folco E and Salerno GL (2005) Sucrose may play an additional role to that of an osmolyte in *Synechocystis* sp. PCC 6803 salt-shocked cells. Plant Physiol Biochem 43: 133–138

Ding DQ, Amino S, Mimura T, Sakano K, Nagata T, Tazawa M (1992) Quantitative analysis of intercellularly-transported photoassimilates in *Chara corallina*. J Exp Bot 43: 1045–1051

Duran WR and Pontis HG (1977) Sucrose metabolism in green algae. The presence of sucrose synthetase and sucrose phosphate synthetase. Mol Cell Biochem 16: 149–152

Echeverria E, Salvucci ME, Gonzalez P, Paris G and Salerno G (1997) Physical and kinetic evidence for an association between sucrose-phosphate synthase and sucrose-phosphate phosphatase. Plant Physiol 115: 223–227

Edelman J (1971) The role of sucrose in green plants. In: Yudkin J, Edelman J and Hough I (eds) Sugar, pp 95–102. Butterworth, London

Edwards J, ap Rees T, Wilson PM and Morrell S (1984) Measurement of the inorganic pyrophosphate in tissues of *Pisum sativum* L. Planta 162: 188–191

Egger B, Einig W, Schlereth A, Wallenda T, Magel E, Loewe A and Hampp R (1996) Carbohydrate metabolism in one- and two-year-old spruce needles, and stem carbohydrates from three months before until three months after bud break. Physiol Plant 96: 91–100

Elbein AD, Pan YT, Pastuszak I and Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiol 13: 17R–27R

Fieulaine S, Lunn JE, Borel F and Ferrer J-L (2005) The structure of a cyanobacterial sucrose-phosphatase reveals the sugar tongs that release free sucrose in the cell. Plant Cell 17: 2049–2058

Fung RWM, Langenkämper G, Gardner RC and MacRae E (2003) Differential expression within an SPS gene family. Plant Sci 164: 459–470

Gibon Y, Bläsing OE, Hannemann J, Carillo P, Höhne M, Hendriks JH, Palacios N, Cross J, Selbig, J and Stitt M (2004a) A robot-based platform to measure multiple enzyme activities in *Arabidopsis* using a set of cycling assays: comparison of changes of enzyme activities and transcript levels during diurnal cycles and in prolonged darkness. Plant Cell 16: 3304–3325

Hagemann M and Marin K (1999) Salt-induced sucrose accumulation is mediated by sucrose-phosphate-synthase in cyanobacteria. J Plant Physiol 155: 424–430

Hampp R, Egger B, Effenberger S and Einig W (1994) Carbon allocation in developing spruce needles – enzymes and intermediates of sucrose metabolism. Physiol Plant 90: 299–306

Hampp R, Schaeffer C, Wallenda T, Stulten C, Johann R and Einig W (1995) Changes in carbon partitioning or allocation due to ectomycorrhiza formation – biochemical evidence. Can J Bot 73: Suppl S548–S556

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA and Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290: 2110–2113

Hawker JS and Smith GM (1984) Occurrence of sucrose phosphatase in vascular and non-vascular plants. Phytochem 23: 245–249

Hendriks JH, Kolbe A, Gibon Y, Stitt M and Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of *Arabidopsis* and other plant species. Plant Physiol 133: 838–849

Hesse H, Sonnewald U and Wilmitzer L (1995) Cloning and expression analysis of sucrose-phosphate synthase from sugar-beet. Mol Gen Genet 247: 515–520

Hincha DK and Hagemann M (2004) Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance. Biochem J 383: 277–283

Holligan PM and Drew EA (1971) Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. 2. Quantitative analysis of standard carbohydrates, and separation and estimation of soluble sugars and polyols from a variety of plant tissues. New Phytol 70: 271–278

Huber SC (1981) Interspecific variation in activity and regulation of leaf sucrose phosphate synthase. Z. Pfanzenphysiol 102: 443–450

Huber SC and Huber JL (1996) Role and regulation of sucrose-phosphate synthase in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47: 431–444

Huynh F (2004) Structure-function relationships of proteins from the Halothermophile *Halothermothrix orenii.* BSc (Hons) thesis (Advanced Studies), p 110. Faculty of Science, Griffith University Queensland, Australia

Ingram J, Chandler JW, Gallagher L, Salamini F and Bartels D (1997) Analysis of cDNA clones encoding sucrose-phosphate synthase in relation to sugar interconversions associated with dehydration in the resurrection plant *Craterostigma plantagineum* Hochst. Plant Physiol 115: 113–121

Iturriaga G, Gaff DF and Zentella R (2000) New desiccation-tolerant plants, including a grass, in the central highlands of Mexico, accumulate trehalose. Aust J Bot 48: 153–158

Karsten U, Barrow KD and King RJ (1993) Floridoside, L-isofloridoside, and D-isofloridoside in the red alga *Porphyra columbina* – seasonal and osmotic effects. Plant Physiol 103: 485–491

Keeling PJ (2004) Diversity and evolutionary history of plastids and their hosts. Am J Bot 91: 1481–1493

Khmelenina VN Kalyuzhnaya MG, Sakharovsky VG, Suzina NE, Trotsenko YA and Gottshalk G (1999) Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch Microbiol 172: 321–329

Khmelenina VN, Sakharovskii VG, Reshetnikov AS and Trotsenk YA (2000) Synthesis of osmoprotectors by halophilic and alkalophilic methanotrophs. Mikrobiologia 69: 465–470

Kirst GO, Janssen MIB, Winter U (1988) Ecophysiological investigations of *Chara vulgaris* L. grown in a brackish water lake – ionic changes and accumulation of sucrose in the vacuolar sap during sexual reproduction. Plant Cell Environ 11: 55–61

Klein RR, Crafts-Brandner SJ and Salvucci ME (1993) Cloning and developmental expression of the sucrose-phosphate synthase gene from spinach. Planta 190**:** 498–510

Komatsu A, Takanokura Y, Omura M and Akihima T (1996) Cloning and molecular analysis of cDNAs encoding three sucrose-phosphate synthase isoforms from citrus fruit (*Citrus unshiu* Marc.). Mol Gen Genet 252: 346–351

Langenkämper G, Fung RWM, Newcomb RD, Atkinson RG, Gardner RG and MacRae EA (2002) Sucrose phosphate synthase genes in plants belong to three different families. J Mol Evol 54: 322–332

Leegood RC (1985) The intercellular compartmentation of metabolites in leaves of *Zea mays*. Planta 164: 163–171

Leloir LF and Cardini CE (1955) The biosynthesis of sucrose phosphate. J Biol Chem 214: 157–165

Lewis LA and McCourt RM (2004) Green algae and the origin of land plants. Am J Bot 91: 1535–1556

Li CR, Zhang XB and Hew CS (2003) Cloning of a sucrose-phosphate synthase gene highly expressed in flowers from the tropical epiphytic orchid *Oncidium* Goldiana. J Exp Bot 54: 2189–2191

Lockhart PJ and Penny D (2005) The place of *Amborella* within the radiation of angiosperms. Trends Plant Sci 10: 201–202

Loewe A, Einig W and Hampp R (1996) Coarse and fine control and annual changes of sucrose-phosphate synthase in Norway spruce needles. Plant Physiol 112: 641–649

Lunn JE (2002) Evolution of sucrose synthesis. Plant Physiol 128: 1490–1500

Lunn JE (2003) Sucrose-phosphatase gene families in plants. Gene 303: 187–196

Lunn JE and Furbank RT (1997) Localisation of sucrose-phosphate synthase and starch in leaves of C_4 plants. Planta 202: 106–111

Lunn JE and Furbank RT (1999) Sucrose biosynthesis in C_4 plants. New Phytol 143: 221–237

Lunn JE and Hatch MD (1997) The role of sucrose-phosphate synthase in the control of photosynthate partitioning in *Zea mays* leaves. Aust J Plant Physiol 24: 1–8

Lunn JE and MacRae EA (2003) New complexities in the synthesis of sucrose. Curr Opin Plant Biol 6: 208–214

Lunn JE, Furbank RT and Hatch MD (1997) Adenosine 5′-triphosphate-mediated activation of sucrose-phosphate synthase in bundle sheath cells of C_4 plants. Planta 202: 249–256

Lunn JE, Price GD and Furbank RT (1999) Cloning and expression of a prokaryotic sucrose-phosphate synthase gene from the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Mol Biol 40: 297–305

Lunn JE, Ashton AR, Hatch MD, Heldt HW (2000) Purification, molecular cloning, and sequence analysis of sucrose-6^F-phosphate phosphohydrolase from plants. Proc Natl Acad Sci USA 97: 12914–12919

Lunn JE, Gillespie VJ and Furbank RT (2003) Expression of a cyanobacterial sucrose-phosphate synthase from *Synechocystis* sp. PCC 6803 in transgenic plants. J Exp Bot 54: 1–15

MacRae EA and Lunn JE (2005) Control of sucrose biosynthesis. In: Plaxton W and McManus MT (eds) Annual Plant Reviews – Control of Primary Metabolism in Plants, pp 234–257. Blackwell, Oxford

Marin K, Zuther E, Kerstan T, Kunert A and Hagemann M (1998) The *ggps* gene from *Synechocytis* sp. strain PCC 6803 encoding glucosyl-glycerol-phosphate synthase is involved in osmolyte synthesis. J Bacteriol 180: 4843–4849

Marin K, Kanesaki Y, Los DA, Murata N, Suzuki I and Hagemann M (2004) Gene expression profiling reflects physiological processes in salt acclimation of *Synechocystis* sp. strain PCC 6803. Plant Physiol 136: 3290–3300

Miao XL, Wu QY, Wu GF and Zhao NM (2003) Sucrose accumulation in salt-stressed cells of *agp* gene deletion-mutant in cyanobacterium *Synechocystis* sp. PCC 6803. FEMS Microbiol Lett 218: 71–77

Mijts BN and Patel BKC (2001) Random sequence analysis of genomic DNA of an anaerobic, thermophilic, halo-

philic bacterium, *Halothermothrix orenii*. Extremophiles 5: 61–69

Müller W and Wegmann K (1978a) Sucrose biosynthesis in *Dunaliella* I. Thermic and osmotic regulation. Planta 141: 155–158

Müller W and Wegmann K (1978b) Sucrose biosynthesis in *Dunaliella* II. Isolation and properties of sucrose phosphate synthetase. Planta 141: 159–163

Neufeld EF and Hassid WZ (1963) Biosynthesis of saccharides from glucopyranosyl esters of nucleotides ("sugar nucleotides"). Adv Carbohydr Chem 18: 309–356

Nielsen TH, Rung JH and Villadsen D (2004) Fructose-2,6-bisphosphate: a traffic signal in plant metabolism. Trends Plant Sci 9: 556–563

Niittylä T, Messerli G, Trevisan M, Chen J, Smith AM and Zeeman SC (2004) A previously unknown maltose transporter essential for starch degradation. Science 303: 87–89

Nishimura M and Beevers H (1979) Subcellular distribution of gluconeogenetic enzymes in germinating castor bean endosperm. Plant Physiol 64: 31–37

Page-Sharp M, Behm CA, Smith GD (1999) Involvement of the compatible solutes trehalose and sucrose in the response to salt stress of a cyanobacterial *Scytonema* species isolated from desert soils. Biochim Biophys Acta 1472: 519–528

Palmer JD, Solti DE and Chase MW (2004) The plant tree of life: an overview and some points of view. Am J Bot 91: 1437–1445

Pontis HG (1977) Riddle of sucrose. In: Northcote DH (ed) Plant Biochemistry II Int rev Biochem, Vol XIII, pp 79–117. University Park Press, Baltimore

Porchia AC and Salerno GL (1996) Sucrose biosynthesis in a prokaryotic organism: presence of two sucrose-phosphate synthases in *Anabaena* with remarkable differences compared with the plant enzymes. Proc Natl Acad Sci USA 93: 13600–13604

Porchia AC, Fiol DF and Salerno GL (1999) Differential synthesis of sucrose and trehalose in *Euglena gracilis* cells during growth and salt stress. Plant Sci 149: 43–49

Pryer KM, Schneider H, Smith AR, Cranfill R, Wolf PG, Hunt JS and Sipes SD (2001) Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. Nature 409: 618–621

Qu Q, Lee S-J and Boos W (2004) TreT, a novel trehalose glycosyltransferring synthase of the hyperthermophilic archaeon *Thermococcus litoralis*. J Biol Chem 279: 47890–47897

Ral JP, Derelle E, Ferraz C, Wattebled F, Farinas B, Corellou F, Buleon A, Slomianny MC, Delvalle D, d'Hulst C, Rombauts S, Moreau H and Ball S (2004) Starch division and partitioning. A mechanism for granule propagation and maintenance in the picophytoplanktonic green alga *Ostreococcus tauri*. Plant Physiol 136: 3333–3340

Reed RH and Stewart WDP (1985) Osmotic adjustment and organic solute accumulation in unicellular cyanobacteria from freshwater and marine habitats. Mar Biol 88: 1–9

Reed RH, Richardson DL, Warr SRC and Stewart WDP (1984) Carbohydrate accumulation and osmotic stress in cyanobacteria. J Gen Microbiol 130: 1–4

Reed RH, Borowitzka LJ, MacKay MA, Chudek JA, Foster R, Warr SRC, Moore DJ and Stewart WDP (1986) Organic solute accumulation in osmotically stressed cyanobacteria. FEMS Microbiol Rev 39: 51–56

Robinson SP and Walker DA (1979) Site of sucrose synthesis in isolated leaf protoplasts. FEBS Lett 107: 295–299

Roser DJ, Melick, DR, Ling HU and Seppelt, RD (1992) Polyol and sugar content of terrestrial plants from continental Antarctica. Antarctic Sci 4: 413–420

Ryu S-I, Park C.S, Cha J, Woo E-J and Lee S-B (2005) A novel trehalose-synthesizing glycosyltransferase from *Pyrococcus horikoshii*: molecular cloning and characterization. Biochem Biophy Res Comm 329: 429–436

Salerno GL (1985a) Measurement of enzymes related to sucrose metabolism in permeabilized *Chlorella vulgaris* cells. Physiol Plant 64: 259–264

Salerno GL (1985b) Occurrence of sucrose and sucrose metabolizing enzymes in achlorophyllous algae. Plant Sci 42: 5–8

Salerno GL and Curatti L (2003) Origin of sucrose metabolism in higher plants: when, how and why? Trends Plant Sci 8: 63–69

Salerno GL, Porchia AC and Sanchez N (1995) Biosynthesis of sucrose in lower organisms. In: Pontis HG, Salerno GL and Echeverria (eds) International Symposium on Sucrose Metabolism, pp 34–39. American Society of Plant Physiologists, Bethesda

Sayago JE, Vattuone MA, Sampietro AR and Isla MI (2001) An invertase inhibitory protein from *Pteris deflexa* Link fronds. J Enz Inhib 16: 517–525

Schilling N and Ehrnsperger K (1985) Cellular differentiation of sucrose metabolism in *Anabaena variabilis*. Z Naturforsch 40: 776–779

Schluepmann H, Pellny T, Van Dijken A, Smeekens S and Paul M (2003) Trehalose 6-phosphate is indispensible for carbohydrate utilization and growth in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100: 6849–6854

Schulte C, Kirst GO and Winter U (1994) Source-sink characteristics of photoassimilate transport in fertile and sterile plants of *Chara vulgaris* L. Bot Acta 107: 362–368

Singer MA and Lindquist S (1998) Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. Mol Cell 1: 639–648

Smirnoff N (1992) The carbohydrates of bryophytes in relation to desiccation tolerance. J Bryol 17: 185–191

Soltis DE and Soltis PS (2004a) The origin and diversification of angiosperms. Am J Bot 91: 1614–1626

Soltis DE and Soltis PS (2004b) *Amborella* not a "basal angiosperm"? Not so fast. Am J Bot 91: 997–1001

Sonnewald U, Quick WP, MacRae E, Krause KP and Stitt M (1993) Purification, cloning and expression of spinach leaf sucrose-phosphate synthase in *Escherichia coli*. Planta 189: 174–181

Stefanovic S, Rice DW and Palmer JD (2004) Long branch attraction, taxon sampling, and the earliest angiosperms: *Amborella* or monocots? BMC Evol Biol 4: 35

Stitt M, Huber S and Kerr P (1987) Control of photosynthetic sucrose formation. In: Hatch MD and Boardman NK (eds) The Biochemistry Of Plants, Vol 10, Photosynthesis, pp 327–409. Academic Press, New York

Strand Å, Zrenner R, Trevanion S, Stitt M, Gustaffson P and Gardeström P (2000) Decreased expression of two enzymes in the sucrose biosynthesis pathway, cytosolic fructose-1,6-bisphosphatase and sucrose phosphate synthase, has remarkably different consequences for photosynthetic carbon metabolism in transgenic *Arabidopsis thaliana.* Plant J 23: 759–770

Sugiharto B, Sakakibara H, Sumadi and Sugiyama T (1997) Differential expression of two genes for sucrose-phosphate synthase in sugarcane: molecular cloning of the cDNAs and comparative analysis of gene expression. Plant Cell Physiol 38: 961–965

Suleiman AAA and Lewis DH (1980) Carbohydrate metabolism in the leafy liverwort *Plagiochila asplenoides* (L) Dum. var Major Nees. New Phytol 84: 45–58

Suleiman AAA, Bacon J, Christie A and Lewis DH (1979) Carbohydrates of the leafy liverwort, *Plagiochila asplenioides* (L.) Dum. New Phytol 82: 439–448

Sun WQ, Li X-P and Ong B-L (1999) Preferential accumulation of D-pinitol in *Acrostichum aureum* gametophytes in response to salt stress. Physiol Plant 105: 51–57

Toroser D and Huber SC (1997) Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach leaves. Plant Physiol 114: 947–955

Touchette BW and Burkholder JM (2000) Overview of the physiological ecology of carbon metabolism in seagrasses. J Exp Mar Biol Ecol 250: 169–205

Tree of Life Web Project, http://tolweb.org (accessed December 2005)

Trevanion SJ, Castleden CK, Foyer CH, Furbank RT, Quick WP and Lunn JE (2004) Regulation of sucrose-phosphate synthase in wheat (*Triticum aestivum*) leaves. Funct Plant Biol 31: 685–695

Turgeon R and Medville R (2004) Phloem loading. A reevaluation of the relationship between plasmodesmatal frequecies and loading strategies. Plant Physiol 136: 3795–3803

Valdez-Alarcon JJ, Ferrando M, Salerno G, Jimenez-Moraila B and Herrera-Estrella L (1996) Characterization of a rice sucrose-phosphate synthase encoding gene. Gene 170: 217–222

Vázquez-Ortíz FA and Valenzuela-Soto EM (2004) HPLC determination of trehalose in *Selaginella lepidophylla* plants. J Liq Chrom Rel Technol 27: 1937–1946

Wera S, de Schrijver E, Geyskens I, Nwaka S and Thevelein JM (1999) Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*. Biochem J 343: 621–626

White D (1995) The Physiology and Biochemistry of Prokaryotes. Oxford University Press, Oxford

Winkenbach F, Grant BR and Bidwell RGS (1972) The effects of nitrate, nitrite, and ammonia on photosynthetic carbon metabolism of *Acetabularia* chloroplast preparations compared with spinach chloroplasts and whole cells of *Acetabularia* and *Dunaliella*. Can J Bot 50: 2545–2551

Winter U. SoulieMarsche I and Kirst GO (1996) Effects of salinity on turgor pressure and fertility in *Tolypella* (Characeae). Plant Cell Environ 19: 869–879

Winter U, Kirst GO, Grabowski V, Heinemann U, Plettner I and Wiese S (1999) Salinity tolerance in *Nitellopsis obtusa*. Aust J Bot 47: 337–346

Worrell AC, Brunneau JM, Summerfelt K, Boersig M and Voelker TA (1991) Expression of maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. Plant Cell 3: 1121–1130

Xiong J, Inoue K and Bauer CE (1998) Tracking molecular evolution of photosynthesis by characterization of a major photosynthesis gene cluster from *Heliobacillus mobilis*. Proc Natl Acad Sci USA 95: 14851–14856

Chapter 28

Sucrose Transport in Higher Plants: From Source to Sink

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Summary

Sucrose transport in plants has been as area of intense interest for many years, particularly following the cloning of the first sucrose/proton symporter, or sucrose transporter, from spinach more than 15 years ago. Much debate and research has focused on phloem loading, particularly the issue of apoplasmic versus symplasmic pathways of loading and in the apoplasmic loaders, on the location of the sucrose transporter protein and mRNA. This chapter focuses on pointing out the remaining unanswered questions in phloem loading and sucrose transport in general rather than extensively reviewing the literature. We discuss in more detail the long-distance transport pathway from source to sink and post-phloem unloading in sink tissue such as dicot seed, cereal grain, sink leaves, roots and tubers.

I. Introduction

The long-distance transport, or translocation, of assimilates from source tissues to sink tissues occurs in the phloem of the vascular bundles. In source leaves, sucrose is synthesized in the cytoplasm of mesophyll cells as the major product of photosynthesis (see Chapter 27). For translocation, sucrose moves into the phloem sieve element (phloem loading), and travels through phloem conduits to other parts of the plant (long-distance transport). In sink tissues such as shoot apices, roots and storage tissues, sucrose moves out of the phloem (phloem unloading) and provides the carbon source for development, growth and storage (summarized in Fig. 28.1).

As for glucose in the animal kingdom, higher plants utilize only a few defined sugar molecules for the long-distance transport of photoassimilate. Sucrose is known as the major translocating sugar in angiosperms (see Section II.B). Also sucrose is the major solute found in the phloem (sieve tube) sap of most plant species, providing not only the carbon source for growth and metabolism but also the driving force for bulk flow of the sap, namely, translocation of all other constituents in the phloem sap, including signaling molecules such as phytohormones (see Turgeon and Wolf, 2008 for further details).

Recent intensive research has made considerable progress in elucidating the mechanism of translocation at the molecular level, particularly in the area of proton-coupled sucrose transport systems across the plasma membrane, and their role in the long-distance transport of sucrose. By way of introduction, we briefly address below some key features of the translocation of sucrose in plants then detail the specific aspects of the transport on a tissue-by-tissue basis.

A. Phloem and Sieve Element/ Companion Cell Complex

The phloem of angiosperms comprises four kinds of tissues, i.e., the sieve elements (SEs), the companion cells (CCs), the phloem parenchyma and the phloem fiber (see Van Bel, 2003 for further details). SEs are longitudinally interconnected via sieve pores at their terminal ends, and serve as the conducting tube or sieve tube, for the phloem sap. Whereas SEs and CCs are formed from a common mother cell by cell division, the two types of cells undergo contrasting differentiation processes during their maturation. SEs lose a large part of the cytoplasmic content such as the nucleus, tonoplast and ribosomes. CCs, on the other hand, retain dense cytoplasm and numerous ribosomes and mitochondria. Since SEs and CCs are closely linked by many plasmodesmata, one important task of CCs is considered to be the supply of compounds necessary for SEs to remain active over the entire growth period. These close ontogenic and functional relationships of the two cells have led to the expression: sieve element/companion cell complex (SE/CC complex), to describe their association.

Abbreviations: CC – Companion cell; CF – Carboxyfluorescein; GFP – Green fluorescent protein; PCMBS – Para-chloromercuribenzylsulphonic acid; RFO – Raffinose-family oligosaccharide; SE – Sieve element; SUT – Sucrose transporter

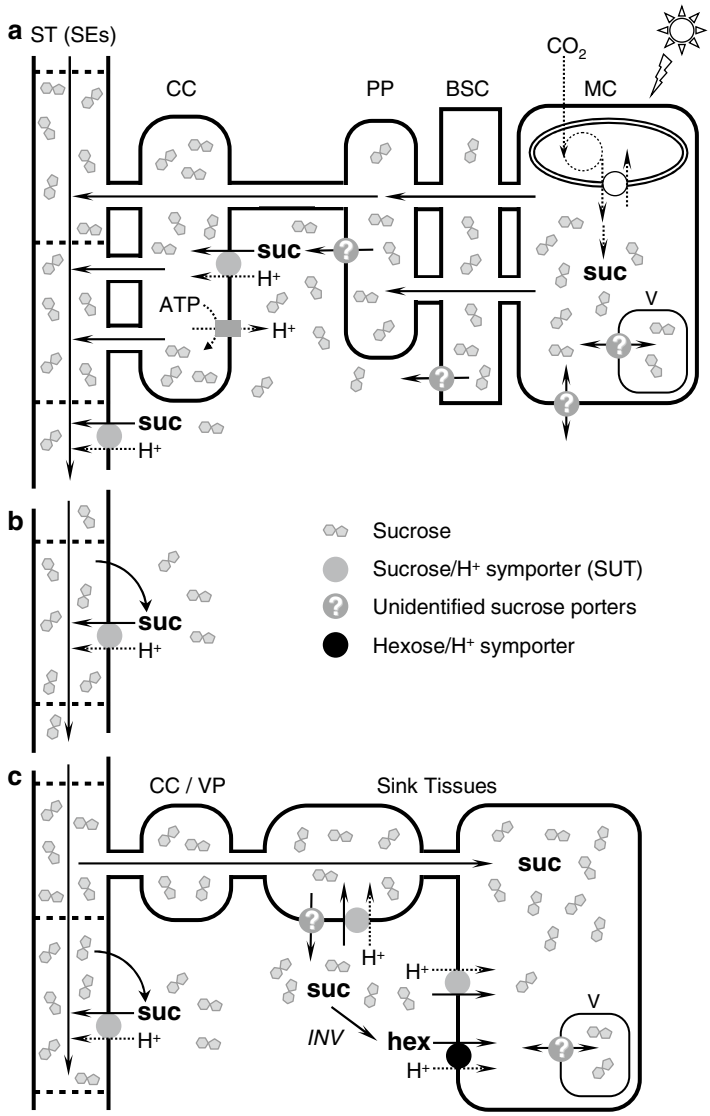

Fig. 28.1. Long-distance transport (Translocation) of sucrose from source leaves to sink tissues. (**a**) Collection phloem (Loading). Sucrose is synthesized in the cytosol of mesophyll cell (*MC*) by photosynthesis. For temporal storage, sucrose is imported into and exported from vacuole (*V*), via unidentified sucrose porter(s). For translocation, sucrose diffuses symplasmically to the bundle sheath cell (*BSC*) and the phloem parenchyma (*PP*) through plasmodesmata. Both symplasmic and apoplasmic routes exit to load the sieve element/companion cell (*SE/CC*) complex. In the latter case, sucrose is released to phloem apoplasm by unascertained mechanisms, and is then taken up actively into the SE/CC complex via a sucrose/H^+ symporter which localizes to the SE or the CC, depending on plant species (see Kühn, 2003; Lalonde et al., 2004). (**b**) Transport phloem. Sucrose is translocated to sink tissues through the sieve tube (ST) continuum. Sucrose/H^+ symporters localize to the SE/CC complex of transport phloem and play a role in retrieving sucrose leaked to the apoplasm of phloem conduits. (**c**) Release phloem (Unloading). Sucrose moves out of the phloem in order to provide carbon source to sink tissues. While unloading of sucrose from phloem is believed to occur symplasmically, both symplasmic and apoplasmic routes have been proposed to exist in the post-phloem transport pathway in sink tissues. In the apoplasmic route, sucrose is released to the apoplasm via unidentified porters (possibly via sucrose/H^+ symporter), and then retrieved into sink tissues via sucrose/H^+ symporter, or via hexose/H^+ symporter following cleavage by cell wall invertase. VP, Vascular parenchyma.

B. Mass Flow Model

To account for the mechanism of long-distance transport of solutes through the phloem continuum, a pressure-driven mass flow model offers the simplest and most widely accepted explanation (see Thorpe and Minchin, 1996; Minchin and Lacointe, 2005). According to this model, solutes (sugars, amino acids, inorganic ions, etc.) move from a source to a sink along an osmotic turgor pressure gradient in the sieve tube. Within source regions, solutes are actively taken up into sieve tubes (phloem loading). This active loading process creates a more negative osmotic potential in the sieve tube. At the sink, solutes are released from sieve tubes (phloem unloading), resulting in a less negative osmotic potential in the sieve tube, allowing water to move out. This hydrostatic pressure drives a bulk flow of phloem sap from source to sink.

C. Sucrose/Proton Symporters (Sucrose Transporters)

Since the cloning by yeast complementation of the first plant sucrose transporter (SUT, Riesmeier et al., 1992), and aided by the sequencing of the *Arabidopsis thaliana* and rice genomes, more than 85 putative SUT sequences from at least 35 species of higher plants have been lodged in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/). All of the SUTs cloned thus far fall into the major facilitator super-family (Marger and Saier, 1993; Saier, 2000) characterized by 12 predicted plasma membrane-spanning helices, arranged in two groups of 6, separated by a cytoplasmic loop (Fig. 28.2). A histidine residue located in the first extracellular loop is conserved in all plant SUTs so far identified and has been shown to be involved in the sucrose transport activity by site-directed mutagenesis (Lu and Bush, 1998). A number of these transporters have been expressed in vitro using yeast and oocyte expression systems (see below) with K_m values for sucrose ranging from 0.2 to 2 mM (see Lemoine, 2000; Kühn, 2003). There is strong evidence that the mechanism of transport is via a 1:1 sucrose/H^+ co-transport, driven by a transmembrane pH gradient supported by the proton pumping ATPase (Boorer et al., 1996). Some SUTs (in particular the SUT2 class cloned by Barker et al., 2000) are characterized by large cytoplasmic loop regions and are reputed to play a role in sugar sensing (see Lalonde et al., 1999; Barker et al., 2000). The role of sucrose transporters in sugar sensing will be reviewed later in this chapter.

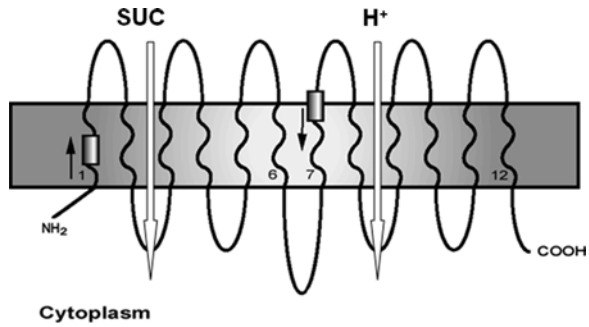

Fig. 28.2. Predicted structural model of plant sucrose/H^+ symporters. The twelve membrane-spanning helices are a typical feature of the major facilitator super-family transporter proteins, with both C and N termini predicted to be intracellular, as is the hydrophilic loop between helices 6 and 7.

Many reports have shown the transcriptional regulation of SUT genes in various developmental processes, by environmental factors such as light and salt stress, and by endogenous signals such as sugars and hormones (reviewed in Lemoine, 2000; Williams et al., 2000; Kühn, 2003; Lalonde et al., 2004; Sauer, 2007). Furthermore, SUT proteins can be phosphorylated (Roblin et al., 1998) and oligomerized in vitro (Reinders et al., 2002; Schulze et al., 2003) and in vivo in a redox-dependent manner (Krügel et al., 2008). It is likely that SUTs are subject to complex regulation at different levels.

For most dicot species, two or more SUT genes have been reported (reviewed by Lemoine, 2000; Williams et al., 2000; Sauer, 2007). In *A. thaliana*, for example, nine SUT genes are found in the genome and their properties have been examined by functional expression in yeast cells (Sauer and Stolz, 1994; Barker et al., 2000; Meyer et al., 2000; Schulze et al., 2000; Weise et al., 2000; Ludwig et al., 2000; Sauer et al., 2004). Intriguingly, two of the *A. thaliana* SUT genes, *AtSUC6* and *AtSUC7*, have been reported to encode aberrant proteins and seem to be pseudogenes (Sauer et al., 2004). In potato and tomato, three different SUT genes, *SUT1*, *SUT2* and *SUT4* have been cloned and characterized (Riesmeier et al., 1993; Barker et al., 2000; Weise et al., 2000). In monocot species, five SUT genes appear to be present in the genome sequence of rice and are expressed in a

variety of tissues (Aoki et al., 2003) while in barley, two genes have been identified (Weschke et al., 2000), three homeologous genes encoding SUT1 have been cloned from wheat (Aoki et al., 2002) and one SUT1-type transporter has been cloned and characterized from maize (Aoki et al., 1999) and sugarcane (Rae et al., 2005). Heterologous expression of monocot SUT genes has proven problematic with low levels of activity achieved in yeast cells for *OsSUT1* and *OsSUT3* (Hirose et al., 1997; Aoki et al., 2003), barley *HvSUT1* and *HvSUT2* (Weschke et al., 2000), and sugarcane *ShSUT1* (Rae et al., 2005); however, recently maize *ZmSUT1*, as well as *HvSUT1* and *ShSUT1*, have been expressed successfully in oocytes (Carpaneto et al., 2005; Sivitz et al., 2005; Reinders et al., 2006). The reason for the difficulty in obtaining good expression of the monocot SUTs in yeast is unknown and not due to the lack of sustained effort (R.T. Furbank, N. Aoki and G.N. Scofield, unpublished observations; N. Sauer, personal communication).

Based on phylogenetic analysis of deduced peptide sequences, SUTs from both dicots and monocots can be classified into three groups (Fig. 28.3; also see Sauer, 2007; Braun and Slewinski, 2009). Type-I SUTs encompass the classic SUT1-type dicot transporters which have often been assigned a role in phloem loading (see Section II.C). Type-II includes all the monocot SUT1 genes, OsSUT3, 4 and 5 and the dicot SUT2 class (the putative "sugar sensors"). Although it is plausible that each SUT group possesses distinctive biochemical properties (see Kühn, 2003; Lalonde et al., 2004; Sauer, 2007), the three SUT groups do not appear, so far, to be functionally distinct, except possibly only on the basis of Km values for sucrose, measured in heterologous expression systems (see Lemoine, 2000; Kühn, 2003; Sauer, 2007). It is very difficult to assign physiological function based on such phylogenetic relationships, as evidenced by the common observation of expression of each of these SUT genes in a variety of tissues during plant development both in dicots and in cereals (reviewed in Kühn, 2003; Sauer, 2007). There also seem to be differences in the structure of the SUT gene family between monocots and dicots; a Type-I SUT sequence is unlikely to exist in the rice genome as it has not been found in any monocots, including expressed sequence tags in public database. The absence of a monocot gene in the classic dicot SUT1 cluster raises interesting questions about phloem loading in cereal species. The physiological role of these SUTs will be the topic of further discussion in the following sections.

II. Phloem Loading

Phloem loading, often defined as the movement and transport of assimilates from the mesophyll cell into the sieve tube, has primary importance as the first step in the translocation of assimilates. Mesophyll cells are closely interconnected by plasmodesmata, and the sucrose synthesized there is believed to move symplasmically via plasmodesmata toward the phloem region of minor veins (see Van Bel, 1993; Lalonde et al., 2003). In the phloem of minor veins, sucrose (or its derivatives) is transported into the SE/CC complex for translocation.

A. Phloem Anatomy and the Mode of Phloem Loading

Usually, SEs have no symplasmic connection with surrounding cells except CCs, while the frequency of plasmodesmata between CCs and surrounding cells vary greatly depending on plant species. Gamalei (1989) has published an extensive survey of phloem anatomy of leaf minor veins in as many as 700 species, and found that they can be classified into two general groups mostly in a family dependent manner: those with numerous plasmodesmata between CCs and the adjacent cells were designated as type 1 (majority of woody plants and some of herbaceous plants, e.g., Cucurbitaceae, Lamiaceae), while those with few or very rare plasmodesmata at this interface as type 2 (mostly herbaceous plants, e.g., Asteraceae, Fabaceae). This suggests that in type 1 species the assimilate could move entirely through symplasm from the mesophyll cells to the SE/CC complex (symplasmic loading; Fig. 28.4), and, alternatively, in type 2 species apoplasmic, carrier-mediated transport is likely to occur around the SE/CC complex (apoplasmic loading; Fig. 28.4). Several lines of evidence in fact support this view: for example, in type 2 species, but not in type 1 species, accumulation in the minor vein and efflux from the cut end of the

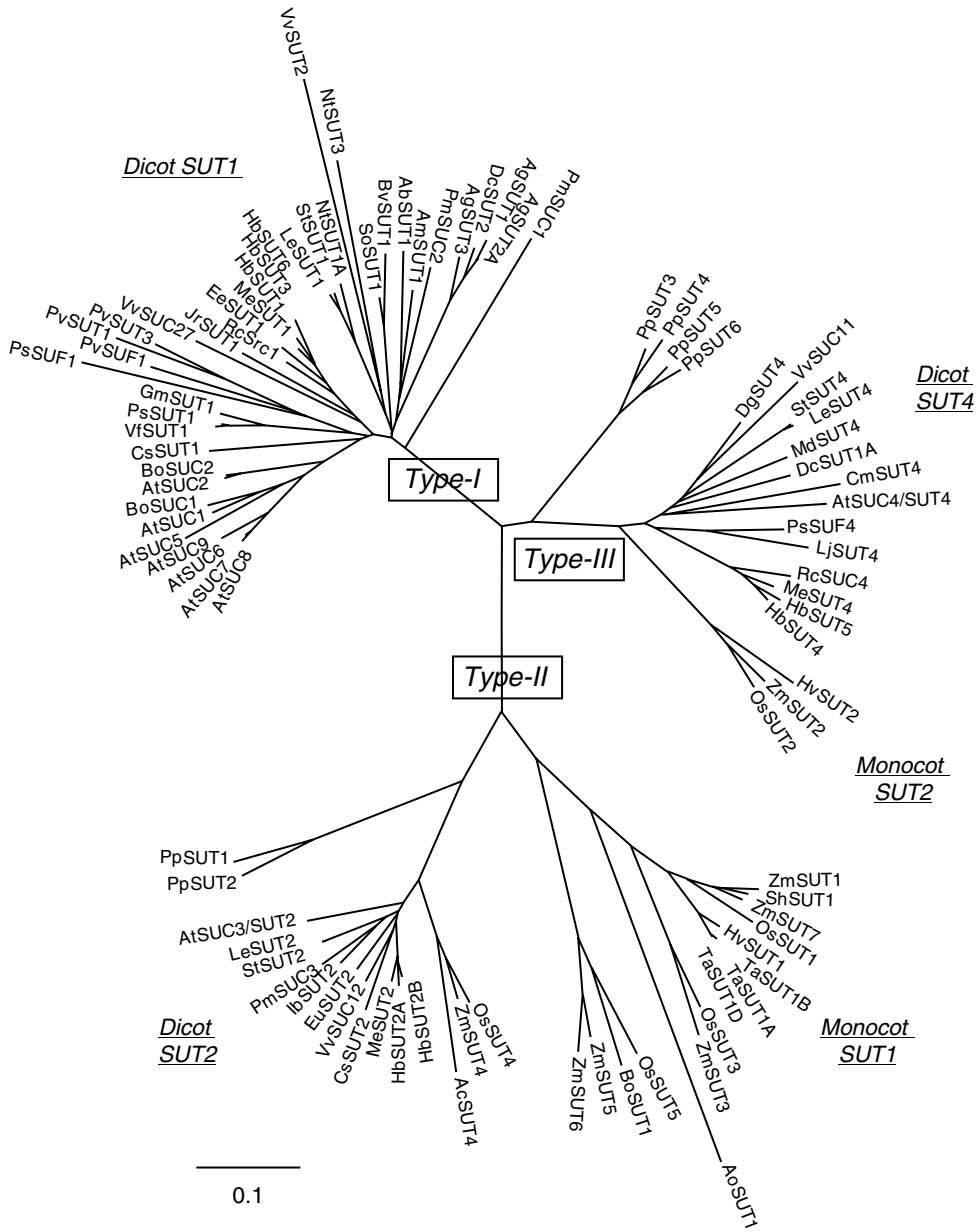

Fig. 28.3. A phylogenetic tree of plant sucrose transporter family, based on deduced amino acid sequences. The CLASTALW program was used to show the relationship between members of this family, and a Neighbor-Joining method was used to construct this unrooted dendrogram. The scale represents 0.1 substitutions per site (one change per ten amino acids). SUT or SUT-like sequences from 38 plant species were retrieved from the GenBank and used for this analysis: Dicot species, *Alonsoa meridionalis* AmSUT1 (accession number AF191025), *Apium graveolens* AgSUT1 (AF063400), AgSUT2A (AF167415), AgSUT3 (DQ286433), *Arabidopsis thaliana* AtSUC1 (At1g71880), AtSUC2 (At1g22710), AtSUC3/SUT2 (At2g02860), AtSUC4/SUT4 (At1g09960), AtSUC5 (At1g71890), AtSUC6 (At5g43610), AtSUC7 (At1g66570), AtSUC8 (At2g14670), AtSUC9 (At5g06170), *Asarina barclaiana* AbSUT1 (AF191024), *Beta vulgaris* BvSUT1 (X83850), *Brassica oleracea* BoSUC1 (AY065839), BoSUC2 (AY065840), *Citrus sinensis* CsSUT1 (AY098891), CsSUT2 (AY098894), *Cucumis melo* CmSUT4 (FJ231518), *Datisca glomerate* DgSUT4 (AJ781069), *Daucus carota* DcSUT1A (Y16766), DcSUT2 (Y16768), *Eucommia ulmoides* EuSUT2 (AY946204), *Euphorbia esula* EeSUT1 (AF242307), *Glycine max* GmSUT1 (AJ563364), *Hevea brasiliensis* HbSUT1 (DQ985466), HbSUT2A (DQ985467), HbSUT2B (DQ985465), HbSUT3 (EF067334), HbSUT4 (EF067335), HbSUT5 (EF067333), HbSUT6 (AM492537), *Ipomoea batatas* IbSUT2 (AY830138), *Juglans regia* JrSUT1 (AY504969), *Lotus japonicus* LjSUT4 (AJ538041), *Lycopersicon esculentum* LeSUT1 (X82275), LeSUT2 (AF166498), LeSUT4 (AF176950),

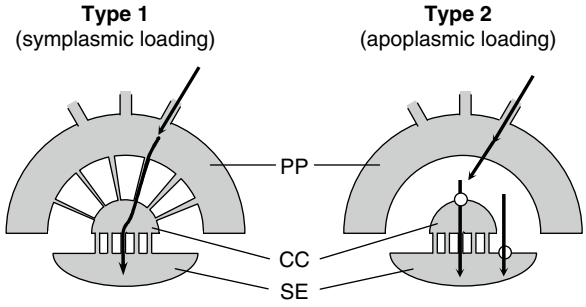

Fig. 28.4. Phloem anatomy and the mode of phloem loading. In type 1 phloem anatomy, there are numerous plasmodesmata between phloem parenchyma (*PP*) and companion cells (*CC*), allowing solutes to move entirely through symplasm from mesophyll cells to sieve element (SE)/CC complex (*arrows*). In type 2, there are few or very rare plasmodesmata between PP and CC, and thus solutes have to move out to apoplasm prior to subsequent uptake into SE/CC complex via plasma membrane carrier protein (*white circles*).

petiole of ^{14}C-photoassimilate are both severely affected by para-chloromercuribenzen sulphonic acid (PCMBS), a potential inhibitor of carrier-mediated sugar uptake from the apoplasm (Van Bel et al., 1994). In addition, molecular biological studies have shown a crucial role of SUTs located in the plasma membrane of CCs or SEs in some type 2 species (see Section II.C). However, the symplasmic continuity presented by type 1 vein structure does not necessarily exclude the co-existence of both loading modes in the SE/CC complex. It has also been reported that some plants have transitional vein anatomy between type 1 and type 2 (type 1-2a, Gamalei, 1991), but their mode(s) of phloem loading remains unclear. In addition, it is feasible that the two modes of phloem loading work within single veins or single SE/CC complexes (Van Bel et al., 1992).

B. Sucrose as Translocating Sugar

Sucrose is contained in the phloem sap of every plant species so far examined and in many of them it is the only detectable sugar molecule. Depending on what kinds of sugars are transported in the phloem, higher plants can be divided into three major groups (Zimmermann and Ziegler, 1975). The first group, most common among higher plants, translocates only sucrose. The second group includes some species in the Cucurbitaceae and Lamiaceae for example, which translocate mainly raffinose-family oligosaccharides (RFOs) such as raffinose, stachyose and verbascose in addition to sucrose. The third group translocates sugar alcohols besides sucrose, for example, mannitol in Apiaceae species and sorbitol in Rosaceae species. Only recently it was demonstrated that a considerable amount of hexoses (e.g., glucose and fructose) could be translocated in the phloem sap of some herbaceous plants (e.g., Paraveraceae, Ranunculaceae); this had hardly been investigated in previous studies (Van Bel and Hess, 2008). Although re-evaluation of a wide-spectrum of plant species is awaited to further address this issue, this new insight suggests that hexose can be regarded as phloem mobile transport sugar, equivalent to sucrose,

Fig. 28.3. (continued) *Malus x domestica* MdSUT4 (AY445915), *Manihot esculenta* MeSUT1 (DQ138374), MeSUT2 (DQ138373), MeSUT4 (DQ138371), *Nicotiana tabacum* NtSUT1 (X82276), NtSUT3 (AF149981), *Pisum sativum* PsSUT1 (AF109921), PsSUF1 (DQ221698), PsSUF4 (DQ221697), *Plantago major* PmSUC1 (X84379), PmSUC2 (X75764), PmSUC3 (AJ534442), *Phaseolus vulgaris* PvSUT1 (DQ221699), PvSUT3 (DQ221701), PvSUF1 (DQ221700), *Ricinus communis* RcSCR1 (Z31561), RcSUC4 (AY725473), *Solanum tuberosum* StSUT1 (X69165), StSUT2 (AY291289), StSUT4 (AF237780), *Spinacia oleracea* SoSUT1 (X67125), *Vicia faba* VfSUT1 (Z93774), *Vitis Vinifera* VvSUC11 (AF021808), VvSUC12 (AF021809), VvSUC27 (AF021810), VvSUT2 (AF439321); Monocot species, *Ananas comosus* AcSUT4 (EF460878), *Asparagus officinalis* AoSUT1 (DQ273271), *Bambusa oldhamii* BoSUT1 (DQ020217), *Hordeum vulgare* HvSUT1 (AJ272309), HvSUT2 (AJ272308), *Oryza sativa* OsSUT1 (X87819), OsSUT2 (AB091672), OsSUT3 (AB071809), OsSUT4 (AB091673), OsSUT5 (AB091674), *Saccharum* hybrid ShSUT1 (AY780256), *Triticum aestivum* TaSUT1A (AF408842), TaSUT1B (AF408843), TaSUT1D (AF408844), *Zea mays* ZmSUT1 (AB008464), ZmSUT2 (AY639018), ZmSUT4 (AY581895), As for maize SUTs, the TIGR Maize Genome Database (http://maize.tigr.org/) was searched, and seven SUT or SUT-like sequences were found and retrieved. For each gene, intron/exon borders could be predicted based on maize or rice SUT cDNA sequences lodged in the GenBank, and peptide sequences were deduced from the probable mRNAs; ZmSUT3 (accession number AZM4_91110), ZmSUT5 (AZM4_71084), ZmSUT6 (AZM4_12743), ZmSUT7 (AZM4_112588). In addition, six SUT-like sequences found in the genome of *Physcomitrella patent*, a non-vascular plant, were also included for comparison: PpSUT1 (XM_001752913), PpSUT2 (XM_001778945), PpSUT3 (XM_001777404), PpSUT4 (XM_001768246), PpSUT5 (XM_001766929), PpSUT6 (XM_001777602). Note that PpSUT1 and PpSUT2, which are closely related to the Type-II clade, possess a long central cytoplasmic loop similar to dicot SUT2 sequences (N. Aoki, unpublished observation).

RFOs, or sugar alcohols, and that a transport mechanism exists for accumulating hexose into sieve tubes.

Intriguingly, this current classification based on the combination of translocating sugars partially correlates with the mode of phloem loading or anatomy of minor vein phloem described above. The RFO-translocating species typically have type 1 minor vein structure, suggesting that RFOs favor symplasmic phloem loading. On the other hand, species with type 2 vein structure generally transport only sucrose, implying apoplasmic sucrose loading.

The hypothesis of symplasmic phloem loading in the RFO-translocating type 1 species became more compelling in light of the following physiological studies. In *Coleus blumei*, a typical RFO-translocating type 1 species, the concentration of solute was estimated to be much higher in SE/CC complexes than in surrounding parenchyma cells (Fisher, 1986), and accumulation of ^{14}C-photoassimilate into minor veins occurred apparently against the concentration gradient and was insensitive to PCMBS (Turgeon and Gowan, 1990). If this is the case, a simple question arises how a high concentration of photoassimilates is maintained in the SE/CC complex, irrespective of frequent plasmodesmatal connections which can facilitate diffusion of solutes toward the surrounding cells. A model to explain this, known as 'polymer trap theory', has been proposed by Turgeon (1991). Sucrose moves symplasmically to CCs where it is converted into RFOs which are too large in size to diffuse back through plasmodesmata toward surrounding parenchyma cells. Several lines of evidence support this hypothesis: RFO synthesis takes place in CCs (Beebe and Turgeon, 1992), and sugar concentrations in micro-dissected leaf tissues are consistent with diffusion of substrates for RFO synthesis (sucrose and galactinol) into CCs, but not with diffusion of RFOs toward the surrounding cells (Haritatos et al., 1996). Furthermore, the importance of RFO synthesis in phloem loading has been demonstrated recently, by RNAi suppression of genes for galactinol synthase (McCaskill and Turgeon, 2007). However, the size exclusion limit of the plasmodesmata connecting the CCs with the adjacent parenchyma cells is not known although it is a key factor of this hypothesis.

It should be noted that all the type 1 species do not necessarily transport RFOs. It has been suggested that transport of RFOs is restricted to the type 1 plants having CCs with a specialized anatomy known as intermediary cells, and other type 1 plants with ordinary CCs transport mostly sucrose (Turgeon and Medville, 2004). It has also been reported as an extreme case that a type 1 species willow translocates only sucrose, and, surprisingly, the sucrose seems to move simply following the concentration gradient from the mesophyll into the transport phloem, suggesting the absence of an active loading mechanism (Turgeon and Medville, 1998). In addition, the loading mechanism of sugar alcohols and the possible relationship with phloem anatomy still remains unclear (see Noiraud et al., 2001), however, genes encoding sugar alcohol transporters have been identified in some plant species (see Juchaux-Cachau et al., 2007; and references therein). In general, it is not possible to predict the mode of phloem loading and/or sugar transport simply from vein anatomy without more comprehensive studies of the physiological and anatomical heterogeneity of the SE/CC complex.

C. Sucrose Transporter as a Phloem Loader

Since the early 1970s, it has been inferred from the following observations that energy-dependent transport is necessary for the apoplasmic loading of sucrose into the SE/CC complex (reviewed in Giaquinta, 1983; Van Bel, 1993). First, as mentioned above, only a small number of sugars including sucrose are found in the phloem sap, implying selective uptake into the SE/CC complex. Second, sucrose concentrations in the phloem sap as high as 200 to 1,600 mM suggest an active transport of sucrose against the concentration gradient. Third, the activity of H^+-ATPase localized in the plasma membrane of SE/CC complex rises during the sink-to-source transition of developing leaves. Fourth, phloem loading of sucrose is markedly inhibited by the thiol group-modifying reagent PCMBS, implying the involvement of membrane protein(s) in the active transport of sucrose. Detailed biochemical studies using plasma membrane vesicles have reinforced this view, and researchers came to infer the presence of a sucrose/H^+ symporter on the plasma membrane of SE/CC complexes by the late 1980s

(see Bush, 1993; Van Bel, 1993). The first cDNA encoding a sucrose transporter protein was isolated from the source leaves of spinach and designated *SoSUT1*, S*pinacia* o*leracea* su*crose* t*ransporter 1* (Riesmeier et al., 1992). In this study a yeast complementation system was used: a yeast strain which cannot absorb sucrose from external media but can metabolize it within the cell was transformed with a spinach cDNA library and selected on the medium containing sucrose as the sole carbon source. The yeast cells expressing *SoSUT1* were shown to take up sucrose (and maltose) selectively from media with an apparent K_m for sucrose of 1.5 mM. The uptake activity is pH-dependent (higher activity in lower pH) and severely inhibited by either thiol modifying reagents or uncouplers. All these biochemical properties of SoSUT1 strongly suggest it to be a H^+-symporter and give striking agreement with the in planta properties of the sucrose/H^+ symporter that had been assumed to be involved in phloem loading. After *SoSUT1*, genes for SUTs have been isolated from many other plant species such as potato (Riesmeier et al., 1993), *A. thaliana* (Sauer and Stolz, 1994), common plantain (Gahrtz et al., 1994) and rice (Hirose et al., 1997), and most of them have been shown to have similar biochemical and structural characteristics.

The first attempt to localize the expression of SUT genes was carried out in the source leaves of potato by in situ hybridization and demonstrated that *StSUT1* mRNA is located in the phloem (Riesmeier et al., 1993). Similarly, the proteins of *A. thaliana* AtSUC2 (Sauer and Stolz, 1994) and common plantain PmSUC2 (Stadler et al., 1995) have been shown to exist in the plasma membrane of CCs by immunolocalization. In addition, antisense suppression of *StSUT1* led to decreased leaf photosynthesis and sugar export, accumulation of soluble sugars and starch in leaves and retardation in growth (Riesmeier et al., 1994; Kühn et al., 1996; Lemoine et al., 1996). Similar phenomena were also observed in the insertion mutant lines of *AtSUC2* (Gottwald et al., 2000) as well as antisense suppression lines of tobacco *NtSUT1* (Bürkle et al., 1998). All these data indicate that the SUTs have a crucial role in the apoplasmic phloem loading of sucrose in the source leaves.

However, it should be noted that phloem loading is not necessarily the sole function of SUTs (see Sections III and IV). Even in the case of a typical "phloem loader" described above the expression of the SUTs has been observed not only in source leaves but also in some sink tissues where sucrose is delivered from the phloem (see Section IV). At the same time, localization of a SUT to the SE/CC complex is not the hallmark of a phloem loader (see Section III.A). Whereas rice OsSUT1 was expressed in SE/CC (Matsukura et al., 2000; Scofield et al., 2007b), antisense suppression of OsSUT1 gave no phenotypic changes supporting its role in phloem loading (Ishimaru et al., 2001; Scofield et al., 2002).

D. Phloem Loading in Grass Species

Most of the discussion on phloem loading described above is based almost exclusively on data from dicot species. To date, our knowledge on the phloem anatomy and the loading mechanism of monocot species is still limited compared to those of dicot species. The following observations from cereal species support the likelihood that apoplasmic loading of sucrose occurs in these agriculturally important crops. (1) In rice, maize, wheat and barley, sucrose is the major sugar found in their phloem sap (Fukumorita and Chino, 1982; Hayashi and Chino, 1986; Ohshima et al., 1990; Winter et al., 1992). (2) In rice, maize and barley, leaf minor veins possess the type 2a anatomy, namely, the CCs are connected with the adjacent cells by only a few plasmodesmata (Evert et al., 1978, 1996; Kaneko et al., 1980; Botha and Cross, 1997). (3) In barley and wheat, symplasmic isolation of source leaf SE/CC complexes has been shown by fluorescent dye transport experiments (Haupt et al., 2001; Aoki et al., 2004). (4) In wheat and maize, assimilate transport from leaves is inhibited by PCMBS (Thompson and Dale, 1981).

As mentioned in Section I.C, involvement of SUTs in phloem loading remains unclear in monocot species. Only a little information is available on cellular localization and physiological function of SUTs in monocot leaves, while SUT mRNAs have been found in source leaves of rice (Hirose et al., 1997; Aoki et al., 2003), maize (Aoki et al., 1999), barley (Weschke et al., 2000), wheat (Aoki et al., 2002) and sugarcane (Rae et al., 2005). Involvement of SUT in assimilate transport in source leaves was previously suggested in maize as the level of *ZmSUT1*

mRNA increases during leaf maturation and with accumulation of carbohydrates in the mature leaf (Aoki et al., 1999). This hypothesis that ZmSUT1 functions in phloem loading in mature maize leaves, has recently been supported by characterizing a knockout mutant in this gene (Slewinski et al., 2009). In mature wheat leaf blades, TaSUT1 protein localizes to the SE of small and medium veins, implying its direct involvement in phloem loading (Aoki et al., 2004). On the other hand, in sugarcane leaves, ShSUT1 protein is not found in the phloem but in the surrounding vascular parenchyma cells and mestome sheath cells (Rae et al., 2005), suggesting its role in retrieval, or possibly release, of sucrose (see Sections II.E and III.A). Furthermore, unlike dicot Type-I SUTs, antisense repression of *OsSUT1* expression in transgenic rice does not appear to affect phloem loading (Ishimaru et al., 2001; Scofield et al., 2002). Although these monocot SUT1 orthologs all fall into the same cluster in the phylogenetic tree of plant SUTs (Fig. 28.3) and appear to possess a similar affinity for sucrose (Weschke et al., 2000; Rae et al., 2005; Carpaneto et al., 2005), their localization and role(s) in the phloem loading pathway may differ depending on species. Further studies are obviously needed in order to evaluate the contribution of SUT-mediated membrane transport to phloem loading in grass species, considering the expression and localization of different SUT isogenes. In fact, all five SUT isogenes appear to be expressed in rice source leaves (Aoki et al., 2003).

Interestingly, a maize mutant *sxd1* (*sucrose export defective1*) exhibits severely impaired growth and accumulates a large amount of starch in its leaves (Russin et al., 1996). This symptom has been attributed to occlusion of plasmodesmata at the bundle sheath/vascular parenchyma interface and the resultant blockage of assimilate export from the source leaves (Russin et al., 1996; Botha et al., 2000). Although neither are directly relevant to the SE/CC complex nor SUTs, this provides valuable information on the physiological role of plasmodesmata in the intercellular transport of assimilates.

Later on, the maize *SXD1* gene has been isolated (Provencher et al., 2001) and surprisingly found to be highly similar to an *A. thaliana* gene *VTE1*, which encodes a tocopherol cyclase, an enzyme essential for vitamin E synthesis (Porfirova et al., 2002). Subsequently, Sattler et al. (2003) have confirmed a tocopherol deficiency in maize *sxd1* leaves and tocopherol cyclase activity with recombinant SXD1 protein. However, an *A. thaliana* tocopherol-deficient line *vte1* having a mutation in *VTE1* did not show any sucrose export phenotype analogous to the maize *sxd1* (Porfirova et al., 2002). In contrast, RNAi-mediated silencing of an *SXD1* ortholog in potato led to a tocopherol deficiency in leaves and impaired photoassimilate export from leaves concomitant with vascular-specific callose deposition in source leaves (Hofius et al., 2004). It appears that the response of assimilate export phenotypes to a deficiency in tocopherol cyclase differs depending upon plant species.

Recently, another interesting maize mutant *tdy1* (*tie-dyed1*) was reported and characterized. The *tdy1* mutant plants develop variegated yellow and green leaf sectors and accumulate starch and sucrose in the yellow sectors (Braun et al., 2006). The starch accumulation of *tdy1* leaves seems to be attributable to impaired sucrose export without reduction of starch breakdown (Slewinski et al., 2008) but by different mechanisms from *sxd1* (Ma et al., 2008). Subsequently, *TDY1* gene was cloned and found that *TDY1* encodes a novel transmembrane protein and is exclusively expressed in phloem of both sink and source tissues (Ma et al., 2009). Fluorescent dye movement experiments revealed that source leaves of *tdy1* plants showed retarded phloem loading, but phloem transport and unloading in sink tissues were unaffected, suggesting that TDY1 functions in carbon partitioning by promoting phloem loading although a detailed mechanism remains unclear. Notably, TDY1 orthologous proteins are found only in grass species to date. This may be a clue to understand the mode and the regulation of phloem loading in grasses.

E. Releasing Sucrose to Phloem Apoplasm

One important issue that has remained unresolved in the apoplasmic phloem loading pathway is where and how sucrose is released to the apoplasm prior to its active uptake into SE/CC complexes. There is no clear answer to this question, although the importance of the apoplasmic sucrose pool in phloem loading has been clearly demonstrated

in vivo in transgenic solanaceous and *A. thaliana* plants expressing a yeast acid invertase in the cell wall (reviewed in Schobert et al., 2000). The overexpression of extracellular invertase led to a large accumulation of carbohydrates, a reduction of photoassimilate export and an inhibition of photosynthesis in the source leaves, resulting from cleavage of sucrose in the leaf apoplasm followed by retrieval of hexoses into mesophyll cells (see Schobert et al., 2000).

Based on physiological studies using leaf tissues and discs and structural observations on plasmodesmatal connectivity in leaf vascular bundles, phloem parenchyma cells (or bundle sheath cells in some cases) are considered to be the most probable site for the sucrose release (see Van Bel, 1993; Beebe and Russin, 1999). In some plant species, the minor vein phloem is surrounded by 'thick-walled' cells, whose cell walls are sometimes heavily lignified or suberized (e.g., bundle sheath cells in C_4 plants). These thickened cell walls surrounding the phloem may be a physical barrier to membrane transport between the two apoplasmic compartments, vascular phloem tissue and non-vascular mesophyll tissue (cf. Section III.B.2). If this is the case, photoassimilate has to move symplasmically via plasmodesmata, to reach phloem (parenchyma) cells. A series of reports on the maize *sxd1* mutant mentioned in Section II.D provides the most promising evidence for this hypothesis (see Schobert et al., 2000).

Mesophyll cells have been demonstrated to be able to release and take up sucrose in a number of studies (see Schobert et al., 2000). Whereas the mechanism of the release (or leakage) still remains unclear, the uptake has been suggested to occur via a H^+-coupled plasma membrane carrier protein (Bush, 1993; Schobert et al., 2000). This release/retrieval in mesophyll is considered to regulate the levels of sugars in the mesophyll apoplasm and/or to achieve an effective channeling to actual releasing cells in the phloem. However, localization of SUT to the plasma membrane of source leaf mesophyll cells has not been shown to date (but cf. Lemoine et al., 1989).

For sucrose release from the cytosol to the apoplasm, a carrier-mediated transport system has been suggested to exist in the plasma membrane of source leaves (see Van Bel, 1993; Schobert et al., 2000; Lalonde et al., 2004). This plasma membrane efflux protein is thought to localize to sucrose-releasing cells in the phloem, most likely in phloem parenchyma cells, and facilitate the export of sucrose from the cells to the phloem apoplasm by an unascertained mechanism, presumably H^+-antiport or uniport, considering acidic pH and low sucrose concentrations in the apoplasm. To date, however, no such efflux protein has been identified in plant cells. *A. thaliana* AtSUC3 and sugarcane ShSUT1 have been shown to localize to the cells adjacent to the phloem in leaves (Meyer et al., 2000; Rae et al., 2005), providing a possible mechanism for the release of sucrose toward the phloem if they were to function bi-directionally. This notion has gained support following the demonstration that the sucrose/H^+ symporter can mediate the efflux of sucrose in *Vicia faba* leaf discs (M'Batchi and Delrot, 1988), in tobacco leaf plasma membrane vesicles (Borstlap and Shuurmans, 2004), and in oocytes when heterologously expressed (Carpaneto et al., 2005). The existence of sucrose effluxers can also be inferred in the post-phloem unloading pathway in sink tissues such as developing seeds (see Section IV.C). In addition, it should be noted that vesicle-mediated transport might possibly be another mechanism for sucrose efflux to the apoplasm when the sucrose stored temporally in vacuoles is remobilized (see Echeverría, 2000). In this case, vacuolar sugar porters may play roles in transporting sucrose across the tonoplast membrane (see Echeverría, 2000; Lalonde et al., 2004). In fact, localization of SUT proteins to the tonoplast membranes of mesophyll cells has recently been reported for barley HvSUT2, *A. thaliana* AtSUC4 and *Lotus japonicus* LjSUT4, all of which are members of Type-III SUTs (Endler et al., 2006; Reinders et al., 2008). However, metabolic and physiological functions of these vacuolar sucrose/H^+ symporters remain unclear.

III. Long-Distance Transport (Translocation) of Sucrose

Following phloem loading in minor veins, sucrose is accumulated in major vein phloem through the leaf vein network, and then translocated to the other parts of plant. The phloem in major veins, petioles, stems and roots functions as pipes to

connect source leaves with terminal sinks such as root tips, shoot apices, flower tissues and developing seeds, and is often termed the 'transport phloem' (see Van Bel, 2003). At present it is generally accepted that not only loading and unloading control the mass flow of phloem sap, but that the transport phloem also plays a role. (see Willenbrink, 2002; Van Bel, 2003; Minchin and Lacointe, 2005)

A. Retrieval Role of Sucrose Transporters in Transport Phloem

As mentioned in Section I, sucrose is the major osmotically active molecule in the phloem sap of most plant species. However, as sieve tubes are essentially leaky pipes, sucrose and other solutes are readily lost from the phloem sap during translocation (reviewed in Van Bel, 2003). In this context, one can presume the necessity of sucrose-retrieving mechanism(s) in the SE/CC complex of transport phloem, in order to return sucrose leaked to the phloem apoplasm (Willenbrink, 2002; Van Bel, 2003).

In fact, Grimm et al. (1990) have reported that the efflux of sucrose in the petioles of *Cyclamen persicum* is compensated by carrier-mediated influx, suggesting the existence of sucrose/H^+ symporter in the phloem. Ayre et al. (2003) compared the distribution of endogenous translocating sugars (sucrose and/or RFOs) to that of exotic solutes (galactinol and octopine) loaded in leaf minor veins, using tobacco, which translocates predominantly sucrose, and *Coleus blumei*, an RFO-translocating species. While either the endogenous solutes or the exotic solutes were efficiently exported from the leaf lamina to the petioles through the sieve tubes, only the exotics were found to accumulate in substantial amount to the petiole apoplasm. From the results, the authors have proposed the existence of retention and retrieval mechanism(s) specific for endogenous translocating sugars in the transport phloem (Ayre et al., 2003).

Despite the fact that mRNAs of SUTs have been found in petioles, stems and/or roots in most plants examined, much less attention has been paid until recently to SUTs in the transport phloem than to those involved in loading and unloading. Localization of SUT proteins in the SE/CC of transport phloem has been reported in a number of plant species. In wheat, for example, TaSUT1 protein has been shown to localize to SEs along the transport phloem of leaf blades, leaf sheaths and peduncles (Aoki et al., 2003). Similar localization patterns of SUT protein in the phloem along the long distance transport pathway has been reported in rice for OsSUT1 (Scofield et al., 2007b). In tomato petioles and stems, the co-localization of three different SUT proteins in the same SE has been shown (Reinders et al., 2002). These three tomato SUTs, LeSUT1, SUT2 and SUT4, possess distinctive structures and show different affinities for sucrose in vitro, suggesting different physiological roles in the transport phloem (Riesmeier et al., 1993; Barker et al., 2000; Weise et al., 2000). It seems likely that the SE/CC complex of transport phloem deploys different SUTs in order to avoid critical losses of sucrose from the phloem sap and to maintain an efficient rate of translocation (Willenbrink, 2002; Van Bel, 2003; Ayre et al., 2003). SUT expression in 'non-loading' phloem has also been shown in the stem and tuber of potato (Kühn et al., 1997, 2003), in the petiole of celery, a mannitol-translocating species (Noiraud et al., 2000, 2001), and in the transport phloem of *Alonsoa meridionalis*, an RFO-translocating, symplasmic phloem loader (Knop et al., 2001, 2004). It is therefore reasonable to assume that 'retrieving apoplasmic sucrose', rather than 'phloem loading', is a more general role for the SUTs in the SE/CC among higher plants.

Moreover, studies using promoter-reporter constructs and specific antibodies have revealed that SUT genes are expressed in a variety of non-phloem cells in both source and sink tissues, including distal cells such as root tips, pollen grains, fiber cells, trichomes and guard cells (Meyer et al., 2000, 2004; Barth et al., 2003; Rae et al., 2005; also see Section IV) and more recently, vessel-associated cells in the xylem of walnut, a woody species (Decourteix et al., 2006). While one can speculate a role of SUT in 'utilizing extracellular sucrose', this may possibly extend the physiological significance of SUT genes beyond translocation and carbon partitioning. This possible diversity in physiological function of SUT may explain why higher plants possess a multi-gene family encoding sucrose/H^+ symporters.

B. Carbohydrate Storage in Stems

Petioles, stems and roots of the plant are also known to function as lateral sinks (or axial sinks) for storing assimilates, as well as pipes for assimilate translocation from source leaves to terminal sinks. Here we discuss the transport of sucrose between phloem and storage cells in stems, focusing on some agriculturally important grass species, in which the stem storage of assimilates has a significant influence on yield.

1. Stem Carbohydrate Reserves in Cereal Species

In some cereal crops, temporal storage of carbohydrate in the stem has been known to contribute substantially to final grain yield (see Cock and Yoshida, 1972; Schnyder, 1993). The stem of grass species comprises the leaf sheaths, the internodes and the peduncle, connecting the nodes. These stem parts save excess photoassimilates when photosynthetic activity is high in source leaves, and accumulate these carbon reserves in the form of polysaccharides such as starch (in rice) or fructan (in wheat and barley). In this 'accumulation' phase, the storage parenchyma cells synthesize and store polysaccharides (starch or fructan) from sucrose in particular organelles (plastid or vacuole, respectively). The stored polysaccharides are remobilized and translocated to filling grains later when the leaf photosynthesis declines due to senescence. In this 'remobilization' phase, the stored polysaccharides are degraded within the organelle, exported into the cytosol, and converted into sucrose. Not surprisingly, changes in carbohydrate metabolism enzymes during this sink-to-source transition in the stem of cereal species have been shown at the biochemical and molecular levels (Hirose et al., 1999, 2006; Wardlaw and Willenbrink, 2000; Van den Ende et al., 2003; Hirose and Terao, 2004; Takahashi et al., 2005; Scofield et al., 2009; and references therein). However, further studies are necessary to clarify the synthesis and degradation pathway of polysaccharides, particularly metabolite transport processes across organelle membranes in storage cells.

Apart from the changes in metabolic pathway within storage cells, intercellular transport of sucrose between phloem SEs and storage cells could also change during the sink-to-source transition; phloem unloading in the sink phase along a sucrose gradient, and reloading in the source phase, presumably against a sucrose gradient. However, little information is available to date on solute transport in mature cereal stems. In wheat internodes, unlike in stems of some dicots (reviewed in Van Bel, 1995), fluorescent dyes can move symplasmically from the phloem to the storage parenchyma, via plasmodesmata (Aoki et al., 2004). Further studies are required on intercellular connections via plasmodesmata, and on expression and localization of transporter proteins, as well as sucrose-related enzymes. Hirose et al. (1999) have reported that *OsSUT1* mRNAs appear to increase during the sink-to-source transition in rice leaf sheaths, in parallel with mRNAs for sucrose biosynthetic enzymes. This SUT may be involved in the transport path to reload sucrose into the phloem in the remobilization phase.

The sink-to-source transition in stems of cereal species, unlike that in developing leaves (see Section IV.A), occurs in the mature tissues that have already completed their development. Thus cereal stems are of particular use for studying the mechanism of this drastic change in carbon partitioning. It would be very interesting to investigate this phenomenon intensively from the perspectives of tissue and cellular anatomy, enzyme equipment for carbohydrate metabolism, symplasmic connections for solute transport, and/or transcript and protein profiles.

2. Sucrose Accumulation in Sugarcane Stems

Sugarcane, another grass species, accumulates a great quantity of sucrose in the internode and thus is the major source of commercial sugar. In maturing sugarcane internodes that are actively accumulating sucrose, it has been suggested that solutes can move symplasmically from the phloem to the storage parenchyma cells (Jacobsen et al., 1992; Rae et al., 2005). In the maturing internodes, cells at the periphery of the vascular bundle have been found to possess lignified and suberized walls, potentially forming a barrier to apoplasmic movement between phloem and storage cells, and these thick-walled cells employ a SUT protein, ShSUT1, presumably in a retrieval role in non-phloem cells (Rae et al., 2005). As well as the

symplasmic pathway of sucrose transport, an apoplasmic pathway has also been proposed where sucrose is released to the internode apoplasm and cleaved by a cell wall invertase, followed by uptake via a hexose transporter and synthesis of sucrose within the cell (Walsh et al., 1996). Moreover, Casu et al. (2003) have reported that maturing internodes express a number of putative sugar transporters, as well as known sucrose and hexose transporters.

IV. Import of Sucrose to Sink Tissues

A. Sink Leaves

It has been elegantly shown by Oparka et al. (1999) that in the young dicot sink leaves but not source leaves, functional symplasmic connections exist between the phloem SEs and the adjacent mesophyll cells. These experiments indicated that the plasmodesmatal connections between the phloem SEs and mesophyll tissues became branched and ceased to pass green fluorescent protein- (GFP-) tagged virus or fluorescent dyes following the developmental transition from sink to source status which occurs in a wave from the base of the leaf to the tip (Oparka et al., 1999). It is not clear how the structural changes in plasmodesmata relate to the gating of these channels or what signal transduction pathway is responsible for the closure of the plasmodesmata, however, it appears that there is a symplasmic pathway present for post-phloem delivery of sucrose to the sink cells of the young leaf. Experiments with tracer dyes (Haupt et al., 2001; Aoki et al., 2004) indicate that a similar symplasmic connection also exists in sink leaf tissue of cereals but not in mature source leaves. Patrick and Offler (1996) support the thesis that in both young leaves and roots, sucrose is delivered symplasmically. These authors point out that by comparing measured sugar transport rates between plant tissues (such as phloem sieve tube to companion cell and seed maternal tissue to filial tissue) it appears that two orders of magnitude higher fluxes are possible through plasmodesmatal connections as compared to apoplasmic active transport (Patrick and Offler, 1996). The existence of a symplasmic pathway for sugar import to source leaves seems at odds, however, with the observation that hexose transporters and cell wall invertase are expressed at high levels in young sink leaf tissue and in roots (reviewed in Roitsch and Tanner, 1996). The presence of these proteins suggests that sucrose could be delivered into the apoplasm from the phloem where it is cleaved by cell wall invertase then taken up actively into the sink cells by hexose transporters. Paradoxically, all 5 *OsSUT* genes in rice are also expressed in young sink leaves (Aoki et al., 2004). The presence of sucrose synthase and SUTs, invertase and hexose transporters and functional plasmodesmata in sink leaves makes it difficult to make a clear pronouncement as to the route of post-phloem carbon import to sink leaf tissues. A likely scenario, however, is that in a similar model to that described above for long-distance transport and storage in stem tissue, symplasmic import maybe the main route for sugar flux, with transporters acting in a retrieval mode to rescue sugars from the apoplasm. This model is supported by the observation that ectopic expression of invertase in the apoplasm of solanaceous species did not appear to affect early leaf development but had profound effects on source leaf gene expression, photosynthesis, carbohydrate composition and senescence (Sonnewald et al., 1991).

B. Floral Tissues

Sugar transport plays a pivotal role at three major stages of flowering and pollen development. Firstly, the developing pollen grain must be nourished with sugars from the maternal tissues of the anther (Dorion et al., 1996; Roitsch et al., 2003), secondly sugar transporters are thought to be important for anther dehiscence (Stadler et al., 1999) and lastly, sugars and SUTs are of pivotal importance in pollen tube growth during pollen germination (Stadler et al., 1999; Hackel et al., 2006; Sivitz et al., 2008). Recently, the pathway of sugar import to pollen grains during development has been studied intensively using a molecular/biochemical approach, driven by a desire to understand the processes underlying environmental stress effects on pollen fertility (Koonjul et al., 2005) and the physiological role of sugar transporters in pollen and anthers (Imlau et al., 1999; Stadler et al., 1999; Lemoine et al., 1999). A pivotal study by Imlau and co-workers has demonstrated the locations of the apoplasmic barriers to solute delivery during pollen development

(Imlau et al., 1999). These authors reported that in *A. thaliana* transformed with GFP driven by the *AtSUC2* promoter, GFP could traffic in the phloem and be unloaded symplasmically in sink tissues such as the anther. This evidence of symplasmic connections between phloem SEs and anther tissue is consistent with dye feeding experiments which suggest that the major barrier to symplasmic transport between the phloem and the pollen occurs at the level of the tapetum. These observations are also consistent with the effects of temperature and drought stress on pollen fertility in rice (Sheoran and Saini, 1996; Oliver et al., 2005) and wheat (Dorion et al., 1996; Koonjul et al., 2005). In cereals, under stress conditions, import of sugars to the pollen grain is impaired due to down regulation of a cell wall invertase and presumably a hexose transporter (Koonjul et al., 2005; Oliver et al., 2005). Sugars and starch build up in the anther and pollen is starved of sugars for starch formation, resulting in low fertility (Sheoran and Saini, 1996). Pollen fertility seems particularly sensitive to this stress around pollen microspore stage, i.e., at meiosis (Oliver et al., 2005). Interestingly, SUTs have also been reported to express in anther tissues of rice and in pollen (Takeda et al., 2001; T. Hirose, G.N. Scofield, N. Aoki and R.T. Furbank, unpublished observations). Whether a switch from the cell wall invertase / hexose transporter couple to the SUT / sucrose synthase sugar import system occurs when pollen develops (similar to that seen in developing seed, see Section IV.C), remains to be elucidated.

C. Developing Seeds

While there still may be controversy concerning the pathway of phloem loading in leaves, there is little doubt that post-phloem transport of sucrose into filial tissue of both monocot and dicot seeds must occur apoplasmically. In all higher plants, the filial and maternal tissues of seeds are separated by the plasma membrane of both tissues with no functional plasmodesmatal connections remaining post-fertilization (see Patrick and Offler, 1995).

1. Dicot Seeds

In dicots, there is strong support for the model that in early seed development, sucrose is delivered from the phloem to the inner seed coat where it passes by processes unknown to the apoplasm and is cleaved by cell wall invertase. Hexoses are subsequently imported to the embryo via hexose transporters (reviewed by Weber et al., 1997; summarized in Fig. 28.5). Following contact of the cotyledons of the embryo with the inner seed

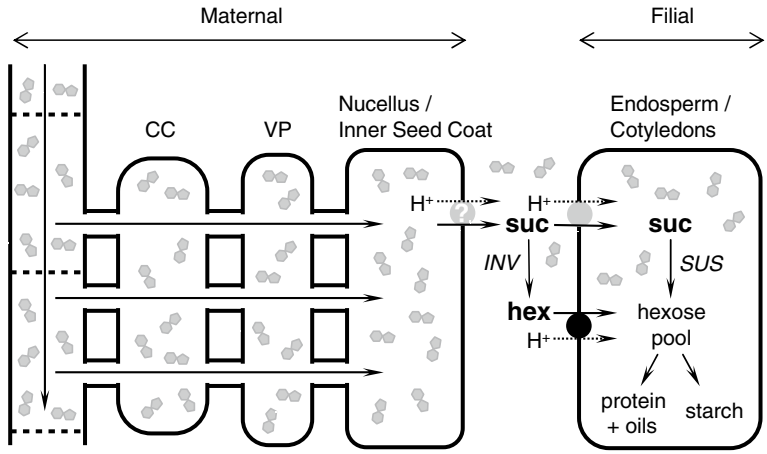

Fig. 28.5. Post-phloem transport of sucrose in developing seeds. Sucrose can move symplasmically from the SE/CC complex to the border cells of maternal tissue (nucellus or inner seed coat), but has to be released to the apoplasm prior to being taken up into filial tissues (endosperm or cotyledon). Sucrose/H^+ symporter expressed in the maternal border cells may be able to act as a sucrose effluxer. In the filial tissues, two different mechanisms have been proposed for the uptake of apoplasmic sucrose; (*1*) sucrose is cleaved by cell wall invertase (*INV*), followed by the uptake via hexose/H^+ symporter, and (*2*) sucrose is directly taken up via sucrose/H^+ symporter and metabolized via sucrose synthase (*SUS*) within the filial cells. Symbols and abbreviations are same as in Fig. 28.1.

coat, invertase and hexose transporter expression are markedly reduced and a SUT is highly expressed in the cotyledonary epidermis, coinciding with the high level expression of sucrose synthase within the cotyledon (reviewed in Weber et al., 1997; summarized in Fig. 28.5). While evidence for this developmental change in sugar import pathways has been generated from the model system *Vicia faba,* it appears from works in common plantain (Gahrtz et al., 1996), canola (King et al., 1997), cotton (Ruan et al., 2001), *A. thaliana* (Ruuska et al., 2002; Baud et al., 2005; Fallahi et al., 2008), that this may be an ubiquitous mechanism in dicot seeds. This transition in sugar import pathways is thought to herald the onset of storage product accumulation and the cessation of cell division. While the other (possibly hormonal) signaling compounds involved in this developmental switch remain unresolved, it has been proposed that sugar signaling, in particular the hexose to sucrose ratio, is pivotal in controlling this process (see Weber et al., 1997). Evidence for the importance of sugar signaling in the transition from cell division to cell expansion has also been reported from measurements of local sugar levels across sections of developing *V. faba* embryos (Borisjuk et al., 1998, 2002) where areas of active cell division correlated with high hexose to sucrose ratios. Also, in transgenic *Vicia narbonensis* seeds, expressing a yeast invertase targeted to the apoplasm, sugar levels were seriously affected and development of the embryo disrupted (Weber et al., 1998; Neubohn et al., 2000). In transgenic chickpeas harboring a similar gene construct, cell number was increased, starch and protein deposition were greatly reduced in high invertase expressing lines and germination was also disrupted, presumably by the persistence of this enzyme activity through desiccation and after rehydration (R.T. Furbank, P. Gremigni, D. Büssis and N. Turner, unpublished observations). It is interesting to note, however, that the developmental switch from hexose import to sucrose import which occurs in legumes in concert with the onset of storage phase may not be pertinent to oilseeds with a persistent endosperm such as tobacco (Tomlinson et al., 2004). In this case, 30-fold overexpression of an invertase in the embryo and seed coat apoplasm, with associated large changes in hexose levels, had no deleterious effects on seed filling (Tomlinson et al., 2004).

In the post-phloem sugar transport pathway of dicot seeds, the step which remains to be resolved is the mechanism of export of sucrose from the maternal seed coat. Sucrose is believed to move symplasmically from the phloem SEs to the cells of the inner seed coat. Sucrose must then move into the apoplasm surrounding the embryo and the inner seed coat cells to be cleared by cell wall invertase or to be actively taken up by the cotyledonary epidermal cells via SUTs (see Fig. 28.5). From the sucrose gradients between maternal tissues and the apoplasm calculated for *Phaseolus vulgaris* and *V. faba* (Patrick and Offler, 1995) simple diffusion could only support 40–50% of the flux in this transport step and carrier mediated efflux has been proposed (Patrick and Offler, 1995). A carrier mediated efflux is also supported by the inhibition of sucrose efflux from seed coats by the sulfhydryl inhibitor PCMBS and the sensitivity of this transport process to inhibition of respiration (reviewed in Patrick and Offler, 1995). Presumably, for sucrose export into the apoplasm, a sucrose/H^+ antiporter would be required. No such protein has been cloned or characterized to date. Intriguingly, SUT protein and RNA have been detected at high levels in the maternal seed coat tissues of a range of dicot seeds (in pea, Tegeder et al., 1999; Zhou et al., 2007; in *V. faba*, Weber et al., 1997; in *P. vulgaris*, Zhou et al., 2007; in common plantain, Lauterbach et al., 2007; and in canola, R.T. Furbank, unpublished observations). It has been postulated (see Patrick and Offler, 1995) that the sucrose/H^+ symporters could operate in the reverse direction as sucrose effluxers in seed coat transfer cells, either actively or as a passive pore. Recently this notion has gained more credence as the maize *ZmSUT1* gene product has been shown to be bidirectional when expressed in oocytes (Carpaneto et al., 2005). More recently, Zhou et al. (2007) discovered novel proteins being classified into either Type-I or Type-II SUTs in seed coats of developing seeds of pea and bean. When expressed in yeast, these SUT-like proteins exhibited kinetic and biochemical properties of pH-independent facilitator, and thus were named SUFs (sucrose facilitators). The SUFs are expressed in cells that are considered responsible for sucrose efflux from seed coats, suggesting that SUFs can play a role in releasing sucrose from seed coat cells by facilitated diffusion according to an outward-directed gradient of sucrose across the plasma membrane (Zhou et al., 2007).

2. Cereal Grains

In contrast to dicot seeds, sugar import to cereal grains received only limited attention until relatively recently. The proposed pathway of import of sugars to cereal endosperm is shown in Fig. 28.5. It has long been known that sugar import from the maternal nucellus tissue to the endosperm requires transport across the apoplasm and that uptake of sugars across the aleurone layer surrounding the endosperm is energy dependent (see Patrick and Offler, 1995). The sugar transporters responsible for this process, however, were not cloned and localized until the last decade. *OsSUT1*, the first cereal SUT cloned (see above and Hirose et al., 1997) has been shown to be highly expressed during grain filling and to be localized to the aleurone/subaleurone and nucellus tissue around the top of the grain, closer to the nucellar projection (Furbank et al., 2001). Antisense suppression of this gene caused carbon starvation of the endosperm and a shriveled grain phenotype (Scofield et al., 2002). Similar expression patterns have been found for *HvSUT1* in barley (Weschke et al., 2000), and for *TaSUT1* in wheat (Bagnall et al., 2000; Aoki et al., 2002).

Whether the proposed dicot model for a switch from an invertase / hexose uptake mechanism early in development to a sucrose uptake mechanism during starch accumulation holds for cereal seeds is still under investigation. It appears from measurements of transcript levels of cell wall invertases and hexose transporters during development of rice grains (Hirose et al., 2002) and barley grains (Weschke et al., 2003) that hexoses are indeed taken up by the endosperm early in grain development. Transcript profiling in barley however, shows that sucrose synthase is also important in early development for providing UDP-glucose required in cellularization of the endosperm (Sreenivasulu et al., 2004).

The pathway of sugar import to maize kernels is less well characterized than in the other cereals and may deviate from the model for rice, wheat and barley. Early in development, hexoses produced by the action of cell wall invertase in the maternal pedicel tissues are taken up by the endosperm basal transfer cells, presumably by a hexose transporter (see Patrick and Offler, 1995). This pathway of early sugar import is supported by analysis of the maize *miniature* mutant, a cell wall invertase knock out, which has smaller grains, lower hexose levels, lower cell numbers and reduced endosperm mitotic activity (Vilhar et al., 2002). Curiously, the maize *sh1* mutant, a sucrose synthase (SS1) knock out with obviously shrunken grain, shows only a mild reduction in starch content but early degradation of endosperm cell integrity (see Chourey et al., 1998; and references therein). The mutant *Sus1-1* (a mutation in sucrose synthase isoform SS2, believed to be responsible for starch biosynthesis), has no obvious phenotype while a double mutant in both isoforms has less than 0.5% of typical sucrose synthase activity in the endosperm (Chourey et al., 1998) but still synthesizes approximately 50% of normal levels of starch (Chourey et al., 1998). This observation implies that the invertase / hexose transport pathway may be active throughout maize kernel development and both sucrose itself and hexoses may be imported during starch biosynthesis.

As with developing dicot seeds, the post-phloem efflux of sucrose from maternal tissues is also somewhat of a mystery in cereal seeds. While the cells of the nucellus in wheat appear to be symplasmically connected and continuous with the phloem (see Patrick and Offler, 1995), the efflux of sucrose from the nucellar projection is sensitive to sulfhydryl inhibitors and uncouplers (Bagnall et al., 2000). Consistent with these observations, SUT1 transcript and protein are detected in the nucellus tissues of both wheat (Bagnall et al., 2000) and rice (Furbank et al., 2001). Once again, in the absence of the discovery of any candidate genes for sucrose effluxers, it is tempting to speculate a role for SUTs in the efflux of sugar from the maternal tissue into the filial apoplasm where it is taken up by the same protein located in the aleurone acting in uptake mode.

Besides carrier-mediated sugar transport, it has been reported recently that inhibitors for endocytosis have been shown to prevent the conversion of ^{14}C-sucrose into starch in developing barley endosperm, suggesting that vesicle-mediated transport of sucrose may contribute to apoplasmic transport of sucrose in grains during starch accumulation (Baroja-Fernandez et al., 2006). The presence of endocytic transport system for sucrose uptake into sink tissues has also been proposed in potato tubers and citrus fruits (Etxeberria et al., 2005; Baroja-Fernandez

et al., 2006). Further studies are required to evaluate whether sugar transport by endocytosis contributes in vivo to the uptake of translocated assimilates into sink cells.

D. "Fleshy" Fruits

The discussion above has focused primarily on the import of photosynthate to the embryo of dicot seeds and the endosperm of cereal seeds: the primary site of storage in many agriculturally important species. Also of interest is the pathway of sugar import to fruiting structures where maternal tissue is the major carbon storage organ. The systems most studied are tomato fruit, grape berry and cotton seed. In tomato fruit, there is a developmental transition from symplasmic continuity between storage parenchyma and phloem SEs early in fruit formation to symplasmic isolation and the expression of a hexose transporter and cell wall invertase in parenchyma tissue (Ruan and Patrick, 1995). This change in transport mechanism may coincide with the transition from starch storage to accumulation of high concentrations of hexoses in the fruit. In grape berry (Coombe, 1992), it appears that sucrose can move symplasmically from the phloem to the vascular parenchyma cells and is then delivered to the apoplasm where it is taken up by the mesocarp cells (see Patrick and Offler, 1996). Comprehensive developmental studies have not been carried out but it appears that this active uptake takes place via cell wall invertase coupled to hexose transporters in the mesocarp flesh cells (Davies and Robinson, 1996; Fillion et al., 1999). However, high-level expression of a SUT in the same tissue is also observed during berry ripening, suggesting that sucrose itself may also be actively transported (Ageorges et al., 2000).

Cotton fiber is an important example of a crop plant where the major storage tissue is maternal in origin. Cotton fiber initiates as a single cell from the ovule epidermis at flowering then elongates to a length of up to 3 cm in the space of 15 to 20 days after flowering (see Ruan et al., 2001). The major storage compound in cotton fiber is cellulose and mature fiber can be comprised of over 90% cellulose by dry weight. Apart from the importance of this fiber in textile production, it has been shown to be a valuable model for cell elongation and solute transport in rapidly elongating tissue (Ruan et al., 2000, 2001). Sugars move into the cotton fiber symplasmically from the seed coat during early development then during the rapid elongation phase, plasmodesmata joining the fiber cells to the underlying seed coat are gated, no longer passing solutes or the fluorescent dye 6-carboxyfluorescein (6-CF), from the seed coat vascular bundle to the fiber (Fig 28.5). Sucrose and potassium transporters are highly expressed at this stage, causing turgor to increase dramatically in the fibers, driving the rapid elongation phase (Ruan et al., 2001). Plasmodesmata then reopen at the end of elongation, followed by a phase of rapid cellulose deposition. The appearance of callose around the neck of the plasmodesmata correlates with this closure and the reopening correlates with the expression of a fiber-specific β-glucanase, thought to degrade the callose constriction of the pore (Ruan et al., 2004, 2005). This is an important example of how the symplasmic pathway of solute transport can be restricted and then resumed during development (see Oparka and Roberts, 2001; Roberts and Oparka, 2003).

E. Roots and Tubers

Sugar import to roots and tubers has been extensively studied in the species potato and sugar beet which store substantial carbon reserves in below ground tissues (see Patrick and Offler, 1995; Bell and Leigh, 1996). Viola et al. (2001) reported evidence that the pathway of post-phloem transport of sugars into the stolon of potato changed with development. These authors observed an apoplasmic step early in stolon swelling and expansion but symplasmic connectivity with the phloem SEs later, during starch deposition (Viola et al., 2001). Localization of cell wall invertase expression suggests that this enzyme may be important early in development and in the apical bud region of the tuber. These observations may explain why overexpression of a yeast invertase in the apoplasm of potato increased tuber size (Sonnewald et al., 1997) if developmental expression patterns of this enzyme are important in determining cell number through sugar signaling early in stolon development and in termination of apical growth.

Post-phloem movement of sucrose into root tissues of non-tuber forming species has been

studied in less detail, but elegant experiments using laser confocal microscopy and the phloem mobile fluorescent dye, 6-CF have shown that in root tips, a symplasmic domain extends from the phloem SEs to the meristematic tissue (Oparka et al., 1994, 1995).

F. Germinating Seeds

Seed germination is an interesting system for the study of sugar transport as the machinery which was involved in carbon storage during seed development becomes source tissue to fuel shoot and root growth in the next generation. The process of seed germination has been extensively studied in cereals from the focus of starch remobilization and hormonal control of gene expression (Bewley, 1997). Until recently, however, little was known about the pathway of sugar movement from the cereal endosperm to the shoot and root or from embryo storage tissues to the embryonic axis and growing tissues of the dicot germinating seed. Relevant to the apoplasmic transport pathway, SUT transcripts had been detected in phloem tissues of castor bean (Bick et al., 1998) and rice (Matsukura et al., 2000), suggesting that sucrose is synthesized from storage reserves and translocated in the phloem of seedling shoots. The role of non-phloem tissues in sucrose transport during seed germination is less certain. In germinating castor bean seeds, epidermal transfer cells of cotyledons abutting the endosperm contain abundant SUT mRNAs (Bick et al., 1998). However, the function of these SUTs in sugar retrieval from the endosperm is unclear as the starch hydrolysis products are generally known to be glucose and maltose. In cereal species, rice *OsSUT1* (Hirose et al., 1997; Matsukura et al., 2000), wheat *TaSUT1* (Aoki et al., 2002) and maize *ZmSUT1* (Aoki et al., 1999) have been found to be highly expressed in germinating seeds. In rice, *OsSUT1* appeared to be expressed dominantly in germinating seeds compared with the other four SUT isogenes (Aoki et al., 2003), and anti-sense suppression lines for *OsSUT1* showed retarded germination (Scofield et al., 2002). These results strongly suggest that expression of the SUT1 ortholog is very important for seed germination in cereal species. However, cellular localization of SUT1 expression in germinating cereal seeds has not been fully investigated. In rice, the expression of *OsSUT1* gene and the localization of OsSUT1 protein in the scutellum phloem of germinating seeds have been reported (Matsukura et al., 2000; Scofield et al., 2007a). Recent evidence from 6-CF feeding suggests that in wheat, the major barrier to symplasmic movement of sugars from the endosperm to the shoot and root is the scutellar epidermis (Aoki et al., 2006). A single symplasmic domain is then present from the scutellar ground cells to the phloem, shoot and root. TaSUT1 is expressed in the scutellar epidermis and ground cells, in addition to the protophloem/phloem SEs. There is good evidence that sucrose is synthesized in the scutellum from glucose released from starch degradation in the endosperm (Edelman et al., 1959). A model of sugar transport during cereal seed germination is shown in Fig. 28.6. Glucose or maltose are transported actively across the scutellar epidermis, then sucrose synthesized in the scutellum can move either symplasmically or apoplasmically (via SUT) to the phloem tissues. Unloading in the root and shoot is assumed to be symplasmic. It is intriguing that *TaSUT1* is expressed in the scutellum epithelial cells which are in contact predominantly with glucose and maltose during the germination process (Aoki et al., 2006). In many species, SUTs will also support maltose transport (see Lemoine, 2000), raising the possibility that SUT proteins are also responsible for maltose uptake into the embryo from the endosperm.

V. Sucrose Transporters as Sucrose Sensors?

There has been a great deal of controversy over the potential "dual function" of SUTs as sugar sensors and sugar transporters (Chiou and Bush, 1998; Lalonde et al., 1999; Barker et al., 2000; Schulze et al., 2000; Barth et al., 2003). Claims that SUTs could act as sucrose sensors gained impetus with the cloning of the dicot SUT2 type transporters, located in SEs of tomato, *A. thaliana* and common plantain (Barker et al., 2000; Schulze et al., 2000; Barth et al., 2003). These SUTs are characterized by extended cytoplasmic domains which show some similarity to the SNF3 and RGT2 yeast sugar sensors (Barker et al., 2000). The solanaceous SUT2 proteins were capable of little or no sucrose transport

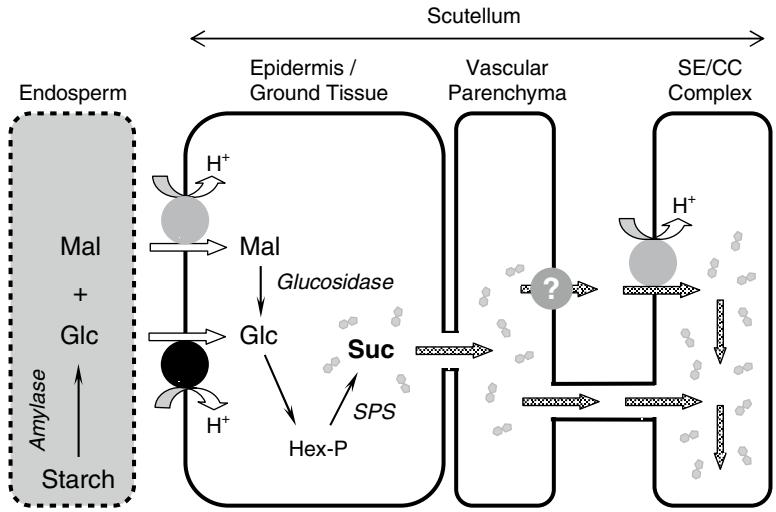

Fig. 28.6. Schematic model of the cellular route of transport in the scutellum of germinating cereal seeds, highlighting potential location of sugar transporters and symplasmic transport. The products of starch degradation in the endosperm, glucose (*Glc*) and maltose (*Mal*), are taken up into scutellum via sugar porters, and then converted into sucrose in scutellar tissues. A hexose/H^+ symporter and a sucrose/H^+ symporter, located in the plasma membrane of scutellar epidermis and ground cells, may be engaged for the uptake of glucose and maltose, respectively. Sucrose (*Suc*) synthesized can be loaded into SE/CC complex either symplasmically or apoplasmically, for translocation to shoot and root apices. In the scutellar vascular bundles, a sucrose/H^+ symporter localizes to SE/CC complex and may play a role in the apoplasmic loading of sucrose, or retrieving sucrose leaked to phloem apoplasm. Symbols are same as in Fig. 28.1. SPS, sucrose-phosphate synthase; Hex-P, hexose phosphate.

after heterologous expression in yeast (Barker et al., 2000; Reinders et al., 2002). These observations, while rather circumstantial in nature, were later supported by evidence that SUT2 co-localized with SUT1 and SUT4 in phloem SEs in potato and SUT1, 4 and 2 show some ability to form homo-oligomers and interact with each other in an in vitro system (Reinders et al., 2002). Doubt has been cast on these conclusions, however, as in common plantain, not only did the SUT2 homolog PmSUC3 not co-localize with the putative phloem loading sucrose porter PmSUC2, but carried out high rates of sucrose transport in yeast cells (Barth et al., 2003). Whether the Solanaceae are unique or data have been subject to overenthusiastic interpretation is yet to be determined.

VI. Concluding Remarks

Sucrose transport in higher plants has been and remains to be a "hot" area of research in plant biology, characterized by a great deal of controversy and debate. Perhaps a more interesting way of dealing with concluding comments from this chapter is to frame some questions, answers and unresolved conundrums. A few are set out below.

How is the phloem loaded? Apoplasmically by SUTs in the easily transformed dicot species but which class of SUT is responsible is still unclear. Loading mechanisms in graminaceous species are still unresolved and the relative role of plasmodesmata in this process remains a mystery. Why are SUTs so ubiquitous throughout plant tissues, both source and sink, which both export and import sucrose, when SUTs are traditionally regarded as sucrose importers? How is the phloem unloaded? Once again, there is often a clear symplasmic pathway in many sink tissues but SUTs are still expressed in these tissues and presumably can function in retrieval to keep sucrose "on track" and out of the apoplasm. How is sucrose released to the apoplasm, via SUTs or specific effluxers? What does the tonoplast sucrose importer/exporter look like? Do vesicle-mediated transport systems, namely exo- and endocytosis, contribute to apoplasmic transport of sucrose in vivo? Do SUTs really form homo- and hetero-oligomeric complexes in vivo and can they act as sensors analogous to the yeast glucose sensors? Why do so many *A. thaliana* sugar transport

knock-outs (including the putative sucrose sensor mutants) show no phenotype and potentially a high level of redundancy? How do higher plants control SUTs and the bulk-flow of phloem sap?

Rarely has an area of research progressed so quickly from a molecular viewpoint but thrown up so many challenges to our understanding of such an important process in higher plants.

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References

Ageorges A, Issaly N, Picaud S, Delrot S and Romieu C (2000) Identification and functional expression in yeast of a grape berry sucrose carrier. Plant Physiol Biochem 38: 177–185

Aoki N, Hirose T, Takahashi S, Ono K, Ishimaru K and Ohsugi R (1999) Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (*Zea mays* L.). Plant Cell Physiol 40: 1072–1078

Aoki N, Whitfeld P, Hoeren F, Scofield G, Newell K, Patrick J, Offler C, Clarke B, Rahman S and Furbank RT (2002) Three sucrose transporter genes are expressed in the developing grain of hexaploid wheat. Plant Mol Biol 50: 453–462

Aoki N, Hirose T, Scofield GN, Whitfeld PR and Furbank RT (2003) The sucrose transporter gene family in rice. Plant Cell Physiol 44: 223–232

Aoki N, Scofield GN, Wang X-D, Patrick JW, Offler CE and Furbank RT (2004) Expression and localization analysis of the wheat sucrose transporter *TaSUT1* in vegetative tissues. Planta 219: 176–184

Aoki N, Scofield GN, Wang, X-D Offler, CE, Patrick JW and Furbank RT (2006) Expression studies indicate sugar transporters play key roles in germinating wheat seeds. Plant Physiol 141: 1255–1263

Ayre BG, Keller F and Turgeon R (2003) Symplastic continuity between companion cells and the translocation stream: long-distance transport is controlled by retention and retrieval mechanisms in the phloem. Plant Physiol 131: 1518–1528

Bagnall N, Wang X-D, Scofield GN, Furbank RT, Offler CE and Patrick JW (2000) Sucrose transport-related genes are expressed in both maternal and filial tissues of developing wheat grains. Aust J Plant Physiol 27: 1009–1020

Barker L, Kühn C, Weise A, Schulz A, Gebhardt C, Hirner B, Hellmann H, Schulze W, Ward JM and Frommer WB (2000) SUT2, a putative sucrose sensor in sieve elements. Plant Cell 12: 1153–1164

Baroja-Fernandez E, Etxeberria E, Muñoz FJ, Morán-Zorzano MT, Alonso-Casajús N, Gonzalez P and Pozueta-Romero J (2006) An important pool of sucrose linked to starch biosynthesis is taken up by endocytosis in heterotrophic cells. Plant Cell Physiol 47: 447–456

Barth I, Meyer S and Sauer N (2003) PmSUC3: characterization of a SUT2/SUC3-type sucrose transporter from *Plantago major*. Plant Cell 15: 1375–1385

Baud S, Wuillème S, Lemoine R, Kronenberger J, Caboche M, Lepiniec L and Rochat C (2005) The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis. Plant J 43: 824–836

Beebe DU and Russin WA (1999) Plasmodesmata in the phloem-loading pathway. In: Van Bel AJE and Van Kesteren WJP (eds) Plasmodesmata: Structure, Function, Role in Cell Communication, pp 261–294. Springer-Verlag, Berlin

Beebe DU and Turgeon R (1992) Localization of galactinol, raffinose, and stachyose synthesis in *Cucrbita pepo* leaves. Planta 188: 354–361

Bell CI and Leigh RA (1996) Differential effects of turgor on sucrose and potassium transport at the tonoplast and plasma membrane of sugarbeet storage root tissue. Plant Cell Environ 19: 191–200

Bewley (1997) Seed germination and dormancy. Plant Cell 9: 1055–1066

Bick J-A, Neelam A, Smith E, Nelson SJ, Hall JL and Williams LE (1998) Expression analysis of a sucrose carrier in the germinating seedling of *Ricinus communis*. Plant Mol Biol 38: 425–435

Boorer KJ, Loo DDF, Frommer WB and Wright EM (1996) Transport mechanism of the cloned potato H^+/sucrose cotransporter StSUT1. J Biol Chem 271: 25139–25144

Borisjuk L, Walenta S, Weber H, Muller-Klieser W and Wobus U (1998) High-resolution histographical mapping of glucose concentrations in developing cotyledons of *Vicia faba* in relation to mitotic activity and storage processes: glucose as a possible developmental trigger. Plant J 15: 583–591

Borisjuk L, Walenta S, Rolletschek H, Muller-Klieser W, Wobus U and Weber H (2002) Spatial analysis of plant metabolism: sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns. Plant J 29: 521–530

Borstlap AC and Shuurmans JAMJ (2004) Sucrose transport into plasma membrane vesicles from tobacco leaves by H^+ symport or counter exchange does not display a linear component. J Membrane Biol 198: 31–42

Botha CEJ and Cross RHM (1997) Plasmodesmatal frequency in relation to short-distance transport and phloem loading in

leaves of barley (*Hordeum vulgare*). Phloem is not loaded directly from the symplast. Physiol Plant 99: 355–362

Botha CEJ, Cross RHM, Van Bel AJE and Peter CI (2000) Phloem loading in the sucrose-export-defective (*SXD-1*) mutant maize is limited by callose deposition at plasmodesmata in bundle sheath-vascular parenchyma interface. Protoplasma 214: 65–72

Braun DM and Slewinski TL (2009) Genetic control of carbon partitioning in grasses: roles of *sucrose transporters* and *tie-dyed* loci in phloem loading. Plant Physiol 149: 71–81

Braun DM, Ma Y, Inada N, Muszynski MG and Baker RF (2006) tie-dyed1 regulates carbohydrate accumulation in maize leaves. Plant Physiol 142: 1511–1522

Bürkle L, Hibberd JM, Quick WPK, Kühn C, Hirner B and Frommer WB (1998) The H^+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. Plant Physiol 118: 59–68

Bush DR (1993) Proton-coupled sugar and amino acid transporters in plants. Annu Rev Plant Physiol Plant Mol Biol 44: 513–542

Carpaneto A, Geiger D, Bamberg E, Sauer N, Fromm J and Hedrich R (2005) Phloem-localized, proton-coupled sucrose carrier ZmSUT1 mediates sucrose efflux under control of sucrose gradient and pmf. J Biol Chem 280: 21437–21443

Casu RE, Grof CPL, Rae AL, McIntyre CL, Dimmock CM and Manners JM (2003) Identification of a novel sugar transporter homologue strongly expressed in mature stem vascular tissues of sugarcane by expressed sequence tag and microarray analysis. Plant Mol Biol 52: 371–386

Chiou TJ and Bush DR (1998) Sucrose is a signal molecule in assimilate partitioning. Proc Natl Acad Sci USA 95: 4784–4788

Chourey PS, Taliercio EW, Carlson SJ and Ruan YL (1998) Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. Mol Gen Genet 259: 88–96

Cock JH and Yoshida S (1972) Accumulation of ^{14}C-labelled carbohydrate before flowering and its subsequent redistribution and respiration in the rice plant. Proc Crop Sci Soc Jpn 41: 226–234

Coombe BG (1992) Research on development and ripening of the grape berry. Am J Enol Vitic 43: 101–110

Davies C and Robinson SP (1996) Sugar accumulation in grape berries: cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissue. Plant Physiol 111: 275–283

Decourteix M, Alves G, Brunel N, Améglio T, Guilliot A, Lemoine R, Pétel G and Sakr (2006) *JrSUT1*, a putative xylem sucrose transporter, could mediate sucrose influx into xylem parenchyma cells and be up-regulated by freeze-thaw cycles over the autumn-winter period in walnut tree (*Juglans regia* L.). Plant Cell Environ 29: 36–47

Dorion S, Lalonde S and Saini HS (1996) Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol 111: 137–145

Echeverría E (2000) Vesicle-mediated solute transport between the vacuole and the plasma membrane. Plant Physiol 123: 1217–1226

Edelman J, Shibko SI and Keys AJ (1959) The role of the scutellum of cereal seedlings in the synthesis and transport of sucrose. J Exp Bot 10: 178–189

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E and Schmidt UG (2006) Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. Plant Physiol 141: 196–207

Etxeberria E, Baroja-Fernandez E, Muños FJ and Pozueta-Romero J (2005) Sucrose-inducible endocytosis as a mechanism for nutrient uptake in heterotrophic plant cell. Plant Cell Physiol 46: 474–481

Evert RF, Eschrich W and Heyser W (1978) Leaf structure in relation to solute transport and phloem loading in *Zea mays* L. Planta 138: 279–294

Evert RF, Russin WA and Botha CEJ (1996) Distribution and frequency of plasmodesmata in relation to photoassimilate pathways and phloem loading in the barley leaf. Planta 198: 572–579

Fallahi H, Scofield GN, Badger MR, Chow WS, Furbank RT and Ruan YL (2008) Localization of sucrose synthase in developing seed and siliques of Arabidopsis thaliana reveals diverse roles for SUS during development. J Ex Bot 59: 3283–3295

Fillion L, Ageorges A, Picaud S, Coutos-Thévenot P, Lemoine R, Romieu C and Derlot S (1999) Cloning and expression of a hexose transporter gene expressed during the ripening of grape berry. Plant Physiol 120: 1083–1093

Fisher DG (1986) Ultrastructure, plasmodesmatal frequency and solute concentration in green areas of variegated *Coleus blumei* Benth. leaves. Planta 169: 141–152

Fukumorita T and Chino M (1982) Sugar, amino acid and inorganic contents in rice phloem sap. Plant Cell Physiol 23: 273–283

Furbank RT, Scofield GN, Hirose T, Wang X-D, Patrick JW and Offler CE (2001) Cellular localisation and function of a sucrose transporter *OsSUT1* in developing rice grains. Aust J Plant Physiol 28: 1187–1196

Gahrtz M, Stolz J and Sauer N (1994) A phloem specific sucrose-H^+ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. Plant J 6: 697–706

Gahrtz M, Schmelzer E, Stolz J and Sauer N (1996) Expression of the *PmSUC1* sucrose carrier gene from *Plantago major* L. is induced during seed development. Plant J 9: 93–100

Gamalei YV (1989) Structure and function of leaf minor veins in trees and herbs. A taxonomic review. Trees 3: 96–110

Gamalei YV (1991) Phloem loading and its development related to plant evolution from trees to herbs. Trees 5: 50–64

Giaquinta RT (1983) Phloem loading of sucrose. Ann Rev Plant Physiol 34: 347–387

Gottwald JR, Krysan PJ, Young JC, Evert RF and Sussman MR (2000) Genetic evidence for the *in planta* role of phloem-specific plasma membrane sucrose transporters. Proc Natl Acad Sci USA 97: 13979–13984

Grimm E, Bernhardt G, Rothe K and Jacob F (1990) Mechanism of sucrose retrieval along the phloem path: a kinetic approach. Planta 182: 480–485

Hackel A, Schauer N, Carrari F, Fernie AR, Grimm B and Kühn C (2006) Sucrose transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in different ways. Plant J 45: 180–192

Haupt S, Duncan GH, Holzberg S and Oparka KJ (2001) Evidence for symplastic phloem unloading in sink leaves of barley. Plant Physiol 125: 209–218

Haritatos E, Keller F and Turgeon R (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. Planta 198: 614–622

Hayashi H and Chino M (1986) Collection of pure phloem sap from wheat and its chemical composition. Plant Cell Physiol 27: 1387–1393

Hirose T and Terao T (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). Planta 220: 9–16

Hirose T, Imaizumi N, Scofield GN, Furbank RT and Ohsugi R (1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.). Plant Cell Physiol 38: 1389–1396

Hirose T, Endler A and Ohsugi R (1999) Gene expression of enzymes for starch and sucrose metabolism and transport in leaf sheaths of rice (*Oryza sativa* L.) during the heading period in relation to the sink to source transition. Plant Prod Sci 2: 178–183

Hirose T, Takano M and Terao T (2002) Cell wall invertase in developing rice caryopsis: molecular cloning of *OsCIN1* and analysis of its expression in relation to its role in grain filling. Plant Cell Physiol 43: 452–459

Hirose T, Ohdan T, Nakamura Y, Terao T (2006) Expression profiling of genes related to starch synthesis in rice leaf sheaths during the heading period. Physiol Plant 128: 425–435

Hofius D, Hajirezaei M-R, Geiger M, Tschiersch H and Melzer M (2004) RNAi-mediated tocopherol deficiency impairs photoassimilate export in transgenic potato plants. Plant Physiol 135: 1256–1268

Imlau A, Truernit E and Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell 11: 309–322

Ishimaru K, Hirose T, Aoki N, Takahashi S, Ono K, Yamamoto S, Wu J, Saji S, Baba T, Ugaki M, Matsumoto T and Ohsugi R (2001) Antisense expression of a rice sucrose transporter OsSUT1 in rice (*Oryza sativa* L.). Plant Cell Physiol 42: 1181–1185

Jacobsen KR, Fisher DG, Maretzki A and Moore PH (1992) Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. Bot Acta 105: 70–80

Juchaux-Cachau M, Landouar-Arsivaud L, Pichaut J-P, Campion C, Porcheron B, Jeauffre J, Noiraud-Romy N, Simoneau P, Maurousset L and Lemoine R (2007) Characterization of AgMaT2, a plasma membrane mannitol transporter from celery, expressing in phloem cells, including phloem parenchyma cells. Plant Physiol 145: 62–74

Kaneko M, Chonan N and Matsuda T (1980) Ultrastructure of the small vascular bundles and transfer pathways for photosynthate in the leaves of rice plant. Japan J Crop Sci 49: 42–50 (In Japanese with English summary)

King SP, Lunn JE and Furbank RT (1997) Carbohydrate content and enzyme metabolism in developing canola siliques. Plant Physiol 114: 153–160

Knop C, Voitsekhovskaja O and Lohaus G (2001) Sucrose transporters in two members of the Scrophulariaceae with different types of transport sugar. Planta 213: 80–91

Knop C, Stadler R, Sauer N and Lohaus G (2004) AmSUT1, a sucrose transporter in collection and transport phloem of the putative symplastic phloem loader *Alonsoa meridionalis*. Plant Physiol 134: 204–214

Koonjul PK, Minhas JS, Nunes C, Sheoran IS and Saini HS (2005) Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat. J Exp Bot 56: 179–190

Krügel U, Veenhoff LM, Langbin J, Wiederhold E, Liesche J, Friedrich T, Grimm B, Martinoia E, Poolman B and Kühn C (2008) Transport and sorting of the *Solanum tuberosum* sucrose transporter SUT1 is affected by posttranscriptional modification. Plant Cell 20: 2497–2513

Kühn C (2003) A comparison of the sucrose trnasporter systems of different plant species. Plant Biol 5: 215–232

Kühn C, Quick WP, Schulz A Riesmeier JW, Sonnewald U and Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. Plant Cell Environ 19: 1115–1123

Kühn C, Franceschi VR, Schulz A, Lemoine R and Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science 275: 1298–1300

Kühn C, Hajirezaei M-R, Fernie AR, Roessner-Tunali U, Czechowski T, Hirner B and Frommer WB (2003) The sucrose transporter *StSUT1* localizes to sieve elements in potato tuber phloem and influences tuber physiology and development. Plant Physiol 131: 102–113

Lalonde S, Boles E, Hellmann H, Barker L, Patrick JW, Frommer WB and Ward JM (1999) A dual function of sugar carriers in transport and sugar sensing. Plant Cell 11: 707–726

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB and Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant Cell Environ 26: 37–56

Lalonde S, Wipf D and Frommer WB (2004) Transport mechanism for organic forms of carbon and nitrogen between source and sink. Annu Rev Plant Biol 55: 341–372

Lauterbach C, Niedermeier M, Besenbeck R, Stadler R and Sauer N (2007) Immunolocalization of the PmSUC1 sucrose transporter in *Plantago major* flowers and reporter-gene analyses of the *PmSUC1* promoter suggest a role in sucrose release from the inner integument. Plant Biol 9: 357–365

Lemoine R (2000) Sucrose transporters in plants: update on function and structure. Biochim Biophys Acta 1465: 246–262

Lemoine R, Delrot S, Gallet O and Larsson C (1989) The sucrose carrier of the plant plasma membrane. II. Immunological characterization. Biochim Biophys Acta 978: 65–71

Lemoine R, Kühn C, Thiele N, Delrot S and Frommer WB (1996) Antisense inhibition of the sucrose transporter in potato: effects on amount and activity. Plant Cell Environ 19: 1124–1131

Frommer WB (1999) Identification of a pollen-specific sucrose transporter-like protein NtSUT3 from tobacco. FEBS Lett 454: 325–330

Lu JM-Y and Bush DR (1998) His-65 in the proton--sucrose symporter is an essential amino acid whose modification with site-directed mutagenesis increase transport activity. Proc Natl Acad Sci USA 95: 9025–9030

Ludwig A, Stolz J and Sauer N (2000) Plant sucrose-H^+ symporters mediate the transport of vitamin H. Plant J 24: 503–509

Ma Y, Baker F, Magallanes-Lundback M, DellaPenna D and Braun DM (2008) *Tie-dyed1* and *Sucrose export defevtive1* act independently to promote carbohydrate export from maize leaves. Planta 227: 527–538

Ma Y, Slewinski TL, Baker RF and Braun DM (2009) *Tie-dyed1* encodes a novel phloem-expressed transmembrane protein that functions in carbohydrate partitioning. Plant Physiol 149: 181–194

Marger MD and Saier MH Jr (1993) A major superfamily of trans-membrane facilitators that catalyze uniport, symport and antiport. Trends Biochem Sci 18: 13–20

Matsukura C, Saitoh T, Hirose T, Ohsugi R, Perata P and Yamaguchi J (2000) Sugar uptake and transport in rice embryo. Expression of companion cell-specific sucrose transporter (*OsSUT1*) induced by sugar and light. Plant Physiol 124: 85–93

M'Batchi B and Delrot S (1988) Stimulation of sugar exit from leaf tissues of *Vicia faba* L. Planta 174: 340–348

McCaskill A and Turgeon R (2007) Phloem loading in Verbascum phoeniceum L. depends on the synthesis of raffinose-family oligosaccharides. Proc Natl Acad Sci USA 104: 19619–19624

Meyer S, Melzer M, Truernit E, Hümmer C, Besenbeck, Stadler R and Sauer N (2000) *AtSUC3*, a gene encoding a new *Arabidopsis* sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. Plant J 24: 869–882

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD and Sauer N (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from Arabidopsis sieve elements and sink tissues. Plant Physiol 134: 684–693

Minchin PEH and Lacointe A (2005) New understanding on phloem physiology and possible consequences for modeling long-distance carbon transport. New Phytol 166: 771–779

Neubohn B, Gubatz S, Wobus U and Weber H (2000) Sugar levels altered by ectopic expression of a yeast-derived invertase affect cellular differentiation of developing cotyledons of *Vicia narbonensis* L. Planta 211: 325–334

Noiraud N, Delrot S and Lemoine R (2000) The sucrose transporter of celery. Identification and expression during salt stress. Plant Physiol 122: 1447–1455

Noiraud N, Maurousset L and Lemoine R (2001) Transport of polyols in higher plants. Plant Physiol Biochem 39: 717–728

Ohshima T, Hayashi H and Chino M (1990) Collection and chemical composition of pure phloem sap from *Zea mays* L.. Plant Cell Physiol 31: 735–737

Oliver SN, Van Dongen J, Alfred SC, Mamun EA, Zhao X, Saini HS, Fernandes SF, Blanchard CL, Sutton BG, Geigenberger P, Dennis ES and Dolferus R (2005) Cold-induced repression of the rice anther-specific cell wall invertase gene *OsINV4* is correlated with sucrose accumulation and pollen sterility. Plant Cell Environ 28:1534–1541

Oparka KJ and Roberts AG (2001) Plasmodesmata: a not so open-and-shut case. Plant Physiol 125: 123–126

Oparka KJ, Duckett CM, Prior DAM and Fisher DB (1994) Real-time imaging of phloem unloading in the root tip of *Arabidopsis*. Plant J 6: 759–766

Oparka, K.J., Prior, D.A.M. and Wright, K.M. (1995) Symplastic communication between primary and developing lateral roots of *Arabidopsis thaliana*. J Exp Bot 46: 187–198

Oparka KJ, Roberts AG, Boevink P, Santa Cruz S, Roberts I, Pradel KS, Imlau A, Kotlizky G, Sauer N and Epel B (1999) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. Cell 97: 743–754

Patrick JW and Offler CE (1995) Post-sieve element transport of sucrose in developing seeds. Aust J Plant Physiol 22: 681–702

Patrick JW and Offler CE (1996) Post-sieve element transport of photoassimilate in sink regions. J Exp Bot 47: 1165–1177

Porfirova S, Bergmüller E, Tropf S, Lemke R and Dörmann P (2002) Isolation of an *Arabidopsis* mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis. Proc Natl Acad Sci USA 99: 12495–12500

Provencher LM, Miao L, Sinha N and Lucas WJ (2001) *Sucrose export defective1* encodes a novel protein implicated in chloroplast-to-nucleus signaling. Plant Cell 13: 1127–1141

Rae AL, Perroux JM and Grof CPL (2005) Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. Planta 220: 817–825

Reinders A, Schulze W, Kuhn C, Barker L, Schulz A, Ward JM and Frommer WB (2002) Protein-protein interactions between sucrose transporters of different affinities colocalized in the same enucleate sieve element. Plant Cell 14: 1567–1577

Reinders A, Sivitz AB, His A, Grof CP, Perroux JM and Ward JM (2006) Sugarcane ShSUT1: analysis of sucrose transport activity and inhibition by sucralose. Plant Cell Environ 29: 1871–1880

Reinders A, Sivitz A, Starker CG, Gantt JS and Ward JM (2008) Functional analysis of LjSUT4, a vacuolar sucrose transporter from *Lotus japonicus*. Plant Mol Biol 68: 289–299

Riesmeier JW, Willmitzer L and Frommer WB (1992) Isolation and characterization of sucrose carrier cDNA from spinach by functional expression in yeast. EMBO J 11: 4705–4713

Riesmeier JW, Hirner B and Frommer WB (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. Plant Cell 5: 1591–1598

Riesmeier JW, Willmitzer L and Frommer WB (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J 13: 1–7

Roberts AG and Oparka KJ (2003) Plasmodesmata and the control of symplastic transport. Plant Cell Environ 26: 103–124

Roblin G, Sakr S, Bonmort J and Delrot S (1998) Regulation of a plasma membrane sucrose transporter by phosphorylation. FEBS Lett 424: 165–168

Roitsch T and Tanner W (1996) Cell wall invertase: bridging the gap. Bot Acta 109: 90–93

Roitsch T, Balibrea ME, Hofmann M, Proels R and Sinha AK (2003) Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 54: 513–524

Ruan Y-L and Patrick JW (1995) The cellular pathway of postphloem sugar transport in developing tomato fruit. Planta 196: 434–444

Ruan Y-L, Llewellyn DJ and Furbank RT (2000) Pathway and control of sucrose import into initiating cotton fibre cells. Aust J Plant Physiol 27: 795–800

Ruan Y-L, Llewellyn DJ and Furbank RT (2001) The control of single-celled cotton fibre elongation by developmentally reversible gating of plasmodesmata and coordinated expression of sucrose and K^+ transporters and expansin. Plant Cell 13: 47–60

Ruan Y-L, Xu S-M, White R and Furbank RT (2004) Genotypic and developmental evidence for the role of plasmodesmatal regulation in cotton fiber elongation mediated by callose turnover. Plant Physiol 136: 4104–4113

Ruan YL, Llewellyn DJ, Furbank RT and Chourey PS (2005) The delayed initiation and slow elongation of fuzz-like short fibre cells in relation to altered pattern of sucrose synthase expression and plasmodesmata gating in a lintless mutant of cotton, J Ex Bot 56: 977–984

Russin WA, Evert RF, Vanderveer PJ, Sharkey TD and Briggs SP (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the *sucrose export defective1* maize mutant. Plant Cell 8: 645–658

Ruuska SA, Girke T, Benning C and Ohlrogge JB (2002) Contrapuntal networks of gene expression during Arabidopsis seed filling. Plant Cell 14: 1191–1206

Saier MH Jr (2000) Families of transmembrane sugar transport proteins. Mol Microbiol 35: 699–710

Sattler SE, Cahoon EB, Coughlan SJ and DellaPenna D (2003) Characterization of tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for tocopherol synthesis and function. Plant Physiol 132: 2184–2195

Sauer N (2007) Molecular physiology of higher plant sucrose transporter. FEBS Lett. 581: 2309–2317

Sauer N and Stolz J (1994) SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. Plant J 6: 67–77

Sauer N, Ludwig A, Knoblauch A, Rothe P, Gahrtz M and Klebl F (2004) *AtSUC8* and *AtSUC9* encode functional sucrose transporters, but the closely related *AtSUC6* and *AtSUC7* genes encode aberrant proteins in different *Arabidopsis* ecotypes. Plant J 40: 120–130

Schnyder H (1993) The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling - a review. New Phytol 123: 233–245

Schobert C, Lucas WJ, Franceschi VR and Frommer WB (2000) Intercellular transport and phloem loading of sucrose, oligosaccharides and amino acids. In: Leegood RC, Sharkey TD and von Caemmerer S (eds) Photosynthesis: Physiology and Metabolism, Advances in Photosynthesis, Vol 9, pp 249–274. Kluwer Academic Publishers, Dordrecht

Schulze W, Weise A, Frommer WB and Ward JM (2000) Function of the cytosolic N-terminus of sucrose transporter AtSUT2 in substrate affinity. FEBS Lett 485: 189–194

Schulze WX, Reinders A, Ward J, Lalonde S and Frommer WB (2003) Interactions between co-expressed Arabidopsis

sucrose transporters in the split-ubiquitin system. BMC Biochem 4: 3

Scofield GN, Hirose T, Gaudron JA, Upadhyaya NM, Ohsugi R and Furbank RT (2002) Antisense suppression of the rice sucrose transporter gene, *OsSUT1*, leads to impaired grain filling and germination but does not affect photosynthesis. Funct Plant Biol 29: 815–826

Scofield GN, Aoki N, Hirose T, Takano M, Jenkins CLD and Furbank RT (2007a) The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants. J Exp Bot 58: 483–495

Scofield GN, Hirose T, Aoki N and Furbank RT (2007b) Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. J Exp Bot 58: 3155–3169

Scofield GN, Ruuska SA, Aoki N, Lewis DC, Tabe LM and Jenkins CLD (2009) Starch storage in the stems of wheat plants: localization and temporal changes. Ann Bot 103: 859–868

Sheoran IS and Saini HS (1996) Drought-induced male sterility in rice: changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. Sex Plant Reprod 9: 161–169

Sivitz AB, Reinders A and Ward JM (2005) Analysis of the transport activity of barley sucrose transporter HvSUT1. Plant Cell Physiol 46: 1666–1673

Sivitz AB, Reinders A and Ward JM (2008) Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose induced anthocyanin accumulation. Plant Physiol 147: 92–100

Slewinski TL, Meeley R and Braun DM (2009) Sucrose transpoter1 functions in phloem loading in maize leaves. J Exp Bot 60: 881–892

Slewinski TL, Ma Y, Baker F, Huang M, Meeley R and Braun DM (2008) Determining the role of *Tie-dyed1* in starch metabolism: epistasis analysis with maize ADP-Glucose pyrophosphorylase mutant lacking leaf starch. J Hered 99: 661–666

Sonnewald U, Brauer M, von Schaewen A, Stitt M and Willmitzer L (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. Plant J 1: 95–106

Sonnewald U, Hajirezaei MR, Kossmann J, Heyer A, Trethewey RN and Willmitzer L (1997) Increased potato tuber size resulting from expression of a yeast invertase. Nat Biotechnol 15: 794–797

Sreenivasulu N, Altschmied L, Radchuk V, Gubatz S, Wobus U and Weschke W (2004) Transcript profiles and deduced changes of metabolic pathways in maternal and filial tissues of developing barley grains. Plant J 37: 539–553

Stadler R, Brandner J, Schulz A, Gahrtz M and Sauer N (1995) Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. Plant Cell 7: 1545–1554

Stadler R, Truernit E, Gahrtz M and Sauer N (1999) The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in *Arabodopsis*. Plant J 19: 269–278

Takahashi S, Ishimaru K, Yazaki J, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T, Kishimoto N and Kikuchi S (2005) Microarray analysis of sink-source transition in rice leaf sheaths. Breed Sci 55: 153–162

Takeda T, Toyofuku K, Matsukura C and Yamaguchi J (2001) Sugar transporters involved in flowering and grain development of rice. J Plant Physiol 158: 465–470

Tegeder M, Wang X-D, Frommer WB, Offler CE and Patrick JW (1999) Sucrose transport into developing seeds of *Pisum sativum* L. Plant J 18: 151–161

Thompson RG and Dale JE (1981) Export of ^{14}C- and ^{11}C-labelled assimilate from wheat and maize leaves: effects of parachloromercuribenzylsulphonic acid and fusicoccin and of potassium deficiency. Can J Bot 59: 2439–2444

Thorpe MR and Minchin PEH (1996) Mechanism of long and short distance transport from sources to sinks. In: Zamski E and Schaffer AA (eds) Photoassimilate Distribution in Plants and Crops: Source and Sink Relations, pp 261–282. Marcel Dekker, New York

Tomlinson KL, McHugh S, Labbe H, Grainger JL, James LE, Pomeroy KM, Mullin JW, Miller SS, Dennis DT and Miki BL (2004) Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: analysis of tobacco seed development and effects of overexpressing apoplastic invertase. J Exp Bot 55: 2291–2303

Turgeon R (1991) Symplastic phloem loading and the sink-source transition in leaves: A model. In: Bonnemain J-L, Delrot S, Lucas WJ and Dainty J (eds) Recent Advances in Phloem Transport and Assimilate Compartmentation, pp 18–22. Ouest Editions, Nantes

Turgeon R and Gowan E (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from apoplast. Plant Physiol 94: 1244–1249

Turgeon R and Medville (1998) The absence of phloem loading in willow leaves. Proc Natl Acad Sci USA 95: 12055–12060

Turgeon R and Medville R (2004) Phloem loading. A reevaluation of the relationship between plasmodesmatal frequencies and loading strategies. Plant Physiol 136: 3795–3803

Turgeon R and Wolf S (2008) Phloem transport: cellular pathway and molecular trafficking. Ann Rev Plant Biol 60: 207–221

Van Bel AJE (1993) Strategies of phloem loading. Ann Rev Plant Physiol Plant Mol Biol 44: 253–281

Van Bel AJE (1995) The low-profile directors of carbon and nitrogen economy in plants: parenchyma cells associated with translocation channels. In: Gartner BL (ed) Plant Stems: Physiology and Functional Morphology, pp 205–222. Academic Press, San Diego

Van Bel AJE (2003) The phloem, a miracle of ingenuity. Plant Cell Environ 26: 125–149

Van Bel AJE and Hess PH (2008) Hexoses as phloem transport sugars: the end of dogma? J Exp Bot 59: 261–272

Van Bel AJE, Gamalei YV, Ammerlaan A and Bik LPM (1992) Dissimilar phloem loading in leaves with symplasmic or apoplasmic minor-vein configurations. Planta 186: 518–525

Van Bel AJE, Ammerlaan A and Van Dijk AA (1994) A three-step screening procedure to identify the mode of phloem loading in intact leaves. Planta 192: 31–39

Van den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A Yoshida M and Kawakami A (2003) Fructan 1-exohydrolases. β-(2,1)-Trimmers during graminan biosynthesis in stems of wheat? Purification, characterization, mass mapping and cloning of two fructan 1-exohydrolase isoforms. Plant Physiol 131: 621–631

Vilhar B, Kladnik A, Blejec A, Chourey PS and Dermastia M (2002) Cytometrical evidence that the loss of seed weight in the *miniature1* seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. Plant Physiol 129: 23–30

Viola R, Roberts AG, Haupt S, Gazzani S, Hancock RD, Marmiroli N, Marchray N and Oparka KJ (2001) Tuberization in potato involves a switch from apoplastic to symplastic phloem unloading. Plant Cell 13: 385–398

Walsh KB, Sky C and Brown SM (1996) Pathway of sucrose unloading from the phloem in sugarcane stalk. In: Wilson JR, Hogarth DM, Campbell JA and Garside AL (eds) Sugarcane: Research Towards Efficient and Sustainable Production, pp 105–107. CSIRO Division of Tropical Crops and Pastures, Brisbane

Wardlaw IF and Willenbrink J (2000) Mobilization of fructan reserves and changes in enzyme activities in wheat stems correlate with water stress during kernel filling. New Phytol 148: 413–422

Weber H, Borisjuk L, Sauer N and Wobus U (1997) A role for sucrose transporters during seed development: molecular characterization of a hexose and a sucrose carrier in Faba bean seeds. Plant Cell 9: 895–908

Weber H, Heim U, Golombek S, Borisjuk L, Manteuffel R and Wobus U (1998) Expression of a yeast-derived invertase in developing cotyledons of *Vicia narbonensis* alters the carbohydrate state and affects storage functions. Plant J 16: 163–172

Weise A, Barker L, Kühn C, Lalonde S, Buschmann H, Frommer WB and Ward JM (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. Plant Cell 12: 1345–1355

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H and Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. Plant J 21: 455–467

Weschke W, Panitz R, Gubatz S, Wang Q, Radchuk R, Weber H and Wobus U (2003) The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J 33: 395–411

Willenbrink J (2002) Assimilate transport in phloem: regulation and mechanism. Russ J Plant Physiol 49: 8–15

Williams LE, Lemoine R and Sauer N (2000) Sugar transporters in higher plants - a diversity of roles and complex regulation. Trends Plant Sci 5: 283–290

Winter H, Lohaus G and Heldt HW (1992) Phloem transport of amino acids in relation to cytosolic levels in barley leaves. Plant Physiol 99: 996–1004

Zhou Y, Qu H, Dibley KE, Offler CE and Patrick JW (2007) A suite of sucrose transporters expressed in coats of developing legume seeds includes novel pH-independent facilitators. Plant J 49: 750–764

Zimmermann MH and Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH and Milburn JA (eds) Encyclopedia of Plant Physiology, New Series Vol 1, Transport in Plants 1, Phloem Transport, pp 480–503. Springer, New York

Part VI

Climate Change and Photosynthesis

Chapter 29

Photosynthesis in a CO_2-Rich Atmosphere

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Summary

The concentration of CO_2 ([CO_2]) in the atmosphere is projected to exceed 550 ppm by 2050. C_3 plants respond directly to growth at elevated [CO_2] by stimulation of photosynthesis and reduced stomatal conductance. The stimulation of photosynthesis is the result of increased velocity of carboxylation of CO_2 by Rubisco and inhibition of the competing oxygenation reaction. Long-term exposure of C_3 plants to elevated [CO_2] can also lead to photosynthetic acclimation in which allocation of resources to components of the photosynthetic machinery, including Rubisco, is altered to optimize metabolic efficiency. The decrease in stomatal conductance that occurs in all plants at elevated [CO_2] can reduce canopy water use and indirectly enhance carbon gain by ameliorating drought stress. However, canopy micrometeorology constrains reductions in water use at the whole-plant level compared to the leaf level. C_4 photosynthesis is not directly stimulated by free-air concentration enrichment (FACE) of CO_2 in the field. However, reduced water use can indirectly enhance carbon gain by ameliorating stress in times and places of drought. There are commonalities and important distinctions between plant responses to growth at elevated [CO_2] under FACE versus controlled environment chambers. In FACE experiments: (1) the enhancement of photosynthesis and productivity by elevated [CO_2] is sustained over time; (2) the decrease in carboxylation capacity and leaf N characteristic of photosynthetic acclimation to elevated [CO_2] is consistent with an optimization of metabolic efficiency rather than a general down-regulation of metabolism, and (3) the enhancement effect of elevated [CO_2] is greatest for photosynthesis, intermediate for biomass accumulation, and lowest for crop yield. Plant responses to elevated [CO_2] have the potential to influence the global carbon cycle and climate in the future, but the complexity of scaling from the leaf to whole plant,

Abbreviations: [CO_2] – CO_2 concentration; A – Rate of photosynthetic CO_2 assimilation; A' – Rate of daily photosynthetic CO_2 assimilation; A_c' – Rate of daily canopy photosynthetic CO_2 assimilation; A/c_i – Photosynthetic CO_2-response curve; A_{sat} – Light-saturated rate of photosynthetic CO_2 assimilation; c_a – Atmospheric CO_2 concentration; c_i – Intercellular CO_2 concentration of leaf; c_i/c_a – Ratio of intercellular to atmospheric CO_2 concentration; E – Leaf transpiration; ET – Evapotranspiration; FACE – Free air concentration enrichment; g_s – Stomatal conductance; g_m – Mesophyll conductance; GPP – Gross primary productivity; J – Rate of electron transport; J_{max} – Maximum apparent electron transport capacity; K_m – Michaelis-Menton constant; k^c_{cat} – Catalytic turnover number of RuBP carboxylation by Rubisco; l – Stomatal limitation to photosynthesis; LAI – Leaf area index; N – Nitrogen; NPP – Net primary productivity; OTC – Open top chamber; PEPc – phosphoenolpyruvate carboxylase; PPFD – Photosynthetic photon flux density; RH – Relative humidity; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – Ribulose 1,5-bisphosphate; T – Temperature; TPU – Triose phosphate utilization; V_{cmax} – Maximum apparent carboxylation capacity of Rubisco; τ – Rubisco specificity for CO_2 relative to O_2

canopy, ecosystem and biosphere make it unclear to what extent this will be realized. Elevated [CO_2] will probably offset some of the future losses in crop yield caused by increased temperature and drought stress, but not to the extent previously thought. Expanding FACE experimentation to consider multiple elements of global change across a wider geographic range and more ecosystem types should be a priority if we are to minimize the problems, and maximize the benefits, of climate change impacts on ecosystem good and services.

I. Introduction

The Intergovernmental Panel on Climate Change Third Assessment Report summarized data on atmospheric CO_2 concentrations ([CO_2]) in the past and projections of atmospheric [CO_2] for the twenty-first century (Fig. 29.1; Prentice et al., 2001; Watson et al., 2001). Geochemical evidence suggests the atmospheric [CO_2] today is higher than at any time during the past 20 million years (Pagani et al., 1999; Pearson and Palmer, 2000). Air bubbles trapped in Antarctic ice over the last millennia contained a relatively stable [CO_2] of ~280 ppm until roughly 1800, at which time an increasingly rapid rise in [CO_2] began (Fig. 29.1; Siegenthaler et al., 1988; Neftel et al., 1994; Barnola et al., 1995; Etheridge et al., 1996). Direct measurements of atmospheric [CO_2] at Mauna Loa and the South Pole began in 1958. Average annual concentrations increased from ~316 ppm in 1958 to ~384 ppm in 2007 (http://cdiac.esd.ornl.gov/ftp/trends/co2/maunaloa.co2). The rise in [CO_2] is projected to continue over this century, although the magnitude of the predicted increase varies among different socio-economic scenarios and different models of global carbon cycling (Fig. 29.1; Prentice et al., 2001; Watson et al., 2001). Scenarios A1 and A2 in Fig. 29.1

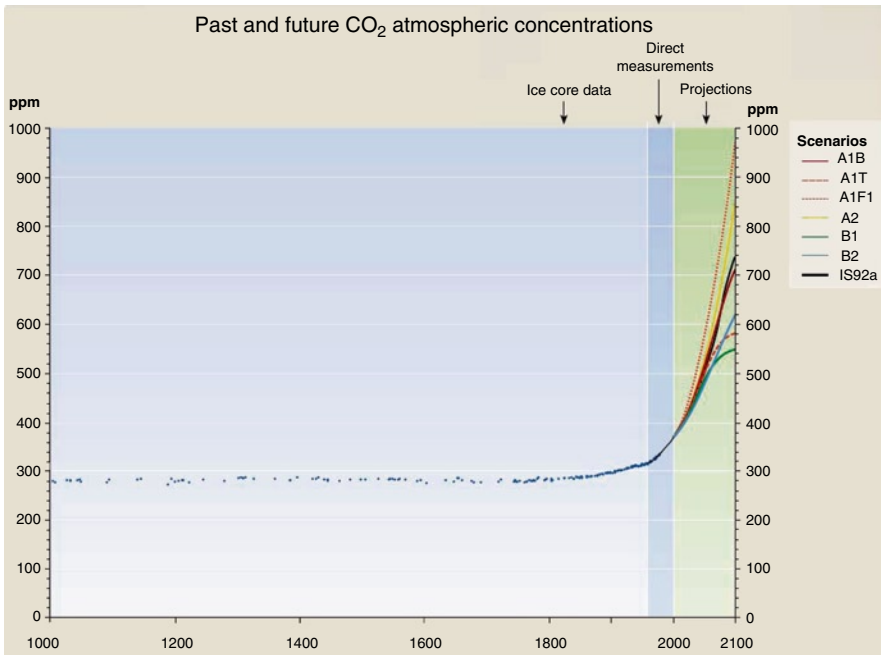

Fig. 29.1. Atmospheric [CO_2] from year 1000 to 2100. Data from 1000 to 1960 are from ice core measurements. Data from 1960 to 2000 are from direct atmospheric measurements. Data from 2000 to 2100 are projections based on the six illustrative SRES scenarios and IS92a from the Second Assessment Report of the Intergovernmental Panel on Climate Change. This figure was reproduced with permission of the Intergovernmental Panel on Climate Change from Figure SPM-10a of the Climate Change 2001: Synthesis Report, Summary for Policymakers by Watson et al. (2001).

represent conditions with a bias towards economic development. This generally leads to higher future [CO_2] than scenarios B1 and B2, which have a bias towards environmentally sensitive development. Development is more globally coordinated in scenarios A1 and B1, which leads to lower [CO_2] than in scenarios A2 and B2 where regional forces exert a stronger influence on development. Heavy dependence on fossil fuels (A1FI) results in [CO_2] of 970 ppm in 2100, versus 581 ppm when alternative energy sources predominate (A1T). The midpoint of the [CO_2] projected by the Integrated Science Assessment Model for 2050 across this range of scenarios is ~550 ppm. By 2100, [CO_2] is projected to be between 549 and 970 ppm. Subsequently many experiments on plant responses to elevated [CO_2] have used 550 or 700 ppm in treatments simulating conditions at the middle to end of this century. While these elevated [CO_2] have been used relatively consistently, the rapid increase in atmospheric [CO_2] in recent decades has caused the [CO_2] in control treatments to increase from ~330 ppm in the 1970s to ~384 ppm in 2007.

The effects of elevated [CO_2] at the middle to end of this century on plant physiology and productivity have been the subject of considerable research during the last 35 years. Most is known about the response of C_3 plants to growth at elevated [CO_2], reflecting the commonality of this photosynthetic pathway and its sensitivity to [CO_2]. Growth at elevated [CO_2] has only two direct effects on C_3 plant physiology and these are the subject of the second section in this chapter.

The first direct effect is on the primary carboxylating enzyme in C_3 photosynthesis, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). Elevated [CO_2] stimulates photosynthesis by (1) increasing the velocity of the carboxylation reaction of Rubisco and (2) reducing CO_2 loss through photorespiration by decreasing the velocity of the competing oxygenation reaction of Rubisco (Fig. 29.2). In addition to these instantaneous effects of [CO_2] on Rubisco kinetics, long-term growth at elevated [CO_2] can lead to photosynthetic acclimation in which the allocation of resources to components of the photosynthetic machinery, including Rubisco, alters to improve metabolic

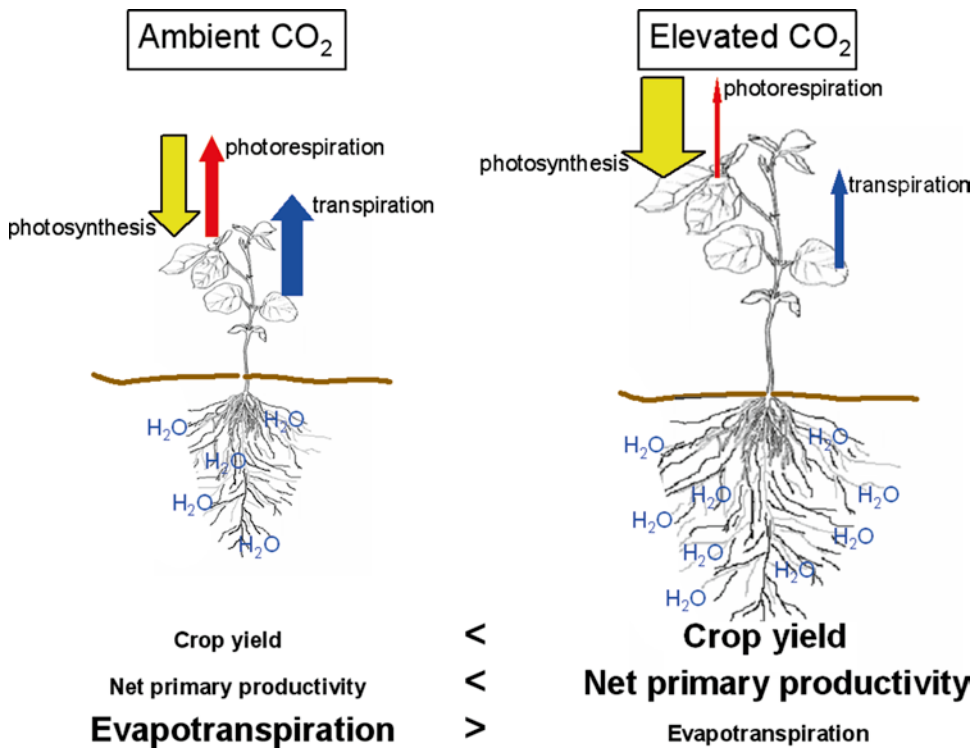

Fig. 29.2. Scheme summarizing the main effects of growth at elevated [CO_2] on C_3 plants.

efficiency at the higher [CO_2]. The second direct effect of elevated [CO_2] on C_3 plants is to reduce stomatal conductance (g_s; Fig. 29.2). Decreased g_s of individual leaves at elevated [CO_2] can integrate across the whole plant to reduce water use and soil moisture depletion, improve water use efficiency while increasing canopy temperatures (Fig. 29.2). This can have complex feedback effects on carbon gain and water relations at elevated [CO_2]. The combination of stimulated photosynthesis and reduced g_s at elevated [CO_2] has significant implications for managed and natural ecosystems as it can result in greater crop yields, greater net primary productivity and reduced evapotranspiration (*ET*) (Fig. 29.2).

The third section of this chapter discusses the effect of growth at elevated [CO_2] on C_4 species. C_4 plants make up <1% of known plant species but are disproportionately important since they include a number of the world's major crops and weeds. C_4 grasslands also contribute ~25–30% of global terrestrial productivity (Gillon and Yakir, 2001). While they have received less attention than C_3 species, three large-scale, free air concentration enrichment (FACE) experiments have recently investigated sorghum, wild grasses, and maize growing at elevated [CO_2]. Elevated [CO_2] does not directly stimulate photosynthesis in C_4 plants, a feature distinguishing them from the C_3 functional group. However, in common with C_3 species, g_s is typically lower at elevated [CO_2] in C_4 species. Reduced g_s can decrease canopy water use and ameliorate water stress. Therefore, in times and places of drought, elevated [CO_2] has the potential to enhance C_4 photosynthesis indirectly.

The range of technology used to fumigate plants with elevated [CO_2] has expanded over the last 35 years from complete enclosure in glasshouses or controlled environment chambers to open-top chambers and FACE experiments. This represents a continuum from pot-based experiments under very controlled, but artificial, micrometeorological conditions to field-based studies of plants with unrestricted rooting that experience real-world conditions of light, temperature, rainfall, humidity, wind, pollutants, pests and diseases. The fourth section of this chapter uses meta-analytic techniques to objectively summarize the results of experimentation on plants at elevated [CO_2]. In addition, it compares the effects of elevated [CO_2] on photosynthesis, biomass accumulation and yield that have been observed in experiments using enclosures versus FACE technology to fumigate plants with elevated [CO_2].

The responses of photosynthesis and g_s to elevated [CO_2] scale up to altered ecosystem water use and net primary productivity. The fifth section of the chapter discusses how this has the potential to feedback on the global carbon cycle and climate, altering the progression of global change.

Making accurate predictions of (1) future crop yield and (2) the degree to which vegetation will act as a sink for carbon from the atmosphere depends on our ability to mathematically model photosynthetic gas fluxes. The sixth section of this chapter demonstrates how a model can be used to identify how photosynthesis might be manipulated to improve performance under future conditions.

The response of plants to growth at elevated [CO_2] generates a number of problems as well as opportunities for the future. In the final section of the chapter we discuss the major gaps in our knowledge and areas in which to focus future research effort. We conclude by reviewing the extent to which elevated [CO_2] may be beneficial and how with further research we could adapt to maximize food supply and energy security.

II. Physiological Responses of C_3 Species to Growth at Elevated [CO_2]

A. [CO_2] and Rubisco Kinetics

The kinetic properties of the competing reactions of ribulose 1,5-bisphosphate (RuBP) carboxylation and RuBP oxygenation catalyzed by Rubisco dominate the response of C_3 photosynthesis to variation in [CO_2]. Rubisco is substrate-limited at current atmospheric [CO_2] and catalyses the only process in the photosynthetic pathway that exerts sufficient control on metabolic flux to alter rates of CO_2 assimilation when [CO_2] is varied at ecologically relevant levels. Development of a robust model of C_3 photosynthesis by Farquhar et al. (1980) has provided a framework within which to interpret photosynthetic responses to elevated [CO_2]. The Farquhar et al. (1980) steady-state leaf biochemical model combines the kinetic properties of Rubisco with a model of the light response of electron transport to allow effective prediction of the light, temperature, CO_2, and O_2 responses

of photosynthesis of C_3 leaves. The robust performance of the model results from the conserved properties of photosynthesis and Rubisco across C_3 plant species. The Farquhar et al. (1980) model assumes that intercellular CO_2 concentration approximates the CO_2 concentration at Rubisco. Based on this assumption along with the theoretical consideration of enzyme kinetics in the photosynthetic carbon reduction cycle, Farquhar et al. (1980) predicted that steady-state photosynthesis may be limited by either the concentration of CO_2 at Rubisco or by the supply rate of RuBP. Due to the low affinity of Rubisco for CO_2, if the enzyme is limited by CO_2 concentration, the rate of photosynthesis will also depend upon the amount of active Rubisco enzyme. The rate of RuBP supply may be limited by either the rate of whole electron transport or by the rate of utilization of triose phosphate (TPU) in the photosynthetic cell (Harley and Sharkey, 1991). Therefore, the potential rate of photosynthesis is the minimum of three rates: the Rubisco limited photosynthesis; the whole chain electron transport limited rate of RuBP regeneration; or the TPU limited rate of RuBP regeneration.

The direct increase in photosynthesis of terrestrial C_3 plants caused by increased [CO_2] results from two properties of Rubisco: (1) CO_2 competitively inhibits the oxygenation reaction, which otherwise produces glycolate leading to photorespiration, and (2) the Michaelis-Menton constant (K_m) for CO_2 is close to the current atmospheric concentration, so elevated [CO_2] increases the velocity of carboxylation.

The suppression of photorespiration by elevated [CO_2] is particularly important because it increases the efficiency of net CO_2 assimilation. Oxygenation of RuBP is the first step of the photosynthetic carbon oxidation, or photorespiratory, pathway. Depending on temperature (Zelitch, 1973), oxygenation by Rubisco decreases the net efficiency of photosynthesis by 20–50%, by utilizing light energy and releasing recently assimilated carbon as CO_2. Carbon dioxide is a competitive inhibitor of the oxygenation reaction, such that a doubling of concentration of CO_2 will roughly halve the rate of oxygenation (Long, 1991). High-CO_2-induced reduction of photorespiration results in an increase in net photosynthesis regardless of whether photosynthesis is Rubisco- or RuBP-limited and regardless of where metabolic control lies. The increase in net CO_2 assimilation resulting from suppression of photorespiration requires no additional light, water, or nitrogen (N), making photosynthesis more efficient with respect to each of these resources.

Rubisco specificity is the ratio of carboxylation to oxygenation activity when the concentrations of CO_2 and O_2 at the Rubisco catalytic site are equal. Rubisco specificity determines the increase in efficiency of photosynthesis with rising atmospheric [CO_2] and varies from 88 to 131 at 25°C across a range of C_3 plants, with an average of about 100 (Bainbridge et al., 1995). Terrestrial C_3 plant Rubisco has a consistently higher specificity factor, in contrast to Rubisco in other photosynthetic groups such as C_4 plants and cyanophyta (Bowes, 1993; von Caemmerer et al., 1994; Delgado et al., 1995). As temperature increases specificity declines due to decreased affinity of Rubisco for CO_2 relative to O_2 and, to a lesser extent, decreased solubility of CO_2 relative to O_2 (Long and Drake, 1991). About 68% of the decline in Rubisco specificity is calculated to result from the change in binding affinity of the protein for CO_2 (Brooks and Farquhar, 1985; Long, 1991). The effect of this temperature-dependent decline in Rubisco specificity is to produce a progressive increase in the stimulation of photosynthesis by elevated atmospheric [CO_2] with increasing temperature. The expected stimulation of RuBP-limited photosynthesis caused by increasing CO_2 from 350 to 700 ppm rises from 4% at 10°C to 35% at 30°C. It also follows from this interaction that the temperature optimum of light-saturated CO_2 assimilation (A_{sat}) must increase with CO_2 by 2°C, 5°C, and 6°C with increase in CO_2 to 450, 550, and 650 ppm (Long et al., 1997). The upper temperature at which a positive A_{sat} may be maintained is similarly increased. These changes in the constraint of photosynthesis by temperature at elevated [CO_2] indicate the importance of considering elevated CO_2 not just as a factor that increases photosynthetic rate, but also as a factor that modifies physiological responses to other abiotic variables. Because increasing CO_2 is predicted to increase leaf temperature, both directly by decreasing latent heat loss and indirectly through radiative forcing of the atmosphere, the interactive effect of CO_2 and temperature has profound importance to future photosynthesis. It also suggests a much greater stimulation of photosynthesis

in hot versus cold climates (Long and Hutchin, 1991; Long et al., 1992; Kirschbaum, 1994).

For a leaf temperature of 25°C, assuming an average specificity and K_m for CO_2 and O_2 for terrestrial plant Rubisco, and a constant ratio of intercellular to external [CO_2] (c_i/c_a) (Long, 1991; Bernacchi et al., 2003), the increase in atmospheric [CO_2] from today's 370 to 550 ppm, as predicted for the middle of this century, would increase Rubisco-limited photosynthesis by 36% and RuBP-limited photosynthesis by 12%. The predicted increase to 700 ppm by the end of the century would cause further increases of Rubisco-limited photosynthesis to 63% and RuBP-limited photosynthesis to 18%. Consequently, the fraction of leaves within a canopy for which photosynthesis is light-saturated (primarily Rubisco-limited) versus light-limited (RuBP-limited) will determine the effect of elevated [CO_2] on whole-plant carbon gain.

B. Acclimation and Optimization of Photosynthesis

A common feature of plants exposed to elevated [CO_2] in chamber experiments is that the initial stimulation of photosynthesis is not sustained over the long-term (Drake et al., 1997). Reduced carboxylation capacity with growth in elevated [CO_2] is well documented (Drake et al., 1997; Rogers and Humphries, 2000) and often associated with a reduction in the amount of Rubisco protein as well as the levels of transcripts for *rbcS* and other photosynthetic genes (Moore et al., 1999). Other nearly universal changes observed in the leaves of C_3 plants grown at elevated [CO_2] are increased carbohydrate content and decreased N content (Drake et al., 1997). Together, these changes have been interpreted as either a general down-regulation of photosynthetic capacity or as an optimization that maximizes carbon gain in tandem with resource use efficiency.

The concept of down-regulation suggests that photosynthetic acclimation at elevated [CO_2] is the result of a general decrease of leaf protein due to reallocation of N within the plant (Makino et al., 1997; Nakano et al., 1997) or, in N-limited plants, earlier leaf senescence (Nie et al., 1995a, b; Stitt and Krapp, 1999). This is consistent with average decreases in leaf N (−17%), total protein (−14%) and Rubisco (−15%) observed in chamber studies (Drake et al., 1997). Under conditions of N limitation, down-regulation under elevated [CO_2] may be more intense and rapid as reductions in leaf protein would significantly limit photosynthesis. It was shown that when growth was restricted by low N, photosynthetic acclimation in elevated [CO_2] could be ameliorated if N was added in direct proportion to plant growth (Farage et al., 1998), suggesting that N dilution, rather than N supply, causes photosynthetic down-regulation.

The alternative concept of optimization considers the trade-off between resource use efficiency and increased carbon gain at elevated [CO_2]. There is evidence to suggest (1) a decreased requirement for Rubisco at elevated [CO_2] and (2) a greater decrease in carboxylation capacity when sink strength is limited. The response of the net rate of photosynthetic CO_2 assimilation (A) to c_i is biphasic, such that as c_i increases from zero, A increases steeply to a transition point beyond which the slope ($\delta A/\delta c_i$) is small and approaches zero (Fig. 29.3). This is because, at low c_i, A is

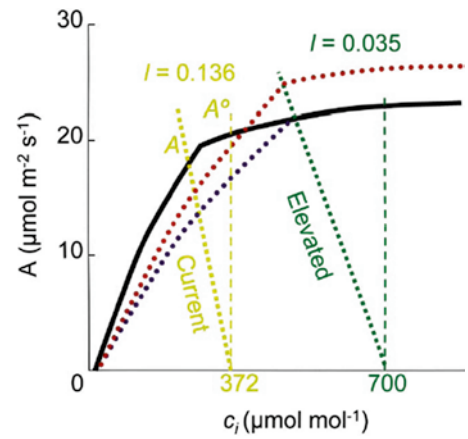

Fig. 29.3. The response of leaf CO_2 uptake (A) to intercellular [CO_2] (c_i), i.e., demand function, as predicted from the leaf biochemical model of photosynthesis of Farquhar et al. (1980). The *dotted black lines* illustrate the decline in c_i that occurs with increasing A_{sat} a constant g_s, i.e., the supply function. This is illustrated both for the current ambient and a future elevated [CO_2]. The *vertical dashed lines* show the supply function if g_s is assumed infinite. The *dotted blue line* illustrates how the demand function would alter if a 30% decrease in Rubisco activity occurred, and the *dotted red line* shows the demand function if a 15% decrease in Rubisco activity and 15% increase in RuBP regeneration capacity occur. I represents stomatal limit at the two CO_2 concentrations. (This figure was reproduced with permission of Annual Reviews from Fig. 29.1 of Long et al., 2004.)

controlled by the amount of active Rubisco, but beyond the transition it is controlled by the rate of RuBP regeneration. At light saturation this transition commonly occurs at the c_i that occurs in the present atmospheric [CO_2]; typically c_i is about 0.7 of atmospheric [CO_2] (Drake et al. 1997). This implies that the amount of active Rubisco and capacity for RuBP regeneration are balanced. If atmospheric [CO_2] increases, c_i is expected to rise proportionately (Drake et al., 1997). As Fig. 29.3 indicates, if c_i increased by 50%, photosynthesis would be limited solely by RuBP regeneration, and a substantial (30%) loss of Rubisco could occur without affecting photosynthesis. This supports the notion of a regulated acclimation, that is optimization, rather than a general down-regulation of photosynthesis. If plants could be engineered to reallocate resources, so that Rubisco was decreased by 15% and the capacity for RuBP regeneration increased by 15% under elevated [CO_2], the increase in A on elevation of [CO_2] could be improved by 40% (Fig. 29.3).

Photosynthetic down-regulation is commonly attributed to an inadequate capacity to utilize photosynthate (sink limitation), which could be due to lack of N to utilize the additional photosynthate produced at elevated [CO_2] (Rogers et al., 1998; Stitt and Krapp, 1999). Since Rubisco accounts for the largest single share of leaf N, reallocation of N from Rubisco to other functions at elevated [CO_2] would maximize the efficiency of use of this commonly limiting resource (Long and Drake, 1991; Woodrow, 1994). On average, results from FACE experiments support this notion. At elevated CO_2 there was a 22% decrease in V_{cmax} under low N compared to a reduction of 12% under high N (Ainsworth and Long, 2005). This corresponded with a decrease in leaf N of 12% under low N, and no change in leaf N under high N supply. Greater down-regulation of photosynthesis can also be observed when factors other than N supply limit sink strength. Comparison of an indeterminate soybean cultivar and an isogenic determinate line of this cultivar derived by a single gene mutation revealed that at the whole-plant level a restricted capacity to utilize photosynthate drives a loss of photosynthetic capacity at elevated [CO_2] (Ainsworth et al., 2004). At ambient [CO_2], the two cultivars had similar photosynthetic rates. At elevated [CO_2], a marked decrease in carboxylation capacity occurred in the determinate mutant while no acclimation occurred in the wild type as it was not genetically limited in its capacity to add "sinks" for photosynthate.

Many sugars, in addition to functioning as substrates, have a regulatory role in the expression of specific plant genes (Koch, 1996). This probably mediates the optimization of photosynthetic capacity at elevated [CO_2]. In source leaves, changes in sucrose, a major form of translocated carbon in most plants and the main substrate for sink metabolism, is an indicator of source/sink balance and can communicate whole-plant carbon flux (Farrar et al., 2000). Therefore, the notion that excess source leaf carbohydrate at elevated [CO_2] feeds back on photosynthetic gene expression and leads to acclimation is attractive (Sheen, 1990; Stitt, 1991; Krapp et al., 1993). However, carbohydrate accumulation at elevated [CO_2] is not always strongly correlated with a loss of photosynthetic capacity (Moore et al., 1998). This is probably due to heterogeneous distribution of sugars within source tissue obstructing meaningful correlation of total carbohydrate content with photosynthetic acclimation (Koroleva et al., 1997, 1998).

When photosynthesis exceeds the capacity for utilization and export of photosynthate, sucrose will accumulate in the leaf phloem and mesophyll vacuoles. Moore et al. (1999) argue that the imbalance between the supply and demand of carbohydrates can be sensed and lead to observed reductions in photosynthetic capacity through the molecular control of Rubisco content at elevated [CO_2] via sucrose cycling (Fig. 29.4). The central premise of this hypothesis is that excess sucrose from photosynthesis is hydrolyzed by vacuolar and apoplastic invertases producing hexoses which are subsequently phosphorylated by hexokinase before being reused to re-synthesize sucrose. Glucose from nocturnal starch hydrolysis (Schleucher et al., 1998; Servaites and Geiger, 2002), and from the amylomaltase-catalyzed conversion of maltose to sucrose, provides substrates for hexokinase, possibly involving a hexokinase bound to the outer envelope of the chloroplast membrane (Wiese et al., 1999), thereby providing 24-h signal production for a hexokinase sensing system (Fig. 29.3). The signal transduction pathway linking hexokinase to photosynthetic acclimation is not fully resolved (reviewed in Smeekens, 2000; Rolland et al., 2002), but there are clearly a number of factors involved including reversible protein

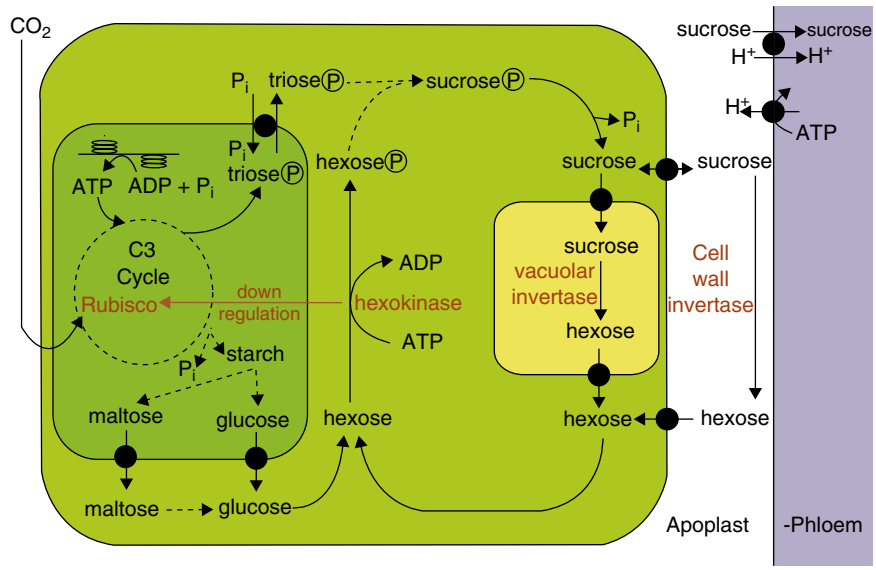

Fig. 29.4. A diagrammatic representation of the hypotheses that seek to describe the mechanism underlying loss of photosynthetic capacity when sucrose accumulates in the mesophyll. Increased levels of sucrose accumulating in source leaves can potentially reduce photosynthetic capacity in both the long term, through down-regulation of Rubisco and other photosynthetic genes, and in the short term, by reducing the capacity for ATP production within the chloroplast. Increased levels of sucrose are sensed via increased sucrose cycling through invertase and perceived by a hexokinase sensing system. Nocturnal degradation of starch will also supply substrates for hexokinase. Depending on the species, the hexokinase-generated signal reduces Rubisco content by down-regulating transcription of the *rbcs* family, translation of the mRNA, and/or affecting assembly of the holoenzyme. *Broken lines* indicate multiple-step processes that have been simplified for clarity. (This figure was reproduced with permission of Annual Reviews from Fig. 29.4 of Long et al., 2004.)

phosphorylation and Ca^{2+} as a second messenger. The control mechanisms that are initiated by sugar signaling vary among species but target the small subunit of Rubisco through transcriptional or translational control (Moore et al., 1999).

Nodulation gives soybean a large additional sink for photosynthate to fix N, which in turn would serve to avoid N-limitation of sink development. This makes it an interesting model species because if decreased carboxylation capacity is observed at elevated [CO_2], when least expected, it indicates the likely widespread nature of the phenomenon. Does down-regulation of photosynthesis and/or optimization of Rubisco occur in soybean grown in elevated [CO_2] under field conditions? When potential Rubisco carboxylation (V_{cmax}) and electron transport through photosystem II (J_{max}) were determined from the responses of A_{sat} to c_i, growth at elevated [CO_2] was found to increase A_{sat} by 15–20%, even though g_s was reduced (Bernacchi et al. 2005a). There was a small, yet statistically significant decrease in V_{cmax} which in turn drove a decrease in V_{cmax}/J_{max}, inferring a shift in resource investment away from Rubisco. Mesophyll conductance (g_m), which is the transfer conductance of CO_2 from the intercellular air-space to the site of carboxylation within the chloroplast, has been shown to restrict photosynthesis by 12% in tobacco plants grown in current atmospheric concentrations of CO_2 (Bernacchi et al., 2002). Any shift in the limitation imposed by g_m on photosynthesis as a result of growth in elevated [CO_2] may also alter the ratio of V_{cmax} to J_{max} as calculated from an A vs. c_i response curve, even in the absence of any true changes in either V_{cmax} or J_{max} (Singsaas et al., 2004). Therefore, it must be considered that an observed apparent down-regulation of V_{cmax} might actually instead arise from a decrease in g_m. Estimates of g_m suggest that it is positively related to A_{sat} (von Caemmerer and Evans, 1991; Loreto et al., 1992), g_s (Loreto et al., 1992), chloroplast surface area exposed to intercellular air spaces (von Caemmerer and Evans, 1991; Evans et al., 1994) and temperature (Bernacchi et al., 2002), but it is not affected by instantaneous change in

[CO_2] (Harley et al., 1992a; Loreto et al., 1992; however, see Flexas et al., 2008). Little is known about the long-term response of g_m to elevated [CO_2], though recent evidence suggests that in general it decreases, varying with species (Singsaas et al., 2004). In soybean under FACE, the observed decrease in V_{cmax}/J_{max} in field grown soybeans at elevated [CO_2] was not an illusion caused by a decrease in g_m (Bernacchi et al. 2005a). Although there was no progressive decline in A_{sat}, analyses of the A versus c_i responses showed that, even in an N-fixing species grown without rooting restriction and under open-field conditions, down-regulation of photosynthesis occurred. This down-regulation is in effect an optimization of photosynthesis to elevated [CO_2] where the decrease in V_{cmax} would result in lower rates of A only when measured at lower [CO_2] with little or no loss at elevated [CO_2].

C. Stomata Sensing CO_2

Stomatal conductance has been shown to be tightly linked with A and has overwhelmingly been shown to decrease with rising [CO_2] (Morison, 1987; Lawlor and Mitchell, 1991; Curtis, 1996; Ainsworth et al., 2002). The responses of guard cells to a range of environmental conditions has been widely reviewed (Jarvis and McNaughton, 1986; Willmer and Fricker, 1996; Medlyn et al., 2001; Vavasseur and Raghavendra, 2005), though mechanisms involved in CO_2 sensing are still not completely understood (Assmann, 1999; Vavasseur and Raghavendra, 2005). Studies on removed epidermal strips show that stomata respond to changes in [CO_2] independent of the mesophyll (Travis and Mansfield, 1979a, b; Schwartz et al., 1988; Leymarie et al., 1999). Further individual guard cells suspended in solution were also shown to shrink and swell based on [CO_2] (Gotow et al., 1982; Fitzsimons and Weyers, 1986). To elucidate where CO_2 sensing occurs, Mott (1988) simultaneously exposed the abaxial vs. adaxial leaf surfaces of two species with amphistomatous leaves to different concentrations of CO_2 while holding c_i constant. The results showed that stomata respond to c_i rather than atmospheric [CO_2] (c_a). This study was instrumental in identifying the general location of CO_2 sensing; however, it did not differentiate the cell type (i.e., mesophyll or guard cell) that controls stomatal responses. Several recent studies have questioned the role of either mesophyll or guard cell photosynthetic processes in stomatal control. For example, Roelfsema et al. (2002) concluded that the red-light response results from reductions in c_i caused by mesophyll photosynthesis. In addition, studies on antisense plants with suppressed expression of Rubisco (von Caemmerer et al., 2004) and Rieske FeS protein (Price et al., 1998) have found little or no difference in stomatal function between wild-type and antisense plants, nullifying the role of either mesophyll or guard cell photosynthesis in the c_i response. Finally, the existence of a CO_2 response in darkness and of functional nonchlorophyllous guard cells in some orchids (Nelson and Mayo, 1975) also brings into question whether photosynthesis plays an essential role. However, Messinger et al. (2006) suggest that there are at least two mechanisms by which stomata respond to CO_2. The first is dependent upon photosynthetic electron transport and is therefore sensitive to the balance between light and dark reactions of photosynthesis, while the other is independent of photosynthetic electron transport and is therefore present in darkness.

The documented responses of stomata to conditions surrounding the leaf, including elevated [CO_2], have led to the development of models that relate g_s to a range of conditions. A notable example is that presented by Ball et al. (1987) which predicts g_s on the basis of photosynthetic rate, relative humidity (RH) and atmospheric [CO_2]. Subsequent modifications of this model have substituted atmospheric vapor pressure deficit in place of RH and incorporated the CO_2 compensation point of photosynthesis (e.g., Leuning, 1995). The modeled responses of g_s to elevated [CO_2] in the short term have been confirmed with multiple species grown under artificial and natural settings (for reviews see Lawlor and Mitchell, 1991; Leuning, 1995; Curtis, 1996; Drake et al., 1997; Field and Avissar, 1998). Several authors have tried to develop more mechanistic models of g_s (Dewar, 2002; Gao et al., 2002; Buckley et al., 2003). The model proposed by Buckley et al. (2003) incorporates whole-plant and epidermal hydromechanics, including water movement through source tissue (e.g., root) to xylem, then to epidermal cells, and finally to the guard cells. In addition, the Buckley model describes a direct link between g_s and photosynthesis as well as incorporating the evaporation

of water vapor from the stomatal cavity to the boundary layer, then to the ambient air. Two major assumptions are used in this model – first, the osmotic gradient across the guard cell membrane is assumed to be proportional to the concentration of ATP in guard cells; and second, the osmotic gradient that can be sustained per unit of ATP was assumed to be proportional to the turgor pressure of adjacent epidermal cells. The g_s of *Vicia faba* L. has been parameterized and tested using the Buckley model. Similar to most other stomatal models, the response of g_s to variations in transpiration rate, irradiance and intercellular CO_2 were correctly predicted along with the transient stomata opening, often observed before conductance declines in response to decreases in humidity, soil water potential, or xylem conductance. This model also correctly predicted the relative insensitivity of stomata to the ambient partial pressure of oxygen, as a result of the assumed dependence of the model on ATP concentration. In the Buckley model, the guard cell [ATP] is calculated using the Farquhar model (Buckley et al., 2003). The major novelty of the Buckley et al. (2003) model is its hydromechanical framework. The direct connection between the hydraulic and photosynthetic properties it uses represent a step towards producing a mechanistic stomatal model, possibly even a dynamic stomatal model.

D. Effects of Elevated [CO_2] on Stomatal Conductance

Growth at elevated [CO_2] anticipated in 2050–2100 leads to lower g_s by ~20%, on average (Drake et al., 1997; Long et al., 2004). However, there are some plant types that are less responsive, with conifers and *Fagus* species often being insensitive (Saxe et al., 1998). The magnitude of the stomatal response generally does not diminish with time in long-term experiments (Lee et al., 2001; Gunderson et al., 2002; Ainsworth et al., 2003). However, it does vary on hourly and daily timescales (Garcia et al., 1998; Rogers et al., 2004). On four dates during 2003, the varying effect of elevated [CO_2] on g_s in soybean fumigated by FACE technology in the field could be predicted with the Ball et al. (1987) model (Leakey et al., 2006a). The dates on which no decrease in g_s at elevated [CO_2] was observed corresponded to periods of low rainfall where soil water depletion may have lead to ABA-signaled stomatal closure in ambient [CO_2]. Soil water depletion in elevated [CO_2] during the corresponding period was probably lower and therefore drought-induced stomatal closure may have been avoided. In combination, these responses would negate the difference in g_s normally observed between ambient and elevated [CO_2], although these would likely return when rainfall replenished soil moisture.

E. Stomatal Acclimation to Elevated [CO_2]

Modeling changes in g_s with rising [CO_2] (e.g., Ball et al., 1987) assumes that the sensitivity of g_s to A, relative humidity and c_a is not affected by long-term growth at elevated [CO_2], i.e., that acclimation of g_s to [CO_2] does not occur. After exposure to ambient or elevated [CO_2] in chambers and branch bags, parameters of the Ball et al. (1987) model were compared for six tree species at four field sites (Medlyn et al., 2001). In only one of eight species/site combinations was there any change with growth [CO_2] that would indicate acclimation. Thus, other than at one water-stressed site, it appears that stomatal acclimation to elevated [CO_2] is unusual in trees. Studies of crops are inconsistent. Parameters of the Ball et al. (1987) model for *Lolium perenne* (rye grass) did not differ when this species was grown at 350 and 600 ppm CO_2 in a pseudo-replicated FACE study (Nijs et al., 1997). In contrast, *Gossypium hirsutum* (cotton) grown at 650 compared to 350 ppm CO_2 in controlled environment cabinets exhibited marginally significant stomatal acclimation (Harley et al., 1992b). In an open top chamber of soybean, sorghum, potato and bean, a substantially lower g_s was reported in soybean at a given A, relative humidity and c_a under elevated [CO_2] (Bunce, 2004). However, in a FACE experiment on soybean there was no change in parameters of the Ball et al. (1987) model at ambient and elevated [CO_2], such that a single parameterization could effectively predict g_s in the field under both treatments (Leakey et al., 2006a).

F. Stomatal Conductance and Transpiration

Plants have dynamic control of water use through modification of the stomatal aperture. However, stomata account for only one of four conductance terms associated with transpiration, where the

others are the intercellular airspace, boundary layer, and cuticle. Growth in elevated [CO_2] may potentially influence any of these other conductances, thereby altering leaf transpiration (*E)* in addition to changes in g_s. While regulated via g_s, *E* is ultimately driven by the physical conditions in the air surrounding the leaf. The effect of elevated [CO_2] on *E* parallels observed responses of g_s. Still, it is important to note that *E* can vary while g_s remains constant due to changes in temperature and/or vapor pressure deficit, both of which may change in future atmospheres (Houghton et al., 2001).

It was long thought that the response of stomata to a range of environmental conditions was dominant in determining the canopy transpiration as a whole. However, as was reviewed by Meinzer (1993), micrometeorological models of canopy transpiration seldom included stomatal components yet were able to predict canopy transpiration with reasonable accuracy. A mechanism to explain the disparity between what happens at the leaf vs. the canopy level includes aerodynamic conductances between the leaf and the atmosphere and changes in leaf temperature that accompany changes in g_s. The relatively still air within the leaf boundary layer becomes more humid as the leaf transpires which results in higher humidity within the plant canopy. The high humidity air within the canopy decreases the humidity gradient from the inside to outside of the leaf. The air within the canopy will mix with the bulk atmosphere, although in doing so it is subjected to numerous resistances (Bazzaz and Sombroek 1996). The barriers slowing the mixing between the humidified air within the canopy and the lower humidity bulk atmosphere can maintain the minimal gradient between humidity inside and outside of the leaf. This phenomenon has been shown to sometimes cause the canopy transpiration of vegetative stands with high water availability to be relatively insensitive to changes in stomatal aperture (McNaughton and Jarvis, 1991).

The impact of uncoupling between the leaf and bulk atmosphere with elevated [CO_2] can be demonstrated from FACE studies in which transpiration at the leaf- and canopy-level are measured. Stomatal conductances for wheat grown in elevated [CO_2] ranged from 27% to 36% lower than control, depending on the time of day (Wall et al., 2000). However, mean daily canopy *ET* was only 7% lower for elevated [CO_2] grown wheat in the same experiment (Kimball et al., 1999). While this difference can be explained based on uncoupling leaf and canopy conductance, the increase in leaf temperature associated with decreased g_s has also been proposed as a mechanism to explain the difference in observations between the leaf and canopy *E* (Idso et al., 1993; Kimball et al., 1995). A higher leaf temperature could potentially lead to an increase in the gradient of vapor pressure deficit between the canopy and the air, thereby increasing transpiration for plant canopies grown in elevated [CO_2] to a rate closer to the control conditions (Polley, 2002). In experiments at SoyFACE, elevated [CO_2] was found to decrease *E* between 9% and 16% depending on year even though there were large increases in photosynthesis and seed yield. Ecosystem *ET* was linked to the g_s of upper canopy leaves such that a 10% decrease in g_s caused 8.6% decrease in *ET* regardless of the growth [CO_2] level. Thus despite system feedbacks, decreased g_s of upper canopy leaves at elevated [CO_2] results in decreased transfer of water vapor to the atmosphere (Bernacchi et al. 2007).

Elevated [CO_2] induced changes in *E* may alter plant responses to changes in their environment. The reduction in *E* associated with growth in elevated [CO_2] has been shown to delay the onset of drought stress and improves the ability of stands of vegetation to withstand drought stress (Rogers et al., 1984; Jones et al., 1985; Allen et al., 1990; Bhattacharya et al., 1990; Chaves and Pereira, 1992; Bowes, 1993; Clifford et al., 1993; Widodo et al., 2003). Evidence also supports greater relative growth stimulation between control and elevated [CO_2] grown plants under water stress compared with well-watered conditions (Clifford et al., 1993).

G. Elevated [CO_2] and Water Use Efficiency

The term water use efficiency is used to express the ratio of transpiration to carbon assimilation. Since both g_s decreases and *A* increases when plants are grown in elevated [CO_2], plants generally become more water-use-efficient. The effects of elevated [CO_2] on water use efficiency have been studied at various scales ranging from the leaf to the ecosystem. As is commonly found with transpiration, effects of elevated [CO_2] on water

use efficiency appear greater at the leaf or plant scale than is commonly seen at the canopy level. However, since the effect of elevated [CO_2] on photosynthesis generally remains higher than control, even in light of photosynthetic down-regulation, the effect of water use efficiency is not usually seen to decrease to the same magnitude over various scales of measurement as transpiration.

H. Stomatal Limitation to Photosynthetic Carbon Assimilation

Stomatal conductance is a finite value; thus it limits the diffusion of CO_2 into the leaf. Quantifying the limitation of g_s on photosynthetic carbon assimilation (l) can be calculated by comparing in situ rates of assimilation with modeled rates of A assuming an infinite g_s (Farquhar and Sharkey, 1982). Since g_s has been consistently shown to decrease with growth in elevated [CO_2], the potential for a higher l may exist. Alternatively, any increases in [CO_2] in the air around the leaf will result in a stronger CO_2 gradient between the atmosphere and the intercellular air spaces. Thus, is the increase in [CO_2] around the leaf sufficiently high to offset the observed decrease in g_s?

Measurements of l made on unstressed soybean plants over two growing seasons revealed l to be 8% under current atmospheric [CO_2] (Bernacchi et al., 2005a). When measured for soybean grown in CO_2 enriched air 50% above current atmospheric concentrations, l was measured at 4.5%, even though g_s was 10% lower in the elevated [CO_2] treatment (Fig. 29.5; Bernacchi et al., 2005a). This decrease in l at elevated [CO_2] is consistent with experiments on aspen (Noormets et al., 2001), oak (Maroco et al., 2002), C_3 and C_4 grasses (Maherali et al., 2003) and sweet gum (Herrick et al., 2004). That l decreases in elevated [CO_2] suggests that decreases in g_s can be achieved without the cost of additional limitation on carbon gain.

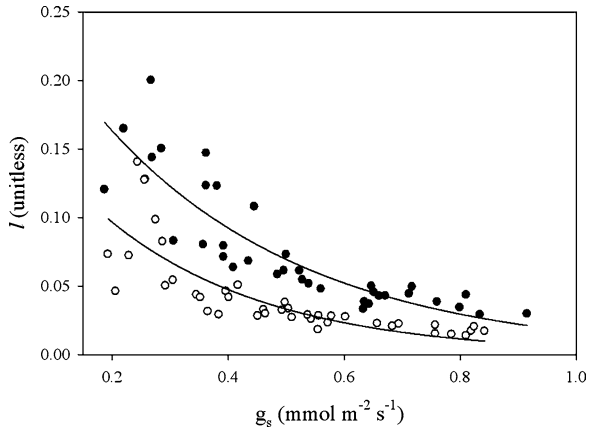

Fig. 29.5. The relationship between stomatal conductance (g_s) and stomatal limitation (l) for soybean (*Glycine max* L. Merr) grown under control (*solid symbols*) and elevated [CO_2] (*open symbols*) at the SoyFACE facility. *Lines* represent non-linear regressions for each treatment.

III. Physiological Responses of C_4 Species to Growth at Elevated [CO_2]

Plants possessing the C_4 photosynthetic pathway are of major economic, ecological and biogeochemical significance. *Zea mays* is the world's most produced crop while sorghum ranks 6th in global food crop production, with almost half of this is located in parts of Nigeria, Mexico, India and Sudan where it is a crucial source of food for large populations because drought stress limits the performance of other crops (USDA, 2005). The C_4 species dominate both tropical and temperate grasslands (Archibold, 1995), contributing ~25–30% of global terrestrial productivity (Gillon and Yakir, 2001). Of the world's 18 worst weeds, 14 possess C_4 photosynthesis (Brown, 1999). The impacts of future elevated [CO_2] on C_4 plants will clearly have important consequences.

Since Riley and Hodges (1969), conflicting evidence has accumulated for the sensitivity (Cure and Acock, 1986; Wand et al., 1999; Anderson et al., 2001) versus insensitivity (Hocking and Meyer, 1991; Ziska et al., 1991; Ghannoum et al., 2000) of C_4 photosynthesis, productivity and yield to elevated [CO_2]. Uncertainty in predictions of future food production (Long et al., 2005), changes in land use resulting from competition between C_3 and C_4 species (Sage and Kubien, 2003), and net primary productivity of grasslands (Coughenour and Chen, 1997) arises from two fundamental and unresolved questions: (1) Does elevated [CO_2] stimulate C_4 photosynthesis directly and (2) Does elevated [CO_2] improve C_4 plant water relations, and thereby indirectly enhance photosynthesis, growth and yield by ameliorating drought stress?

If only the latter mechanism impacts performance in the field, then the "fertilization" effect of CO_2 on C_4 crop yield, species competitiveness and grassland productivity will be limited to times or places of water stress, and will be qualitatively rather than just quantitatively different to that of C_3 plants.

These questions are re-examined here, and in more detail by Leakey (2009), in response to new data from recent FACE studies. The FACE studies are valuable because their fully-open air simulations of future, elevated [CO_2] under field conditions, allow experimentation on large plots of plants without disturbance of the plant-soil-atmosphere continuum. In other words, rooting volume is not restricted the micrometeorological controls on field-scale water relations are not modified as they are in enclosure studies. Free air concentration enrichment studies allow an update to the conceptual model of C_4 plant responses to elevated [CO_2] presented by Ghannoum et al. (2000). The experimental evidence is consistent with a modified scheme (Fig. 29.6) in which growth at elevated [CO_2] consistently decreases g_s and increases c_i in C_4 species. This can slow the depletion of soil moisture during drought and delay stress. In addition, greater c_i can counteract the reduction in c_i arising during drought stress as a result of low soil moisture and low humidity effects on g_s. This will impact photosynthesis

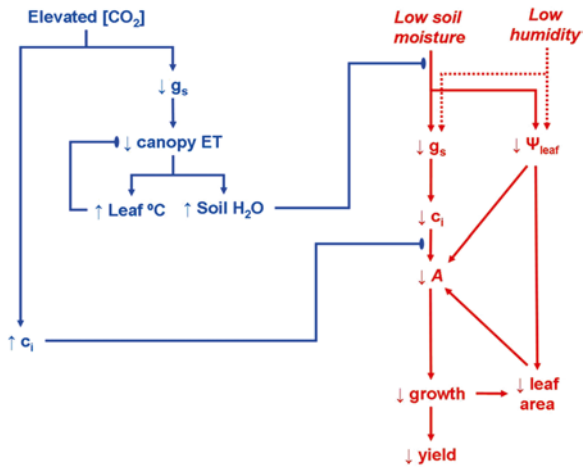

Fig. 29.6. Scheme describing the mechanism by which C_4 plant responses to growth at elevated [CO_2] (*blue lines*) interact with plant responses to drought in order to ameliorate stress (*red lines*). (This figure is reproduced from Leakey, 2009.)

only if c_i is below the inflexion point of the C_4 A/c_i curve. This means that elevated [CO_2] can ameliorate the negative effects of drought stress on carbon gain and growth. In cases where the benefits from elevated [CO_2] are of sufficient magnitude or frequency it can also offset yield losses to drought. The following sections discuss the evidence from FACE experiments for this scheme.

A. Does Elevated [CO_2] Directly Stimulate Photosynthesis?

Elevated [CO_2] has two direct effects on C_3 plants, it decreases stomatal aperture and it stimulates photosynthesis by decreasing photorespiration and increasing the velocity of carboxylation at Rubisco (Long et al., 2004). The C_4 plants similarly show a decrease in g_s (Ainsworth and Long, 2005), but because they use a different primary carboxylase, phosphoenolpyruvate carboxylase (PEPc), for which oxygen is not a competing substrate, they avoid photorespiration and are typically CO_2 saturated at the current atmospheric [CO_2]. Therefore, in theory, C_4 photosynthesis should not show a direct response to increased [CO_2]. A number of enclosure studies support this contention (Hocking and Meyer, 1991; Ziska et al., 1991; Ghannoum et al., 1998, 2000). However, stimulation of C_4 photosynthesis under elevated [CO_2] in growth cabinet experiments, where plants were well watered and drought stress was not intended to occur, has also been reported (Knapp et al., 1993; Amthor et al., 1994; Poorter et al., 1996; Wand et al., 1999; Anderson et al., 2001). Such stimulation has been attributed to a number of mechanisms, including: (1) direct effects on CO_2 saturation (Wong, 1979; Watling and Press, 1997; Ziska and Bunce, 1997); (2) altered bundle sheath leakiness (Saliendra et al., 1996; Watling et al., 2000); (3) direct CO_2 fixation in the bundle sheath (Moore et al., 1986), and (4) C_3-like photosynthesis in young C_4 leaves (Dai et al., 1995; Ziska et al., 1999), as previously reviewed by Ghannoum et al. (2000).

C_4 species have been studied at three large-scale FACE experiments, which support the hypothesis that elevated CO_2 has little or no direct effect on C_4 photosynthesis. In Maricopa, Arizona during 1998 and 1999, *Sorghum bicolor* was grown at ambient (~370 ppm) and elevated [CO_2] (~570 ppm),

with either sufficient irrigation to meet crop water demand or a simulated drought. While there was some evidence for photosynthetic responses to growth at elevated [CO_2] in young tissue and leaves (Cousins et al., 2001, 2003), it was reported that the direct effects of elevated [CO_2] on photosynthesis were "nominal" (Wall et al., 2001). Given sufficient water, in vivo photosynthesis of the youngest fully expanded leaf at midday was unaffected by growth [CO_2] on 13 of 17 measurement dates, across the two growing seasons (Wall et al., 2001). Photochemical light energy usage was stimulated by growth at elevated [CO_2], but, only during periods of drought or at midday when all treatments suffered short-term water stress due to high atmospheric vapor pressure deficits and temperatures (Cousins et al., 2002). Collectively, these results show that C_4 photosynthesis in sorghum was most likely indirectly stimulated via amelioration of water stress and not directly stimulated via biochemical mechanisms. The possibility remains that photosynthesis in young leaves of sorghum was directly stimulated by elevated [CO_2], but if so the response was not of significant magnitude to drive a statistically significant stimulation of biomass accumulation or yield under well-watered conditions (Ottman et al., 2001).

A mixed species, managed grassland including a C_4 species (*Paspadalum dilatatum*) was studied using FACE technology in New Zealand. A/c_i curve measurements indicated: (1) *P. dilatatum* grown at both ambient (~360 ppm) and elevated [CO_2] (~475 ppm) were operating at, or close to, saturating c_i (2) no evidence of photosynthetic acclimation (von Caemmerer et al., 2001) and C_4 photosynthesis was not stimulated by elevated [CO_2].

At Champaign Illinois, in the U.S. Corn Belt, *Zea mays* was grown at ambient (~370 ppm) and elevated [CO_2] (~550 ppm) during the growing seasons of 2002 and 2004. On 5 dates in each season, the in situ gas exchange of the youngest fully expanded leaves was measured every ~2 h from sunrise until sunset. On 7 out of 10 dates, there was no CO_2 effect on photosynthesis (Leakey et al., 2004, 2006b). This corresponded with when the crop was not experiencing drought stress. The lack of photosynthetic stimulation resulted from the fact that the operating c_i of plants growing at both ambient and elevated [CO_2] was above the inflexion point of the A c_i curve, where photosynthesis is CO_2-saturated. Also, there was no evidence of any photosynthetic acclimation to elevated [CO_2] in terms of in vivo or in vitro photosynthetic enzyme capacities, leaf carbohydrates or leaf N (Leakey et al., 2006b). In 2004, the crop did not experience drought stress at any time and, as was observed for well-watered sorghum in Arizona, there was no stimulation of biomass accumulation or yield due to growth at elevated [CO_2].

There are a number of factors which may have contributed to the apparent sensitivity of C_4 photosynthesis to elevated [CO_2] observed in some enclosure studies versus the insensitivity of C_4 photosynthesis observed in other enclosure studies as well as the FACE experiments. In order to meet the water requirements of the shoot, the roots of the major C_4 crops can often extend to depths of 1–2 m (Allen et al., 1998; Carcova et al., 2000). This greatly increases capacity for water uptake over that possible in the smaller soil volume of pots used in some enclosure experiments (e.g., 3.5 L (Ziska and Bunce, 1997; Ziska et al., 1999); 5 L (Maroco et al., 1999; Wong, 1979)). Even when well-watered, the smaller soil volume of pots may limit water supply to the shoot. If this were the case, reduced demand for water resulting from lower g_s at elevated [CO_2] could alleviate this limitation and increase productivity, which could easily be misinterpreted as the result of direct stimulation of C_4 photosynthesis by elevated [CO_2] under well-watered conditions. Growing plants in open top chambers (OTCs), which can provide unrestricted rooting depths, should eliminate this problem (Amthor et al., 1994). However, air inside OTC's can be more than 4°C warmer and 0.8 kPa drier than outside the enclosure, when PPFD is high (>1600 μmol m^{-2} s^{-1}; Whitehead et al., 1995). This would likely inhibit photosynthesis, by promoting water stress during the middle of the day. Again, reduced g_s at elevated [CO_2] could alleviate such stress and support greater rates of photosynthesis, which could be misinterpreted as a result of direct stimulation of C_4 photosynthesis by elevated [CO_2]. In addition, canopy photosynthesis of C_4 crops can reduce the daytime ambient [CO_2] in an enclosure. For example, sorghum reduced the atmospheric [CO_2] from 350 to 330 ppm in a controlled environment cabinet (Watling et al., 2000). This sub-ambient [CO_2] could be sufficient to drop c_i below the inflexion

point of the A/c_i curve, which would reduce photosynthesis. Photosynthetic draw down of $[CO_2]$ would be unlikely to drop c_i below the inflexion point of the A/c_i curve from plants grown at 550 ppm or greater. However, the difference between photosynthesis in these high and low $[CO_2]$ conditions would result from the direct effect of sub-ambient $[CO_2]$, not elevated $[CO_2]$, on photosynthesis. Only one C_4 species has been studied in each of the FACE experiments to date, and all three studies have examined NADP-malic enzyme type C_4 photosynthesis. C_4 subtypes show variation in their N use efficiency (Ghannoum et al., 2005), but no consistent differences in their sensitivity to elevated $[CO_2]$ (Wand et al., 2001). Clearly, further field studies are needed to demonstrate that the conclusions drawn from FACE experiments to date are consistent across a broader range of genetic and environmental variation.

B. Does Elevated $[CO_2]$ Improve C_4 Plant Water Relations, and then Indirectly Stimulate Photosynthesis, Growth and Yield by Reducing Drought Stress?

It has been commonly suggested that lower g_s at elevated $[CO_2]$ will consistently lead to less total plant water use, conservation of soil moisture and the potential for amelioration of drought stress (Ghannoum et al., 2000; Long et al., 2004). Evidence for the operation of this mechanism has come from enclosure studies, with elevated $[CO_2]$ reducing water use in maize (−25% to 30%; Samarakoon and Gifford, 1996), *Panicum coloratum* (approx. −17%; (Seneweera et al., 1998) and *Bouteloua gracilis* (Morgan et al., 1998). As a result, plant water status, photosynthesis and growth in all three species were enhanced under conditions of water stress. Also, the enhancement of biomass accumulation by C_4 species under elevated $[CO_2]$ is greater under drought stress than well watered conditions (Owensby et al., 1997; Seneweera et al., 1998). Impairment of shoot growth by atmospheric water deficit has also been shown to be alleviated by elevated $[CO_2]$ (Seneweera et al., 1998).

Nonetheless, several factors can interfere with amelioration of water stress by elevated $[CO_2]$ in the field. First, Stomatal conductance can, at least to some degree, be uncoupled from whole-plant transpiration (Jarvis and McNaughton, 1986; Collatz et al., 1991; Meinzer et al., 1997). Second, the impacts of elevated $[CO_2]$ on soil water status can be exceeded by other influences on soil water such as extreme droughts or rain events. The recent FACE experiments allowed these issues to be addressed.

Elevated $[CO_2]$ significantly reduced the g_s of *S. bicolor* grown under FACE with both adequate (−32%) and inadequate irrigation (−37%) (Wall et al., 2001). Whole-plant water use was also reduced by elevated $[CO_2]$, but to a lesser extent (Triggs et al., 2004). On average over 2 years, elevated $[CO_2]$ reduced soil water depletion by 10% under ample irrigation and 4% under severe drought stress (Conley et al., 2001). As described earlier, the semi-desert growing conditions in Maricopa, Arizona could cause transient drought stress, even in plants that were well irrigated (Cousins et al., 2002). As a consequence, photosynthesis of amply irrigated sorghum was occasionally stimulated by growth at elevated $[CO_2]$ – leading to 9% greater midday photosynthesis over two seasons (Wall et al., 2001). By comparison, leaf water potential of sorghum under severe drought stress was regularly improved by growth at elevated $[CO_2]$ and this allowed a 23% enhancement of midday photosynthesis, on average over two seasons.

The growth of maize in 2002 and 2004 at SoyFACE allowed the effects of growth at elevated $[CO_2]$ to be compared between an "average" year that featured episodic drought stress (2002) and an "atypical" year of ideal growing conditions without drought stress (2004) (Leakey et al., 2004, 2006b). Elevated $[CO_2]$ reduced the g_s over the diurnal period of *Z. mays* 23% and 29% in 2002 and 2004, respectively. Consistent with results from the sorghum FACE experiment, this resulted in a smaller, but significant, decrease in whole-plant water use. Growth of *Z. mays* at elevated $[CO_2]$ resulted in up to 31% greater soil moisture between 5 and 25 cm depth, and up to 11% greater soil moisture between 25 and 55 cm depth, when it was measured in 2004 (Leakey et al., 2006b). Low rainfall did result in a period of drought stress during the 2002 growing season. During this time, photosynthesis was greater under elevated $[CO_2]$ than ambient $[CO_2]$. This was driven by amelioration of drought stress under elevated $[CO_2]$ that resulted from conservation of soil water and maintenance of higher g_s and photosynthesis.

Elevated [CO_2] can increase canopy temperatures as a function of lowering stomatal conductance. This can be visualized as the difference in canopy temperature between inside elevated [CO_2] plots and outside the plots where plants are growing at ambient [CO_2]. For example, maize canopy temperature can be 1.1°C greater at elevated [CO_2] compared to ambient [CO_2] (Fig. 29.7).

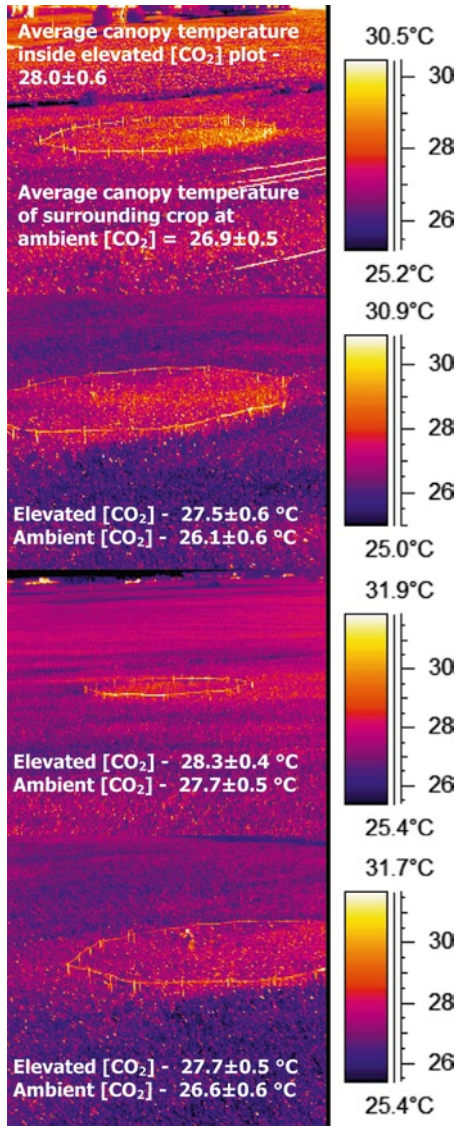

Fig. 29.7. Canopy temperature of maize growing inside (elevated [CO_2]) and outside (ambient [CO_2]) a FACE plot at SoyFACE, Illinois at 15:30 on 15 July 2004. The average temperature of the canopy inside the four replicate elevated [CO_2] plots was 27.9±0.2°C. This was significantly higher than the temperature of the canopy outside the plots under ambient [CO_2] (26.8±0.3°C; p=0.03). (This figure is reproduced from Leakey, 2009.)

By comparison, elevated [CO_2] increased canopy temperature of sorghum by 1.47°C and 1.85°C in well-watered and droughted treatments of sorghum, respectively (Triggs et al., 2004). Greater leaf temperature at elevated [CO_2] will increase transpiration, counteracting reduced g_s to some extent. This has the potential to increase or decrease photosynthesis, depending on what growth temperatures are relative to photosynthetic temperature optima. Further work is needed to determine the importance of this mechanism to plant productivity and crop yield.

Elevated [CO_2] consistently reduces g_s in C_4 species and has the potential to thereby lower plant water use, ameliorate drought stress and stimulate photosynthesis. However, the degree to which this mechanism operates in different environmental conditions (e.g., varying water supply, nutrient availability or temperature) and among different species or C_4 sub-types is still poorly understood. This is important because predictions of ecosystem productivity and crop production in future CO_2-rich atmospheres are uncertain as a consequence.

IV. Are Expectations from Enclosure Studies Met in Open-Air Field Conditions?

Thousands of experiments using different experimental platforms have evaluated the response of photosynthesis to increases in atmospheric [CO_2]. Early experiments used greenhouses, artificially illuminated controlled environmental chambers, or transparent enclosures or open-top enclosures in the field. Many of these experiments used plants grown in pots, and focused on the early stages of plant growth. Free air concentration enrichment technology was developed as a means to investigate plant responses to elevated [CO_2] under fully open-air conditions at an ecologically and agronomically relevant scale. This section describes the different experimental platforms for measuring photosynthesis in a [CO_2]-rich atmosphere, and then reviews the results of investigation of photosynthesis from thousands of controlled environment studies and 15 years of FACE experimentation.

A. Enclosures vs. FACE

Experiments examining the effects of elevated [CO_2] on photosynthesis have been conducted in laboratory growth chambers, greenhouses, closed-top field chambers, open-top field chambers, and most recently using FACE facilities. Temperature, light quantity and quality, humidity, air supply and movement, and CO_2 are controlled by the experimenter in laboratory growth chambers and greenhouses (Tibbitts and Langhans 1993), but conditions rarely match field conditions (Lawlor and Mitchell, 1991). Light levels are lower in controlled environments, and the spectrum is often significantly different from natural sunlight. Air-flow is typically vertical, and must be rapid and constant in order to maintain chamber temperature (Tibbitts and Langhans 1993). By necessity, plants are grown in pots, which can have an important impact in elevated [CO_2] experiments. Arp (1991) showed that rooting volume suppressed the response of plants to elevated [CO_2], and that many reports of acclimation of photosynthesis were artifacts of pot size. Drake et al. (1997) compared 103 studies performed at a low rooting volume (<10 L) with 59 high rooting volume (>10 L) studies, and confirmed that acclimation of photosynthesis to elevated [CO_2] was greater at lower rooting volume. Therefore, results of elevated [CO_2] experiments from controlled environments must be interpreted with care, as measured responses may be determined by other factors in the controlled environment, not just elevation of [CO_2].

Transparent enclosures and open top chambers have been used in the field in order to eliminate many of the limitations of growth chamber studies. Several designs of OTCs have been described (reviewed by Whitehead et al., 1995) that allow plants to be rooted in the ground and to experience natural fluctuations in light intensity and temperature. While OTCs offer a cost-effective method of CO_2 fumigation of plants rooted in the ground, OTCs also have some important limitations. Chambers reduce transmittance of solar irradiation, alter the ratio of diffuse to total solar irradiance, and increase air temperature and air water vapor saturation deficit (Whitehead et al., 1995). Chambers also block wind and the dispersal of pests or pathogens, interfere with rainfall, and alter plant-atmosphere coupling (Long et al., 2004). Therefore, the effect of the OTC can exceed the effect of elevated [CO_2] (Drake et al., 1989; Day et al., 1996).

Free air concentration enrichment technology was developed as a means to investigate plant responses to elevated [CO_2] under fully open-air conditions at an ecologically and agronomically relevant scale. A network of FACE facilities has been established around the globe (for a detailed map, see the Carbon Dioxide Information Analysis Center website: http://cdiac.esd.ornl.gov/programs/FACE/whereisface.html). The FACE approach uses a vertical or horizontal array of vent pipes to release jets of CO_2-enriched air or pure CO_2 gas at the periphery of vegetation plots, and relies on natural wind and diffusion to disperse CO_2 across the experimental area. Wind direction and velocity are measured along with [CO_2] in the center of each vegetation plot. A computer-controlled system then adjusts CO_2 flow rate, controlled by a mass-flow control valve, to maintain the target high [CO_2] (Long et al., 2004). Only pipes on the upwind side of the plots release CO_2, unless wind speed is less than 0.4 m s^{-1}, in which case CO_2 is released from adjacent release points (McLeod and Long, 1999). Because FACE relies on wind to carry the gas, strong prevailing winds can create a dilution gradient across the plot. The center of a plot may be close to the target [CO_2], while the upwind side may be above the target and the downwind side below the target. However, isotopic analysis of the carbon composition of vegetation exposed to ^{13}C and ^{14}C showed remarkable uniformity, suggesting that over growing seasons, dilution gradients in [CO_2] are not significant (Leavitt et al., 1996). A second potential disadvantage is that fumigation is compromised during still conditions. In FACE systems that use blowers, pumping air into the plot disrupts temperature inversions (Pinter et al., 2000), and in FACE systems without blowers, fumigation is impossible under perfectly still conditions (McLeod and Long, 1999). Despite these limitations, FACE likely offers the best technology for understanding the effects of elevated [CO_2] on vegetation, and large-scale FACE facilities (8–30 m diameter) have been established on forest, grassland, desert and agricultural lands (reviewed by Long et al., 2004; Ainsworth and Long, 2005). The majority of these experiments have used a target [CO_2] between 550 and 600 ppm, the [CO_2]

predicted for the middle of this century (Prentice et al., 2001).

B. Photosynthetic Response to Elevated [CO_2] in Enclosures: Results and Predictions

One of the paradigms to develop from early elevated-[CO_2] studies was that the initial stimulation of photosynthesis would not persist in the long term as nutrient limitation at the ecosystem scale developed over time and limited plant response to elevated [CO_2] (Tissue and Oechel 1987; Diaz et al., 1993; Oechel et al., 1994). Tissue and Oechel (1987) reported that *Erioporum vaginatum*-dominated tussock tundra physiologically adjusted to elevated [CO_2] (680 ppm) within 3 weeks, such that plants at ambient and elevated [CO_2] had similar photosynthetic rates. The authors suggested that carbohydrate sufficiency provided one explanation for the rapid ecosystem-level homeostatic adjustment to elevated [CO_2] (Oechel et al., 1994). Further mesocosm studies by Diaz et al. (1993) reported that even on productive soils, elevated [CO_2] led to nutrient deficiency in leaves due to increased N sequestration in soil microorganisms. These early, high-profile studies provided the early groundwork for the paradigm that stimulation of photosynthesis in elevated [CO_2] would be short-lived.

As described in our discussion of photosynthetic acclimation/optimization, a second paradigm from enclosure studies was that down-regulation of photosynthesis was correlated to an increase in leaf carbohydrate concentration and a subsequent dilution of leaf N concentration and protein concentration (Stitt and Krapp, 1999). Down-regulation was also attributed to rooting volume limitation, which limited sink demand and exacerbated nutrient limitation (Arp, 1991). In a survey of chamber experiments, Drake et al. (1997) found that a doubling of [CO_2] resulted in equal reductions of leaf protein concentration and Rubisco concentration. Therefore, the question of whether down-regulation would occur in the field remained, and there was little evidence for a selective acclimation of Rubisco to elevated [CO_2].

A third paradigm to emerge from chamber studies was that the direct effects of elevated [CO_2] on photosynthesis were much greater than the downstream effects on growth and yield (Grashoff et al., 1995; Wand et al., 1999). In a quantitative review of trees, Curtis and Wang (1998) reported a 54% stimulation of photosynthesis in trees grown under roughly twice current atmospheric [CO_2], greater than the 31% increase in leaf biomass and the 29% increase in total biomass. A quantitative review of 111 soybean studies reported that elevated [CO_2] (689 ppm on average) stimulated leaf CO_2 assimilation by 39%, total dry weight by 37% and seed yield by 24% (Ainsworth et al., 2002). Therefore, even in agronomic species selected for maximum investment in yield, several feedbacks appear to prevent full investment in reproduction, and considerable room for improvement exists (Ainsworth et al., 2002).

C. Results from FACE – Confirmatory and Surprising

Are these paradigms developed from studies in controlled environments supported by FACE studies? There is little evidence from FACE experiments on forests, deserts and perennial grasslands that the initial stimulation of photosynthesis is diminishing with time. Using a database of results from large-scale FACE experiments, the average response of A_{sat} in perennial species to elevated [CO_2] was calculated (for primary references, see Ainsworth and Long, 2005). Average stimulation of A_{sat} across these experiments ranged from 33% to 39% in elevated [CO_2] (grey bar in Fig. 29.8a), and there was no sign that this stimulation was diminishing over 10 years of experimentation (Fig. 29.8a). This general trend for all species is supported by work with individual species. Light-saturated photosynthesis of sun and shade leaves of over story *Liquidambar styraciflua* (L.) trees growing at the FACTS 1 FACE facility (Duke Forest, Orange County, NC, USA) was measured over the first 6 years of the experiment (Fig. 29.8b; Herrick and Thomas, 2001; Springer et al., 2005). The range of stimulation at elevated [CO_2] varies with position in the canopy and month of measurement, yet there is no indication that the response is weakening with time (Fig. 29.8b). The most comprehensive study of the effect of long-term growth at elevated [CO_2] comes from a 10-year FACE study in Eschikon, Switzerland of *Lolium perenne* grown under two N fertilization levels, and abrupt changes in source:sink balance following periodic harvests (Ainsworth et al., 2003).

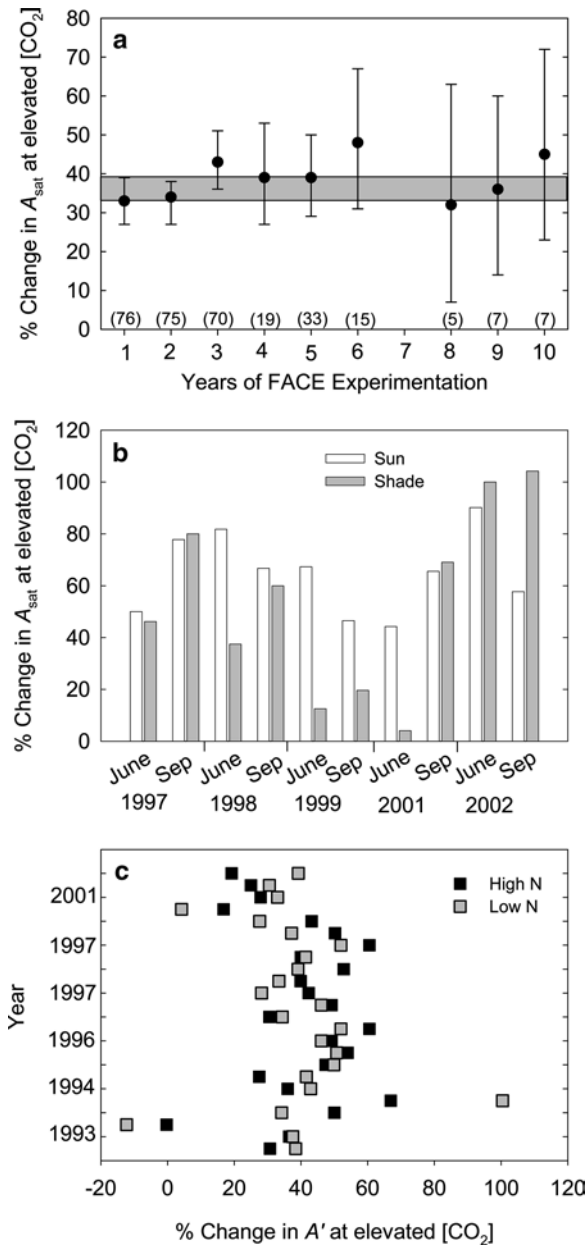

Fig. 29.8. Evidence that the increase in photosynthesis with growth at elevated [CO_2] is sustained over time. (**a**) Results from a meta-analysis of 12 large-scale FACE experiments investigating changes in A_{sat} over multiple years of experimentation. The mean ±95% confidence intervals are shown for each year. Degrees of freedom for each year are in parenthesis. The *grey bar* represents the overall 10-year average ±95% confidence interval. Note that there is no trend towards a decrease in stimulation of A_{sat} over time. (**b**) The percent change in light-saturated photosynthesis of *Liquidambar styraciflua* sun and shade leaves growing at the Duke FACE site from 1997 to 2002 (Adapted from Herrick and Thomas, 2001; Springer et al., 2005). (**c**) The percent change in the daily integral of carbon uptake in *Lolium perenne* grown under elevated [CO_2] at the Swiss FACE experiment from 1993 to 2001. (Adapted from Ainsworth et al., 2003.)

More than 3,000 measurements characterized the response of leaf photosynthetic rate to elevated [CO_2] over the 10 years of the experiment. On average, growth at elevated [CO_2] (600 ppm) increased the rate of light-saturated photosynthesis by 43% and increased the daily photosynthetic carbon gain (A') by 37% (Fig. 29.8c). The magnitude of stimulation in A' varied with time after harvests and with N fertilization treatment; however, the experiment provided no evidence of significant decline in leaf photosynthetic stimulation across the 10 year period (Fig. 29.8c) Therefore, the evidence from FACE suggests that stimulation of photosynthesis at elevated [CO_2] will not be short-lived.

Chamber studies have suggested that there is a general down-regulation of photosynthesis in plants grown at elevated [CO_2] in the long term (Drake et al., 1997). This concept is still invoked to explain ecosystem responses at elevated [CO_2] (Nowak et al., 2004). However, as described earlier, evidence is emerging to support the notion that decreased carboxylation capacity at elevated [CO_2] represents a physiological optimization (see Section II.B, above). This issue was re-addressed using a database of gas exchange studies from FACE experiments (Ainsworth and Long, 2005). On average, V_{cmax} was 12% lower in elevated [CO_2] (n = 273), while J_{max} was reduced by only 3% (n = 207) (Table 29.1). Thus, there was a small (5%) but significant reduction in the ratio of V_{cmax}:J_{max} (n = 136). Further, Rubisco content (mass/unit area) was reduced by 19% in elevated [CO_2], while N content (mass/unit area) was reduced by 5% (Ainsworth and Long, 2005; Table 29.1). Assuming Rubisco accounts for 25% of leaf N (Spreitzer and Salvucci, 2002), the 20% decrease in Rubisco could account for the small decrease in total N content. Therefore, evidence from FACE studies suggests that the decrease in Rubisco is specific, and not part of a general decrease in leaf protein.

Generally the increase in photosynthesis in FACE studies was greater than increases in biomass or yield (Nowak et al., 2004; Ainsworth and Long, 2005), yet the magnitude of the stimulations was lower than predicted. The average increase in light-saturated photosynthesis in 45 species measured at 11 different FACE studies was 31% (Table 29.1), while above-ground production increased by 17% and yield increased by 16% at elevated [CO_2] (Table 29.1). This trend held true

Table 29.1. Results of the meta-analysis of FACE effects on light-saturated CO_2 uptake (A_{sat}), maximum in vivo carboxylation rate (V_{cmax}), maximum rate of electron transport (J_{max}), ratio of V_{cmax}: J_{max}, Rubisco content (mass/unit area), N content (mass/unit area), above-ground dry matter production, and yield

Variable	df	Number of species	Effect size (E)	Lower 95% CI	Upper 95% CI
A_{sat}	414	43	1.31	1.28	1.34
C_3	380	38	1.33	1.30	1.36
C_4	33	5	1.09	1.02	1.17
V_{cmax}	272	26	0.88	0.86	0.91
J_{max}	206	20	0.96	0.94	0.99
V_{cmax}: J_{max}	135	19	0.96	0.95	0.97
Rubisco content	23	7	0.81	0.69	0.94
N content	123	21	0.95	0.92	0.98
Dry matter production	178	25	1.17	1.15	1.20
C_3	133	20	1.20	1.17	1.23
C_4	12	5	1.03	0.96	1.11
Yield	35	7	1.16	1.10	1.22
C_3	23	5	1.20	1.14	1.27
C_4	11	2	1.05	0.97	1.13

df degrees of freedom, *CI* confidence interval

for both species with the C_3 photosynthetic pathway, where photosynthesis is directly stimulated by increased [CO_2], and species with the C_4 pathway, where photosynthesis is only indirectly stimulated by high [CO_2] under conditions of water stress (Leakey et al. 2004, 2006b; Table 29.1). Increased photosynthesis does not necessarily translate into increased biomass and yield in plants grown at elevated [CO_2] in FACE experiments, supporting the notion that there is considerable scope for improving the capacity of plants to utilize the potential benefits of high [CO_2], both in terms of carbon uptake and storage in ecosystems and crop yields. However, there is a significant energy (i.e., respiratory) cost associated with the increase in carbon gain at elevated [CO_2] (Leakey et al. 2009) that ultimately limits the efficiency with which the increase in carbon gain can be converted to biomass and yield.

D. Direct Comparison of FACE and Chambers

A meta-analysis of 111 elevated [CO_2] chamber studies with soybean (Ainsworth et al., 2002) and results from the SoyFACE experiment (Ainsworth et al., 2004; Rogers et al., 2004; Bernacchi et al., 2005a) provide a direct comparison of FACE and chamber studies in a single, agronomically important species. When limiting the database of chamber studies to those with elevated [CO_2] treatments between 450 and 550 ppm, the average increase in A_{sat} was 24% and decrease in g_s was 8%, although that decrease was not statistically significant (Fig. 29.9). Bernacchi et al. (2005a) measured A_{sat} every 2 weeks throughout the growing season at SoyFACE in 2001 and 2002 and reported a slightly smaller 18% increase in elevated [CO_2]. A meta-analysis of SoyFACE results indicates an average 16% increase in A_{sat} across a variety of cultivars, which is smaller than suggested by chamber studies (Fig. 29.9). At SoyFACE, the largest stimulation in A_{sat} occurred during grain-filling (Bernacchi et al., 2005a); however, there was no significant difference in the stimulation of photosynthesis at different developmental stages in the chamber studies, averaging 22% during vegetative growth, 29% during pod-fill and 26% during flowering. Over the course of the first growing season at SoyFACE, midday g_s was reduced by 21.9% in elevated [CO_2] (Rogers et al. 2004). Stomatal conductance under saturating light conditions was reduced on average by 14% at SoyFACE (Fig. 29.9; Ainsworth et al., 2004; Bernacchi et al., 2005a). Canopy photosynthesis was stimulated by 59% in soybeans grown under elevated [CO_2] in controlled

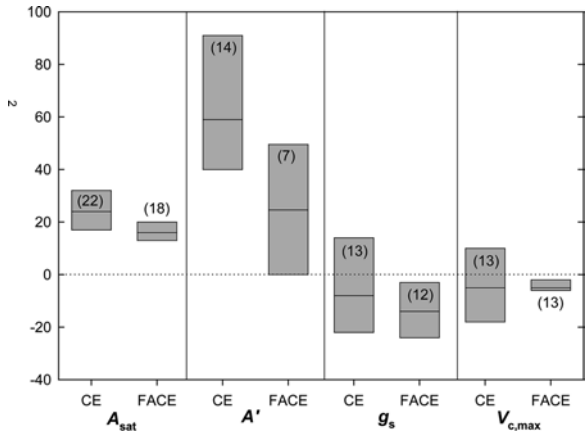

Fig. 29.9. A comparison of light-saturated photosynthesis (A_{sat}), daily carbon uptake (A'), stomatal conductance (g_s), and in vivo maximum carboxylation rate (V_{cmax}) from a meta-analysis of controlled environment (CE) studies of soybean grown at elevated [CO_2] (Adapted from Ainsworth et al., 2002) and soybeans grown at elevated [CO_2] in a Free Air Concentration Enrichment (FACE) experiment (Ainsworth et al., 2004; Rogers et al., 2004; Bernacchi et al., 2005b). The mean ± 95% confidence intervals are shown in the *box plots* and the sample size for each observation is given in parenthesis. *Boxes* overlapping 0 indicate no significant change at elevated [CO_2].

environments, with an average elevated [CO_2] treatment of 761 ppm. Although the SoyFACE treatment was somewhat lower (550 ppm), the degree of enhancement in daily carbon uptake at SoyFACE was considerably lower, only 25% over the course of the first growing season (Rogers et al., 2004), perhaps reflecting differences in reduction of g_s. The database of chamber studies suggests that photosynthetic capacity does not change in soybeans grown under elevated [CO_2]. Neither V_{cmax} nor the apparent quantum yield of photosynthesis was altered under elevated [CO_2] (Fig. 29.9). However, there was a small, yet statistically significant decrease in V_{cmax} at SoyFACE, which in turn drove a decrease in the V_{cmax}:J_{max} inferring a shift in resource investment away from Rubisco (Bernacchi et al., 2005a). In summary, there was rough agreement in the response of light-saturated photosynthesis to elevated [CO_2] in FACE and chamber studies, but there were significant differences in the magnitude of the response of g_s to elevated [CO_2] and acclimation of photosynthetic capacity to elevated [CO_2].

V. Influence of Plant Responses to Elevated [CO_2] on Global Carbon Cycle and Climate

A. Photosynthetic Feedback on the Carbon Cycle

Extensive evidence suggests that photosynthesis will be stimulated as atmospheric [CO_2] rises in the future, and this will impact plant function in both natural and managed ecosystems. Less well understood is how plant responses to elevated [CO_2] will feedback to impact the future global carbon cycle. Terrestrial gross primary productivity of ~120 petagrams of carbon per year (Pg C/yr) means that ~16% of the total pool of carbon in the atmosphere (730 Pg C) is photosynthetically assimilated by terrestrial plants each year (Prentice et al., 2001). Meanwhile, respiration from autotrophic and heterotrophic terrestrial organisms releases almost as much carbon back into the atmosphere each year. These fluxes are large compared to an annual release of carbon from fossil fuel burning of ~8.4 Pg C/yr (Canadell et al., 2007). Gross primary productivity (GPP) was greater than total respiration during the last 20 years of the twentieth century, making the terrestrial biosphere a carbon sink (Schimel et al., 2001). If elevated [CO_2] increases photosynthesis and in turn the magnitude of GPP relative to gross terrestrial respiration, the growing terrestrial carbon sink will get larger. The potential for such carbon sequestration has been studied by measuring how the difference between GPP and plant respiration, which is defined as net primary productivity (NPP), responds when FACE technology is used to fumigate whole ecosystems with elevated [CO_2]. Forest ecosystems are responsible for ~80% of terrestrial NPP (Field et al., 1998). On average, NPP was increased 23% by growth at [CO_2] of 550 versus 372 ppm in temperate forest ecosystems that had reached canopy closure and were dominated by aspen and birch, pine, poplar or sweet gum (Norby et al., 2005). Agricultural ecosystems have a lower NPP than forests but with appropriate management can represent a small carbon sink (Schimel et al., 2000; Bernacchi et al., 2005b). Above-ground NPP of soybean increased by 17% when grown at elevated [CO_2] compared to ambient [CO_2] (Morgan et al., 2005),

suggesting modest potential for greater carbon sequestration in the future. However, NPP only represents the net flux of carbon into an ecosystem. The carbon must be stored in plant biomass or the soil if it is to be sequestered in the long-term rather than re-released to the atmosphere via respiration. The degree to which this occurs is currently unclear (Thompson et al., 2004; Norby et al., 2005). Pine trees growing at elevated [CO_2] sequester carbon primarily in larger stems, while sweet gum allocates more carbon to fine root biomass (Norby et al., 2005). This suggests that there will be variation in the amount of carbon sequestered by ecosystems even when elevated [CO_2] stimulates NPP to a similar extent. Measurements of carbon storage in soils under elevated [CO_2] in FACE experiments have produced mixed results (Hungate et al., 1996; Van Kessel et al., 2000; Schlesinger and Lichter, 2001; Van Groenigen et al., 2002). Stimulation of NPP by elevated [CO_2] was sustained over 6 years in a pine plantation and lead to greater inputs of litter-fall and fine root turnover into soil organic matter (Lichter et al., 2005). Nonetheless, the soil fractions receiving larger inputs of carbon had short turnover times, while more recalcitrant soil fractions received smaller inputs of carbon. This was interpreted to suggest that forests would not sequester sufficient carbon to offset anthropogenic emissions (Lichter et al., 2005). Elevated [CO_2] stimulates photosynthesis of C_3 species in all functional groups (Ainsworth and Long, 2005). However, FACE experiments have not been conducted in tropical or boreal forests and the impact of growth at elevated [CO_2] on carbon cycling in these systems has not been determined. However, a modeling analysis suggests the potential for variation in response between biomes is high, given that the stimulation of NPP in tropical forests might be more than twice as great as in boreal forests (Hickler et al., 2008). The stimulation of photosynthesis, and therefore GPP, will saturate at some elevated [CO_2] in the future (Farquhar et al., 1980; Cao and Woodward, 1998). A climate-carbon cycle model has been used to compare scenarios in which (1) stimulation of NPP by rising [CO_2] is sustained and the terrestrial biosphere remains a carbon sink for the remainder of this century, and (2) NPP becomes saturated with respect to [CO_2] in 2000 and the terrestrial biosphere becomes a carbon source in 2050 (Thompson et al., 2004). The latter scenario results in 40% higher atmospheric [CO_2] in 2100. Reality is likely to fall somewhere between these extreme scenarios, so further work is needed to constrain the estimates of how enhanced photosynthesis at elevated [CO_2] will scale to greater NPP and how much of this carbon will remain in plant and soil pools. It has been hypothesized that this will depend on whether microbial activity and nutrient turnover at the ecosystem scale can be maintained and not start to limit productivity (Zak et al., 2000; Luo et al., 2004). Even after 5 years of growth at elevated [CO_2], progressive N-limitation was not observed in a pine plantation (Finzi et al., 2006). The potential for enhanced photosynthesis at elevated [CO_2] to feedback and reduce the rate at which atmospheric [CO_2] is rising is clear, but whether this potential will be realized depends on long-term phenomena and is still unclear.

B. Stomatal Feedbacks to Climate

Stomatal responses at the leaf level are well-documented but, as stated above, the extent of our knowledge is poorer at the canopy and field/ecosystem levels. Nevertheless, the potential exists for stomatal effects to influence the climate of certain regions. Locally, higher canopy temperatures and higher humidity associated with growth in elevated [CO_2] atmospheres implies that the temperature of the air column above plant canopies will generally increase. As a result, warming due to elevated [CO_2] may be greater than that previously predicted (Houghton et al., 2001) due to the direct effects of the greenhouse gases on the physiology of plants. Therefore, regardless of global warming, there may be shifts in the optimal geographic regions for the production of major crops, as well as shifts in natural ecosystems. The decrease in canopy-scale E with elevated [CO_2] may also affect regional precipitation patterns, particularly in intercontinental regions where transpiration from vegetation may account for a large fraction of the moisture in the atmosphere (Bernacchi et al. 2007). Therefore, there is strong reason to believe that vegetation responses to future atmospheric conditions will act to decrease precipitation, as suggested by previous model predictions (Sellers et al., 1997).

VI. Lessons from Modeling Photosynthesis

A. Modifying the Kinetics of Rubisco for Higher CO_2 Uptake Rate

Over evolutionary time, adaptation occurs to maintain photosynthetic efficiency or to take advantage of the environmental conditions. An adaptation of plant structure related to photosynthesis is exemplified by the 'Kranz structure' of C_4 plants, which evolved in multiple, independent events in response to a reduction in atmospheric CO_2 levels that began during the Cretaceous and continued until the Miocene (Ehleringer et al., 1991). The kinetic parameters of enzymes in photosynthesis show adaptation over a geological time scale as well as the different kinetic parameters of Rubiscos from organisms of different ecological niches (Bainbridge et al., 1995; Zhu et al., 2004). In addition to changes in the N distribution between Rubisco and capacity of RuBP regeneration, what are the optimum kinetic properties for Rubisco for higher photosynthetic CO_2 uptake rate under both current and future elevated CO_2 conditions?

It has long been recognized that the genetic modification of Rubisco to increase the specificity for CO_2 relative to O_2 (τ) would decrease photorespiration and potentially increase C_3 crop productivity. However, when the kinetic properties of Rubisco across photosynthetic organisms are compared, it appears that the forms with high τ have low maximum catalytic rates of carboxylation per active site (k^c_{cat}) (Bainbridge et al., 1995). Theoretical considerations also suggest that increased τ might only be achieved at the expense of k^c_{cat}. If a fixed inverse relationship between k^c_{cat} and τ implied from measurements is assumed and if increased concentration of Rubisco per unit leaf area is not an option, will increased τ result in increased leaf and canopy photosynthesis? Zhu et al. (2004) used a mathematical model to explore this question. From τ and k^c_{cat} reported for Rubiscos from diverse photosynthetic organisms, an inverse relationship of k^c_{cat} on τ was defined. Following the steady-state biochemical model of leaf photosynthesis (Farquhar et al., 1980), A is either limited by V_{cmax} or the rate of regeneration of RuBP, and in turn, the rate of whole chain electron transport (J). If J is limiting, an increase in τ will increase net CO_2 uptake due to products of the electron transport chain being partitioned away from photorespiration into photosynthesis. The effect of an increase in τ on Rubisco-limited photosynthesis depends on both k^c_{cat} and $[CO_2]$. There are conflicting consequences at the level of the canopy when τ is modified: increased τ would increase light-limited photosynthesis, while the associated decrease in k^c_{cat} would lower the light-saturated rate of photosynthesis. Zhu et al. (2004) simulated the consequences of variation in τ assuming an inverse change in k^c_{cat} for carbon gain by crop canopies. Increase in τ results in an increase in leaf CO_2 uptake at low light, but decreases CO_2 uptake in high light. Over the course of a day, total crop canopy CO_2 uptake (A_c') results from significant amounts of both light-limited and light-saturated photosynthesis. Simulation of A_c' suggests that the present average τ found in C_3 terrestrial plants is supra-optimal for the present atmospheric $[CO_2]$ of 370 ppm, but would be optimal for 200 ppm, a value remarkably close to the average of the last 400,000 years. This suggests that Rubisco in higher terrestrial plants has adapted to the past atmospheric $[CO_2]$, but further adaptation has been slow and has failed to change in response to the relatively rapid rise in $[CO_2]$ that has occurred since the start of the Industrial Revolution.

The theory that increased $[CO_2]$ favors the selection of Rubiscos with increased k^c_{cat} and decreased τ is consistent with the observation that Rubisco from C_4 plants, where the enzyme functions in high $[CO_2]$, typically has a higher k^c_{cat} and lower τ than in C_3 land plants (Seemann et al. 1984; Sage, 2002). Galmes et al. (2005) observed that lower $[CO_2]$ is found in the mesophyll of plants from saline and arid habitats, providing evidence for selection of higher τ forms of Rubisco in some C_3 species. Modeling by Zhu et al. (2004) showed that if Rubisco from the non-green algae *Griffithsia monilis* could be expressed in place of present C_3 crop Rubiscos, then canopy carbon gain could be increased by 27% (Table 29.2). These simulations suggest that very substantial increases in crop carbon gain could result if exotic forms of Rubisco could be successfully expressed in C_3 plants. However, if specificity can only be increased at the expense of catalytic rate, a decrease in specificity rather than an increase will improve C_3 crop carbon gain. Ideally a crop would

Table 29.2. Estimates of the daily canopy carbon gain (A_c') after Zhu et al. (2004) and assuming the hypothetical replacement of the average form of Rubisco from C_3 crop species with Rubiscos from other species. Reported values for k^c_{cat} of these species (Jordan and Ogren, 1984; Seeman et al., 1984; Whitney et al., 2001) are listed. The final row extends on the results of Zhu et al. (2004) to simulate the gain that could be achieved if a form of Rubisco with a high k^c_{cat} (*Amaranthus edulis*) could be expressed in the sunlit leaves and a form with high τ (current C_3 average) in the shade leaves

Species	A_c' (mmol m^{-2} day^{-1})	(%)	A_{sat} (μmol m^{-2} s^{-1})
Current average C_3 crop	1,040	100	14.9
(k^c_{cat} = 2.5, τ = 92.5)			
Griffithsia monilis	1,430	127	21.5
(k^c_{cat} = 2.6, τ = 167)			
Amaranthus edulis	1,250	117	28.3
(k^c_{cat} = 7.3, τ = 82)			
Amaranthus edulis/current	1,360	131	28.3
(k^c_{cat} = 7.3, τ = 82)			
(k^c_{cat} = 2.5, τ = 92.5)			

express a high k^c_{cat} Rubisco in the upper canopy leaves exposed to full sunlight and a high τ Rubisco in the shaded lower canopy leaves. Most C_3 annual crop canopies form leaves at progressively higher levels, such that leaves start life at the top of the canopy and become progressively shaded as new leaves form above. Shading is sensed in plant leaves by the balance of red/far-red light via the phytochrome system (Gilbert et al., 2001). One possibility would be for plants to trigger the replacement of a high k^c_{cat} Rubisco with a high τ form as the leaf acclimates to shade. Table 29.2 shows that such a system could increase A_c' by 31%, by comparison to an equivalent crop canopy with the current typical Rubisco of C_3 crops. This would increase A_c' both by decreasing photorespiratory losses in the lower canopy and increasing the light-saturated rate of photosynthesis in the upper canopy.

VII. Conclusion

Large-scale FACE experiments provide the best approach for studying the effects of atmospheric change on plant performance (see Section IV). Therefore, throughout this chapter data from FACE experiments are reviewed to provide an estimate of how elevated [CO_2] will affect photosynthesis in the future. However, data from past and present FACE experiments are limited in eight regards. First, deployment of large-scale FACE experiments has been limited to temperate croplands, grasslands, young forests and a temperate desert site. There have been no tropical FACE experiments despite the fact that future food supply and human well-being will depend heavily on crop performance in the tropics, and >50% of global terrestrial net primary productivity comes from tropical forests and grasslands (Prentice et al., 2001). New FACE experiments are needed in these biomes to facilitate reliable estimates of food security and biogeochemistry at the global scale. Second, FACE experiments have been established at very few, or in most cases, only one site for each crop species or ecosystem under investigation, so additional sites are needed to account for field and regional scale variation in abiotic and biotic conditions. Third, the longest running FACE experiment to date was only 10 years in duration (Ainsworth et al., 2003). Models suggest that nutrient limitation at the ecosystem scale will limit stimulation of productivity by elevated [CO_2] in the long term (Luo et al., 1994). Therefore, long-term experiments are essential if this important prediction is to be empirically tested, especially in forest ecosystems. Long-term experiments are also needed because inter-annual variation in climate significantly impacts plant responses to elevated [CO_2] (Moore et al., 2006), meaning that multi-year studies can reveal different findings to shorter-term experiments at the same site (Oren et al., 2001).

Fourth, most FACE experiments have investigated only the interaction of CO_2 with treatments of one or two other treatment variables, and only in 2 × 2 factorial or 2 factor split-plot designs. However, global climate change is a multifaceted phenomenon including changes in [CO_2], tropospheric [O_3], rainfall, temperature, N deposition and pest invasion, to name a few. Determining the combined effects of these changes is a high priority that could be achieved by expanding the size of FACE facilities. While additional treatments cannot be progressively added to studies of perennial ecosystems, existing experiments on annual crops could be expanded without compromising experimental design or committing the resources to build an entirely new facility. Fifth, the expense of FACE experiments has restricted replication to 3–4 plots of each CO_2 treatment. This may limit the power of experiments to detect subtle but important responses of plant or ecosystem processes to elevated [CO_2] particularly in natural ecosystems. Sixth, FACE studies to date have only compared photosynthesis at ambient [CO_2] with photosynthesis at a single elevated [CO_2], projected to occur in the middle of this century. Multiple elevated [CO_2] treatments would be invaluable for parameterization and validation of physiological and ecosystem models. These first six limitations are the result of the high cost of construction and operation of FACE experiments, but these could be addressed by an increased priority for funding of FACE research. The seventh and eighth limitations of FACE experiments are harder to overcome and are shared by experiments using other fumigation technologies, at least to some extent. While climate change involves a gradual increase in [CO_2] over years, FACE experiments impose a step increase in [CO_2]. This may constitute a perturbation that initially leads to a burst of carbon sequestration and N utilization, which then diminishes over time (Luo and Reynolds, 1999). While this may not be as serious a concern in annual ecosystems where the plants experience elevated [CO_2] throughout their life times, it is a concern in short-term studies of perennial systems fumigated by either FACE or enclosures. Finally, most elevated [CO_2] experiments do not control the [CO_2] in the ambient [CO_2] treatment. Therefore, the ambient [CO_2] has risen from ~330 ppm in the 1970s to ~380 ppm in 2006. The relative magnitude of photosynthetic stimulation at 550 ppm compared to ambient [CO_2] will therefore decline in experiments conducted at a later date. This creates some difficulties for synthesis of data across experiments and would be expected to diminish treatment effects over time if individual FACE studies are successfully operated over decades (Rogers et al., 2006). Some enclosure studies have fumigated plants with a mixture of [CO_2] free-air and a controlled injection of [CO_2] to a set-point matching ambient [CO_2]. However, this becomes increasingly challenging and expensive in larger facilities and open-air sites. In summary, while much high-quality data is now available from 19 years of FACE experimentation, advances in scale and technology are required to establish understanding of processes key to future food supply and ecosystem function.

Despite the limited scope of the existing dataset, the investment in conducting FACE experiments to simulate future atmospheric conditions in large plots, under field conditions with minimal microclimate perturbation is justified by the important differences in plant responses to elevated [CO_2] that they reveal, compared to enclosure studies. Long et al. (2006) and Ainsworth et al. (2008a) compared results from enclosure studies of yield response to elevated [CO_2] in soybean (115 studies), wheat (211 studies) and the C_4 crops maize and sorghum (14 studies) with results from the corresponding FACE experiments. Soybean yield at elevated [CO_2] (550 ppm) was stimulated relative to ambient [CO_2] by 32% in chamber studies but only 14% in FACE studies. Wheat yield at elevated [CO_2] (550 ppm) was stimulated relative to ambient [CO_2] by 31% in chamber studies but only 13% in FACE studies. The crop yield of C_4 plants at elevated [CO_2] (550 ppm) was stimulated relative to ambient [CO_2] by 18% in chamber studies but not stimulated in FACE studies. This suggests model predictions of future global food supply may be overly optimistic as they include CO_2 effects based on data from chamber studies, which were summarized in literature reviews during the 1980s (Kimball 1983; Cure and Acock, 1986; Allen et al., 1987). In addition, the smaller stimulation of daily photosynthetic carbon gain, biomass accumulation and yield by elevated [CO_2] in FACE experiments than enclosure studies (Table 29.3) suggests output from models of canopy, ecosystem and global

Table 29.3. Percentage increases in yield, biomass and photosynthesis of crops grown at elevated [CO_2] (550 μmol mol^{-1}) relative to ambient [CO_2] in enclosure studies versus FACE experiments. (Data were summarized by Kimball, 1983; Cure and Acock, 1986; Allen et al., 1987 and Long et al., 2006.)

	Yield			
Source	Rice	Wheat	Soybean	Maize
Enclosure studies in Kimball (1983)	19	28	21	
Enclosure studies in Cure and Acock (1986)	11	19	22	27
Enclosure studies in Allen et al. (1987)			26	
Enclosure studies in Long et al. (2006)		31	32	18
FACE studies in Long et al. (2006)	***12***	***13***	***14***	***0***[a]
Biomass				
Enclosure studies in Cure and Acock (1986)	21	24	30	8
Enclosure studies in Allen et al. (1987)			35	
FACE studies in Long et al. (2006)	***13***	***10***	***25***	***0***[a]
Photosynthesis				
Enclosure studies in Cure and Acock (1986)	35	21	32	4
FACE studies in Long et al. (2006)	***9***	***13***	***19***	***6***

Mean response ratios from these reviews were adjusted to an elevated [CO_2] of 550 μmol mol^{-1} using the non-rectangular hyperbolic functions for C_3 and C_4 species in Long et al., 2006

[a]Data from only 1 year in Leakey et al., 2006b

carbon balance may need to be re-assessed, and that the terrestrial carbon sink may not act to decrease atmospheric [CO_2] as much as previously thought. On the other hand, g_s is decreased more by growth at elevated [CO_2] in FACE experiments than enclosure studies (Long et al., 2004). This corresponds with observations of savings in plant water use, and in turn indirect enhancement of carbon gain through amelioration of drought stress. Likewise, enhanced leaf area index (LAI) under elevated [CO_2] has not been consistently observed in FACE experiments, but in accordance with results from enclosure experiments (Saralabai et al., 1997) is assumed to occur as a consequence of greater photosynthesis in models of biosphere carbon balance (e.g., Foley et al., 1996). This could lead to errors in fluxes of carbon, water and energy between vegetation and the atmosphere.

The smaller than expected benefits of plant growth at elevated [CO_2] under field conditions have unfavorable implications when put in context of the perturbation to food supply and ecosystem services that is likely to result from future changes in tropospheric ozone, temperature, rainfall and N. However, since the stimulation of photosynthesis in FACE experiments is smaller not only than enclosure studies, but also than theoretical expectation (Long et al., 2006) it suggests manipulation of plant physiology by traditional breeding or genetic modification may enhance the positive effects of elevated [CO_2] on photosynthesis, productivity and yield (Ainsworth et al., 2008). At the leaf level, reallocation of resources within the leaf to decrease V_{cmax} while increasing J_{max} could theoretically increase the stimulation of photosynthesis at elevated CO_2 (Fig 29.3). The absence of an increase in LAI in herbaceous plants grown at elevated [CO_2] limits the enhancement of carbon gain at the canopy scale. Changes in plant allometry, which bypass this limitation, might also be possible. As plant material becomes an increasingly important fuel source (Perlack et al., 2005), development of biomass crops, which convert sunlight into biomass with maximum efficiency, will have a significant impact on energy security and sustainable development. Critically, harvest index, or the ratio of harvestable mass to total plant biomass, often decreases at elevated [CO_2] (e.g., Ainsworth et al., 2002; Morgan et al., 2005) indicating that although more carbon is assimilated there is a bottleneck in the translocation of carbon to seeds. Having identified these constraints on enhancement of plant performance under elevated [CO_2] there is an opportunity to gain further understanding of the mechanism behind them and then act to circumvent them.

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References

Ainsworth EA and Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. New Phytol 165: 351–371

Ainsworth EA, Davey PA, Bernacchi CJ, Dermody OC, Heaton EA and Moore DJ, Morgan PB, Naidu SL, Ra HSY, Zhu XG, Curtis PS, Long SP (2002) A meta-analysis of elevated [CO_2] effects on soybean (*Glycine max*) physiology, growth and yield. Glob Change Biol 8: 695–709

Ainsworth EA, Leakey ADB, Ort DR and Long SP (2008a) FACE-ing the facts: inconsistencies and interdependence among field, chamber and modeling studies of elevated [CO_2] impacts on crop yield and food supply. New Phytol 179: 5–9

Ainsworth EA, Rogers A, Blum H, Nosberger J and Long SP (2003) Variation in acclimation of photosynthesis in *Trifolium repens* after eight years of exposure to free air CO_2 enrichment (FACE). J Exp Bot 54: 2769–2774

Ainsworth EA, Rogers A and Leakey ADB (2008) Targets for crop biotechnology in a future high-CO_2 and high-O_3 world. Plant Physiol 147:13–19

Ainsworth EA, Rogers A, Nelson R and Long SP (2004) Testing the "source-sink" hypothesis of down-regulation of photosynthesis in elevated CO_2 in the field with single gene substitutions in *Glycine max*. Ag Forest Meteorol 122: 85–94

Allen LH, Boote KJ, Jones JW, Jones PH, Valle RR, Acock B, Rogers HH and Dahlman RC (1987) Response of vegetation to rising carbon dioxide: Photosynthesis, biomass and seed yield of soybean. Glob Biogeochem Cycles 1: 1–14

Allen RG, Pereira LS, Raes D and Smith M (1998) Crop evapotranspiration - guidelines for computing crop water requirements - FAO irrigation and drainage paper 56. In: Food and Agriculture Organisation of the United Nations, Rome

Allen SG, Idso SB and Kimball BA (1990) Interactive effects of CO_2 and environment on net photosynthesis of water-lily. Ag Ecosyst Environ 30: 81–88

Amthor JS, Mitchell RJ, Runion GB, Rogers HH, Prior SA and Wood CW (1994) Energy content, construction cost and phytomass accumulation of *Glycine max* (L) Merr and *Sorghum bicolor* (L) Moench grown in elevated CO_2 in the field. New Phytol 128: 443–450

Anderson LJ, Maherali H, Johnson HB, Polley HW and Jackson RB (2001) Gas exchange and photosynthetic acclimation over subambient to elevated CO_2 in a C_3-C_4 grassland. Glob Change Biol 7: 693–707

Archibold OW (1995) Ecology of world vegetation. Chapman and Hall, London

Arp WJ (1991) Effects of source-sink relations on photosynthetic acclimation to elevated CO_2. Plant Cell Environ 14: 869–875

Assmann SM (1999) The cellular basis of guard cell sensing of rising CO_2. Plant Cell Environ 22: 629–637

Bainbridge G, Madgwick P, Parmar S, Mitchell R, Paul M, Pitts J, Keys AJ and Parry MAJ (1995) Engineering Rubisco to change its catalytic properties. J Exp Bot 46: 1269–1276

Ball JT, Woodrow IE and Berry JA (1987) A model predicting stomatal conductance and its contribution to the control of photosynthesis under different environmental conditions. In: Biggins J (ed) Progress in Photosynthesis Research, pp 221–224. Martinus-Nijhoff Publishers, Dordrecht

Barnola JM, Anklin M, Porcheron J, Raynaud D, Schwander J and Stauffer B (1995) CO_2 evolution during the last millennium as recorded by Antarctic and Greenland ice. Tellus B 47: 264–272

Bazzaz FA, Sombroek WG (1996) Global climate changes and agricultural production: an assessment of current knowledge and critical gaps. In: Bazzaz F, Sombroek W (eds) Global climate changes and agricultural production, pp 319–330. John Wiley and Sons, Chichester

Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S and Long SP (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiol 130: 1992–1998

Bernacchi CJ, Calfapietra C, Davey PA, Wittig VE, Scarascia-Mugnozza GE, Raines CA and Long SP (2003) Photosynthesis and stomatal conductance responses of poplars to free-air CO_2 enrichment (PopFACE) during the first growth cycle and immediately following coppice. New Phytol 159: 609–621

Bernacchi CJ, Hollinger SE and Meyers T (2005b) The conversion of the corn/soybean ecosystem to no-till agriculture may result in a carbon sink. Glob Change Biol 11: 1867–1872

Bernacchi CJ, Morgan PB, Ort DR and Long SP (2005a) The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing in vivo Rubisco capacity. Planta 220: 434–446

Bernacchi CJ, Kimball BA, Quarles DR, Long SP, Ort DR (2007) Decreases in stomatal conductance of soybean under open air elevation of [CO_2] are closely coupled with decreases in ecosystem evapotranspiration. Plant Physiology 143: 134–144

Bhattacharya N, Hileman D, Ghosh P, Musser R, Bhattacharya S and Biswas P (1990) Interaction of enriched CO_2 and water-stress on the physiology of and biomass production in sweet-potato grown in open-top chambers. Plant Cell Environ 13:933–940

Bowes G (1993) Facing the inevitable - plants and increasing atmospheric CO_2. Annu Rev Plant Physiol 44: 309–332

Brooks A and Farquhar G (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. Planta 165: 397–406

Brown RH (1999) Agronomic implications of C_4 photosynthesis. In: Sage RF and Monson RK (eds) C_4 Plant Biology, pp 473–503, Academic Press Limited, London

Buckley TN, Mott KA and Farquhar GD (2003) A hydromechanical and biochemical model of stomatal conductance. Plant Cell Environ 26: 1767–1785

Bunce JA (2004) Carbon dioxide effects on stomatal responses to the environment and water use by crops under field conditions. Oecologia 140: 1–10

Canadell JG, Le Quere C, Raupach MR, Field CB, Buitenhuis ET, Ciais P, Conway TJ, Gillett NP, Houghton RA and Marland G (2007). Contributions to accelerating atmospheric CO_2 growth from economic activity, carbon intensity, and efficiency of natural sinks. Proc Nat Acad Sci USA 104:18866–18870

Cao MK and Woodward FI (1998) Net primary and ecosystem production and carbon stocks of terrestrial ecosystems and their responses to climate change. Glob Change Biol 4: 185–198

Carcova J, Maddonni GA and Ghersa CM (2000) Long-term cropping effects on maize: Crop evapotranspiration and grain yield. Agron J 92: 1256–1265

Chaves M and Pereira J (1992) Water-stress, CO_2 and climate change. J Exp Bot 43: 1131–1139

Clifford S, Stronach I, Mohamed A, Azamali S and Crout N (1993) The effects of elevated atmospheric carbon-dioxide and water-stress on light interception, dry-matter production and yield in stands of groundnut (*Arachis-Hypogaea* L). J Exp Bot 44: 1763–1770

Collatz GJ, Ball JT, Grivet C and Berry JA (1991) Physiological and environmental-regulation of stomatal conductance, photosynthesis and transpiration - a model that includes a laminar boundary-layer. Agr Forest Meteorol 54: 107–136

Conley MM, Kimball BA, Brooks TJ, Pinter PJ, Hunsaker DJ, Wall GW, Adam NR, LaMorte RL, Matthias AD, Thompson TL, Leavitt SW, Ottman MJ, Cousins AB and Triggs JM (2001) CO_2 enrichment increases water-use efficiency in sorghum. New Phytol 151: 407–412

Coughenour MB and Chen DX (1997) Assessment of grassland ecosystem responses to atmospheric change using linked plant-soil process models. Ecol Appl 7: 802–827

Cousins AB, Adam NR, Wall GW, Kimball BA, Pinter PJ, Leavitt SW, LaMorte RL, Matthias AD, Ottman MJ, Thompson TL and Webber AN (2001) Reduced photorespiration and increased energy-use efficiency in young CO_2-enriched sorghum leaves. New Phytol 150: 275–284

Cousins AB, Adam NR, Wall GW, Kimball BA, Pinter PJ, Ottman MJ, Leavitt SW and Webber AN (2002) Photosystem II energy use, non-photochemical quenching and the xanthophyll cycle in *Sorghum bicolor* grown under drought and free-air CO_2 enrichment (FACE) conditions. Plant Cell Environ 25: 1551–1559

Cousins AB, Adam NR, Wall GW, Kimball BA, Pinter PJ, Ottman MJ, Leavitt SW and Webber AN (2003) Development of C_4 photosynthesis in sorghum leaves grown under free-air CO_2 enrichment (FACE). J Exp Bot 54: 1969–1975

Cure JD and Acock B (1986) Crop responses to carbon-dioxide doubling - a literature survey. Agr Forest Meteorol 38: 127–145

Curtis PS (1996) A meta-analysis of leaf gas exchange and nitrogen in trees grown under elevated carbon dioxide. Plant Cell Environ 19: 127–137

Curtis PS and Wang XZ (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. Oecologia 113: 299–313

Dai ZY, Ku MSB and Edwards GE (1995) C_4 photosynthesis - the effects of leaf development on the CO_2-concentrating mechanism and photorespiration in maize. Plant Physiol 107: 815–825

Day FP, Weber EP, Hinkle CR and Drake BG (1996) Effects of elevated atmospheric CO_2 on fine root length and distribution in an oak-palmetto scrub ecosystem in central Florida. Glob Change Biol 2: 143–148

Delgado E, Medrano H, Keys AJ and Parry MAJ (1995) Species variation in Rubisco specificity factor. J Exp Bot 46: 1775–1777

Dewar RC (2002) The Ball-Berry-Leunning and Trdieu-Davis stomata models: synthesis and extension with a spatially aggregated picture of guard cell function. Plant Cell Environ 25: 1383–1398

Diaz S, Grime JP, Harris J and McPherson E (1993) Evidence of a feedback mechanism limiting plant-response to elevated carbon-dioxide. Nature 364: 616–617

Drake BG, Leadley PW, Arp WJ, Nassiry D and Curtis PS (1989) An open top chamber for field studies of elevated atmospheric CO_2 concentration on saltmarsh vegetation. Funct Ecol 3: 363–371

Drake BG, GonzalezMeler MA and Long SP (1997) More efficient plants: A consequence of rising atmospheric CO_2? Annu Rev Plant Physiol 48: 609–639

Ehleringer JR, Sage RF, Flanagan LB and Pearcy RW (1991) Climate change and the evolution of C_4 photosynthesis. Trends Ecol Evol 6: 95–99

Etheridge DM, Steele LP, Langenfelds RL, Francey RJ, Barnola JM and Morgan VI (1996) Natural and anthropogenic changes in atmospheric CO_2 over the last 1000 years from air in Antarctic ice. J Geophys Res-Atmos 101: 4115–4128

Evans JR, von Caemmerer S, Setchell BA and Hudson GS (1994) The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. Aust J Plant Physiol 21: 475–495

Farage PK, McKee IF and Long SP (1998) Does a low nitrogen supply necessarily lead to acclimation of photosynthesis to elevated CO_2? Plant Physiol 118: 573–580

Farquhar GD and Sharkey TD (1982) Stomatal conductance and photosynthesis. Annu Rev Plant Physiol 33: 317–345

Farquhar GD and von Caemmerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149: 78–90

Farrar J, Pollock C and Gallagher J (2000) Sucrose and the integration of metabolism in vascular plants. Plant Sci 154: 1–11

Field CB and Avissar R (1998) Bidirectional interactions between the biosphere and the atmosphere - Introduction. Glob Change Biol 4: 459–460

Field CB, Behrenfeld MJ, Randerson JT and Falkowski P (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281: 237–240

Finzi AC, Moore DJP, DeLucia EH, Lichter J, Hofmockel KS, Jackson RB, Kim HS, Matamala R, McCarthy HR, Oren R, Pippen JS and Schlesinger WH (2006) Progressive nitrogen limitation of ecosystem processes under elevated CO_2 in a warm-temperate forest. Ecology 87: 15–25

Fitzsimons PJ and Weyers JDB (1986) Volume changes of *Commelina communis* guard-cell protoplasts in response to K^+, light and CO_2. Physiol Plantarum 66: 463–468

Flexas J, Ribas-Carbo M, Diaz-Espej A, Galmes J and Medrano H (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. Plant Cell Environ 31: 602–621

Foley JA, Prentice IC, Ramankutty N, Levis S, Pollard D, Sitch S and Haxeltine A (1996) An integrated biosphere model of land surface processes, terrestrial carbon balance, and vegetation dynamics. Glob Biogeochem Cycles 10: 603–628

Galmes J, Flexas J, Keys AJ, Cifre J, Mitchell RAC, Madgwick PJ, Haslam RP, Medrano H and Parry MAJ (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant Cell Environ 28: 571–579

Gao Q, Zhao P, Zeng X, Cai X and Shen W (2002) A model of stomata conductance to quantify the relationship between leaf transpiration, microclimate, and soil water stress. Plant Cell Environ 25: 1373–1381

Garcia RL, Long SP, Wall GW, Osborne CP, Kimball BA, Nie GY, Pinter PJ, Lamorte RL and Wechsung F (1998) Photosynthesis and conductance of spring-wheat leaves: field response to continuous free-air atmospheric CO_2 enrichment. Plant Cell Environ 21: 659–669

Ghannoum O, Siebke K, von Caemmerer S and Conroy JP (1998) The photosynthesis of young Panicum C_4 leaves is not C_3-like. Plant Cell Environ 21: 1123–1131

Ghannoum O, Caemmerer S, Ziska LH and Conroy JP (2000) The growth response of C_4 plants to rising atmospheric CO_2 partial pressure: a reassessment. Plant Cell Environ 23: 931–942

Ghannoum O, Evans JR, Chow WS, Andrews TJ, Conroy JP and von Caemmerer S (2005) Faster rubisco is the key to superior nitrogen-use efficiency in NADP-malic enzyme relative to NAD-malic enzyme C_4 grasses. Plant Physiol 137: 638–650

Gilbert I, Jarvis P and Smith H (2001) Proximity signal and shade avoidance differences between early and late successional trees. Nature 411: 792–795

Gillon J and Yakir D (2001) Influence of carbonic anhydrase activity in terrestrial vegetation on the O-18 content of atmospheric CO_2. Science 291: 2584–2587

Gotow K, Kondo N and Syono K (1982) Effect of CO_2 on volume change of guard-cell protoplast from *Vicia-faba* L. Plant Cell Physiol 23: 1063–1070

Grashoff C, Dijkstra P, Nonhebel S, Schapendonk A and VandeGeijn S (1995) Effects of climate change on productivity of cereals and legumes: model evaluation of observed year-to-year variability of the CO_2 response. Glob Change Biol 1:417–428

Gunderson CA, Sholtis JD, Wullschleger SD, Tissue DT, Hanson PJ and Norby RJ (2002) Environmental and stomatal control of photosynthetic enhancement in the canopy of a sweetgum (*Liquidambar styraciflua* L.) plantation during 3 years of CO_2 enrichment. Plant Cell Environ 25: 379–393

Harley PC and Sharkey TD (1991) An improved model of C_3 photosynthesis at high CO_2: reversed O_2 sensitivity explained by lack of glycerate reentry into the chloroplast. Photosynth Res 27: 169–178

Harley PC, Loreto F, Dimarco G and Sharkey TD (1992a) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. Plant Physiol 98: 1429–1436

Harley PC, Thomas RB, Reynolds JF and Strain BR (1992b) Modeling photosynthesis of cotton grown in elevated CO_2. Plant Cell Environ 15: 271–282

Herrick JD and Thomas RB (2001) No photosynthetic downregulation in sweetgum trees (*Liquidambar styraciflua* L.) after three years of CO_2 enrichment at the Duke Forest FACE experiment. Plant Cell Environ 24: 53–64

Herrick JD, Maherali H and Thomas RB (2004) Reduced stomatal conductance in sweetgum (*Liquidambar styraciflua*) sustained over long-term CO_2 enrichment. New Phytol 162: 387–396

Hickler T, Smith B, Prentice IC, Mjofors K, Miller P, Arneth A and Sykes MT (2008) CO_2 fertilization in temperate face experiments not representative of boreal and tropical forests. Global Change Biology 14(7): 1531–1542

Hocking PJ, Meyer CP (1991) Effects of CO_2 enrichment and nitrogen stress on growth, and partitioning of dry-matter and nitrogen in wheat and maize. Aust J Plant Physiol 18: 339–356

Houghton JT, Ding Y, Griggs DJ, Noguer M, Van der Linden PJ and Xiaosu D (eds) (2001) Climate Change 2001: The scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental

Panel on Climate Change. Cambridge University Press, New York

Hungate BA, Jackson RB, Field CB and Chapin FS (1996) Detecting changes in soil carbon in CO_2 enrichment experiments. Plant Soil 187: 135–145

Idso SB, Kimball BA, Akin DE and Kridler J (1993) A general relationship between CO_2-induced reductions in stomatal conductance and concomitant increases in foliage temperature. Environ Exp Bot 33: 443–446

Jarvis PG and McNaughton KG (1986) Stomatal control of transpiration - scaling up from leaf to region. Adv Ecol Res 15: 1–49

Jones P, Jones J and Allen L (1985) Seasonal carbon and water balances of soybeans grown under stress treatments in sunlit chambers. Trans Asae 28: 2021–2028

Jordan D B and Ogren W L (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Dependence on ribulose bisphosphate concentration, pH and temperature. Planta 161:308–313

Kimball BA (1983) Carbon dioxide and agricultural yields: an assemblage and analysis of 430 prior observations. Agron J 75: 779–788

Kimball BA, Pinter PJ, Garcia RL, LaMorte RL, Wall GW, Hunsaker DJ, Wechsung G, Wechsung F and Kartschall T (1995) Productivity and water use of wheat under free-air CO_2 enrichment. Glob Change Biol 1: 429–442

Kimball BA, LaMorte RL, Pinter PJ, Wall GW, Hunsaker DJ, Adamsen FJ, Leavitt SW, Thompson TL, Matthias AD and Brooks TJ (1999) Free-air CO_2 enrichment and soil nitrogen effects on energy balance and evapotranspiration of wheat. Water Resour Res 35: 1179–1190

Kirschbaum MUF (1994) The sensitivity of C_3 photosynthesis to increasing CO_2 concentration - a theoretical-analysis of its dependence on temperature and background CO_2 concentration. Plant Cell Environ 17: 747–754

Knapp AK, Hamerlynck EP and Owensby CE (1993) Photosynthetic and water relations responses to elevated CO_2 in the C_4 grass *Andropogon-Gerardii*. Int J Plant Sci 154: 459–466

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol 47: 509–540

Koroleva OA, Farrar JF, Tomos AD and Pollock CJ (1997) Patterns of solute in individual mesophyll, bundle sheath and epidermal cells of barley leaves induced to accumulate carbohydrate. New Phytol 136: 97–104

Koroleva OA, Farrar JF, Tomos AD and Pollock CJ (1998) Carbohydrates in individual cells of epidermis, mesophyll, and bundle sheath in barley leaves with changed export or photosynthetic rate. Plant Physiol 118: 1525–1532

Krapp A, Hofmann B, Schafer C and Stitt M (1993) Regulation of the expression of Rbcs and other photosynthetic genes by carbohydrates - a mechanism for the sink regulation of photosynthesis. Plant J 3: 817–828

Lawlor DW and Mitchell RAC (1991) The effects of increasing CO_2 on crop photosynthesis and productivity - a review of field studies. Plant Cell Environ 14: 807–818

Leakey ADB, Bernacchi CJ, Dohleman FG, Ort DR and Long SP (2004) Will photosynthesis of maize (*Zea mays*) in the US Corn Belt increase in future CO_2 rich atmospheres? An analysis of diurnal courses of CO_2 uptake under free-air concentration enrichment (FACE). Glob Change Biol 10: 951–962

Leakey ADB, Bernacchi CJ, Ort DR and Long SP (2006a) Long-term growth of soybean at elevated [CO_2] does not cause acclimation of stomatal conductance under fully open-air conditions. Plant Cell Environ 29:1794–1800

Leakey ADB, Uribelarrea M, Ainsworth EA, Naidu SL, Rogers A, Ort DR and Long SP (2006b) Photosynthesis, productivity, and yield of maize are not affected by open-air elevation of CO_2 concentration in the absence of drought. Plant Physiol 140: 779–790

Leakey ADB (2009) Rising atmospheric carbon dioxide concentrations and the future of C_4 crops for food and fuel. Proc Royal Soc B 276: 2333–2343.

Leakey, A.D.B., Xu, F., Gillespie, K.M., McGrath, J.M. Ainsworth, E.A., Ort, D.R., (2009) The genomic basis for stimulated respiration by plants growing under elevated carbon dioxide. Proc Nat Acad Sci USA 106: 3597–3602

Leavitt SW, Paul EA, Galadima A, Nakayama FS, Danzer SR, Johnson H and Kimball BA (1996) Carbon isotopes and carbon turnover in cotton and wheat FACE experiments. Plant Soil 187: 147–155

Lee TD, Tjoelker MG, Ellsworth DS and Reich PB (2001) Leaf gas exchange responses of 13 prairie grassland species to elevated CO_2 and increased nitrogen supply. New Phytol 150: 405–418

Leuning R (1995) A critical-appraisal of a combined stomatal-photosynthesis model for C_3 plants. Plant Cell Environ 18: 339–355

Leymarie J, Lasceve G and Vavasseur A (1999) Elevated CO_2 enhances stomatal responses to osmotic stress and abscisic acid in *Arabidopsis thaliana*. Plant Cell Environ 22: 301–308

Lichter J, Barron SH, Bevacqua CE, Finzli AC, Irving KE, Stemmler EA and Schlesinger WH (2005) Soil carbon sequestration and turnover in a pine forest after six years of atmospheric CO_2 enrichment. Ecology 86: 1835–1847

Long SP (1991) Modification of the response of photosynthetic productivity to rising temperature by atmospheric CO_2 concentrations - has its importance been underestimated? Plant Cell Environ 14: 729–739

Long SP and Drake BG (1991) Effect of the long-term elevation of CO_2 concentration in the field on the quantum yield of photosynthesis of the C_3 sedge, *Scirpus-olneyi*. Plant Physiol 96: 221–226

Long SP and Hutchin PR (1991) Primary production in grasslands and coniferous forests with climate change - an overview. Ecol Appl 1: 139–156

Long SP, Nie GY, Baker NR, Drake BG, Farage PK, Hendrey G, Lewin KH (1992) The implications of concurrent increases in temperature, CO_2 and tropospheric O_3 for terrestrial C_3 photosynthesis. Photosynth Res 34: 108–108

Long S, Osborne C, Humphries S (1997) Photosynthesis, rising atmospheric CO_2 concentration and climate change. In: Bremeyer A, Hall D, and Melillo J (eds) Scope 56: Global Change. Wiley, Chichester

Long SP, Ainsworth EA, Rogers A and Ort DR (2004) Rising atmospheric carbon dioxide: Plants face the future. Annu Rev Plant Biol 55: 591–628

Long SP, Ainsworth EA, Leakey ADB and Morgan PB (2005) Global food insecurity. Treatment of major food crops with elevated carbon dioxide or ozone under large-scale fully open-air conditions suggests recent models may have overestimated future yields. Philos T Roy Soc B 360: 2011–2020

Long SP, Ainsworth EA, Leakey ADB, Nosberger J and Ort DR (2006) Food for thought: lower than expected crop yield stimulation with rising carbon dioxide concentrations. Science 312: 1918–1921

Loreto F, Harley PC, Dimarco G and Sharkey TD (1992) Estimation of mesophyll conductance to CO_2 flux by 3 different methods. Plant Physiol 98: 1437–1443

Luo Y, Field CB and Mooney HA (1994) Predicting responses of photosynthesis and root fraction to elevated CO_2: Interaction among carbon, nitrogen and growth. Plant Cell Environ 17: 1195–1204

Luo YQ and Reynolds JF (1999) Validity of extrapolating field CO_2 experiments to predict carbon sequestration in natural ecosystems. Ecology 80: 1568–1583

Luo Y, Su B, Currie WS, Dukes JS, Finzi A, Hartwig U, Hungate B, McMurtrie RE, Oren R, Parton WJ, Pataki DE, Shaw MR, Zak DR and Field CB (2004) Progressive nitrogen limitation of ecosystem responses to rising atmospheric carbon dioxide. Bioscience 54: 731–739

Maherali H, Johnson HB and Jackson RB (2003) Stomatal sensitivity to vapour pressure difference over a subambient to elevated CO_2 gradient in a C_3/C_4 grassland. Plant Cell Environ 26: 1297–1306

Makino A, Harada M, Sato T, Nakano H and Mae T (1997) Growth and N allocation in rice plants under CO_2 enrichment. Plant Physiol 115: 199–203

Maroco JP, Breia E, Faria T, Pereira JS and Chaves MM (2002) Effects of long-term exposure to elevated CO_2 and N fertilization on the development of photosynthetic capacity and biomass accumulation in *Quercus suber* L. Plant Cell Environ 25: 105–113

Maroco JP, Edwards GE and Ku MSB (1999) Photosynthetic acclimation of maize to growth under elevated levels of carbon dioxide. Planta 210: 115–125

McLeod AR and Long SP (1999) Free-air carbon dioxide enrichment (FACE) in global change research: A review. In: Advances in Ecological Research, Vol 28, pp 1–56

McNaughton K and Jarvis P (1991) Effects of spatial scale on stomatal control of transpiration. Agr Forest Meteorol 54: 279–301

Medlyn BE, Barton CVM, Broadmeadow MSJ, Ceulemans R, De Angelis P, Forstreuter M, Freeman M, Jackson SB, Kellomaki S, Laitat E, Rey A, Roberntz P, Sigurdsson BD, Strassemeyer J, Wang K, Curtis PS and Jarvis PG (2001) Stomatal conductance of forest species after long-term exposure to elevated CO_2 concentration: a synthesis. New Phytol 149: 247–264

Meinzer FC (1993) Stomatal control of transpiration. Trends Ecol Evol 8: 289–294

Meinzer FC, Andrade JL, Goldstein G, Holbrook NM, Cavelier J and Jackson P (1997) Control of transpiration from the upper canopy of a tropical forest: the role of stomatal, boundary layer and hydraulic architecture components. Plant Cell Environ 20: 1242–1252

Messinger SM, Buckley TN and Mott KA (2006) Evidence for involvement of photosynthetic processes in the stomatal response to CO_2. Plant Physiol 140: 771–778

Moore BD, Cheng SH and Edwards GE (1986) The influence of leaf development on the expression of C_4 metabolism in *Flaveria trinervia*, a C_4 dicot. Plant Cell Physiol 27: 1159–1167

Moore BD, Cheng SH, Rice J and Seemann JR (1998) Sucrose cycling, Rubisco expression, and prediction of photosynthetic acclimation to elevated atmospheric CO_2. Plant Cell Environ 21: 905–915

Moore BD, Cheng SH, Sims D and Seemann JR (1999) The biochemical and molecular basis for photosynthetic acclimation to elevated atmospheric CO_2. Plant Cell Environ 22: 567–582

Moore DJP, Aref S, Ho RM, Pippen JS, Hamilton J and DeLucia EH (2006) Annual basal area increment and growth duration of *Pinus taeda* in response to eight years of free-air CO_2 enrichment. Global Change Biology. 12: 1367–1377

Morgan JA, LeCain DR, Read JJ, Hunt HW and Knight WG (1998) Photosynthetic pathway and ontogeny affect water relations and the impact of CO_2 on *Bouteloua gracilis* (C_4) and *Pascopyrum smithii* (C_3). Oecologia 114: 483–493

Morgan PB, Bollero GA, Nelson RL, Dohleman FG and Long SP (2005) Smaller than predicted increase in aboveground net primary production and yield of field-grown soybean under fully open-air [CO_2] elevation. Glob Change Biol 11: 1856–1865

Morison JIL (1987) Climatology, plant-growth and CO_2 history. Nature 327: 560–560

Mott KA (1988) Do stomata respond to CO_2 concentrations other than intercellular. Plant Physiol 86: 200–203

Nakano H, Makino A and Mae T (1997) The effect of elevated partial pressures of CO_2 on the relationship between photosynthetic capacity and N content in rice leaves. Plant Physiol 115: 191–198

Neftel A, Friedli H, Moor E, Lötscher H, Oeschger H, Siegenthaler U, Stauffer B (1994) Historical CO_2 record from the Siple station ice core. In: Boden T, Kaiser D, Sepanski R and Stoss F (eds) Trends '93: A Compendium of Data on Global Change, pp 11–14. Carbon Dioxide Inf. Anal. Cent., Oak Ridge

Nelson SD and Mayo JM (1975) The occurrence of functional non-chlorophyllous guard cells in *Paphiopedilum spp*. Can J Bot 53: 1–7

Nie GY, Hendrix DL, Webber AN, Kimball BA and Long SP (1995a) Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO_2 concentration in the field. Plant Physiol 108: 975–983

Nie GY, Long SP, Garcia RL, Kimball BA, Lamorte RL, Pinter PJ, Wall GW and Webber AN (1995b) Effects of free-air CO_2 enrichment on the development of the photosynthetic apparatus in wheat, as indicated by changes in leaf proteins. Plant Cell Environ 18: 855–864

Nijs I, Ferris R, Blum H, Hendrey G and Impens I (1997) Stomatal regulation in a changing climate: a field study using free air temperature increase (FATI) and free air CO_2 enrichment (FACE). Plant Cell Environ 20: 1041–1050

Noormets A, Sober A, Pell EJ, Dickson RE, Podila GK, Sober J, Isebrands JG and Karnosky DF (2001) Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (*Populus tremuloides* Michx.) clones exposed to elevated CO_2 and/or O_3. Plant Cell Environ 24: 327–336

Norby RJ, DeLucia EH, Gielen B, Calfapietra C, Giardina CP, King JS, Ledford J, McCarthy HR, Moore DJP, Ceulemans R, De Angelis P, Finzi AC, Karnosky DF, Kubiske ME, Lukac M, Pregitzer KS, Scarascia-Mugnozza GE, Schlesinger WH and Oren R (2005) Forest response to elevated CO_2 is conserved across a broad range of productivity. Proc Natl Acad Sci USA 102: 18052–18056

Nowak RS, Ellsworth DS and Smith SD (2004) Functional responses of plants to elevated atmospheric CO_2 - do photosynthetic and productivity data from FACE experiments support early predictions? New Phytol 162: 253–280

Oechel WC, Cowles S, Grulke N, Hastings SJ, Lawrence B, Prudhomme T, Riechers G, Strain B, Tissue D and Vourlitis G (1994) Transient nature of CO_2 fertilization in Arctic tundra. Nature 371: 500–503

Oren R, Ellsworth DS, Johnsen KH, Phillips N, Ewers BE, Maier C, Schafer KVR, McCarthy H, Hendrey G, McNulty SG and Katul GG (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO_2-enriched atmosphere. Nature 411: 469–472

Ottman MJ, Kimball BA, Pinter PJ, Wall GW, Vanderlip RL, Leavitt SW, LaMorte RL, Matthias AD and Brooks TJ (2001) Elevated CO_2 increases sorghum biomass under drought conditions. New Phytol 150: 261–273

Owensby CE, Ham JM, Knapp AK, Bremer D and Auen LM (1997) Water vapour fluxes and their impact under elevated CO_2 in a C_4-tallgrass prairie. Glob Change Biol 3: 189–195

Pagani M, Freeman KH and Arthur MA (1999) Late Miocene atmospheric CO_2 concentrations and the expansion of C_4 grasses. Science 285: 876–879

Pearson PN and Palmer MR (2000) Atmospheric carbon dioxide concentrations over the past 60 million years. Nature 406: 695–699

Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ Erbach DC (2005) Biomass as a feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. Report DOE/GO-102005–2315, Oak Ridge National Laboratory, Oak Ridge.

Pinter PJ, Kimball BA, Wall GW, LaMorte RL, Hunsaker DJ, Adamsen FJ, Frumau KFA, Vugts HF, Hendrey GR, Lewin KF, Nagy J, Johnson HB, Wechsunge F, Leavitt SW, Thompson TL, Matthias AD and Brooks TJ (2000) Free-air CO_2 enrichment (FACE): blower effects on wheat canopy microclimate and plant development. Agr Forest Meteorol 103: 319–333

Polley H (2002) Implications of atmospheric and climatic change for crop yield and water use efficiency. Crop Sci 42: 131–140

Poorter H, Roumet C and Campbell BD (1996) Interspecific variation in the growth response of plants to elevated [CO_2]: A search for functional types. In: Korner C, and Bazzaz FA (eds) Carbon Dioxide, Populations, and Communities, pp 375–412, Academic Press, New York

Prentice IC, Farquhar GD, Fasham MJR, Goulden ML, Heimann M, Jaramillo VJ, Kheshgi HS, Le Qu´er´e C, Scholes RJ and Wallace DWR (2001) The carbon cycle and atmospheric carbon dioxide. In; Houghton JT, Ding Y, Griggs DJ, Noguer M, Van der Linden PJ, Dai X, Maskell K and Johnson CA (eds) Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change, pp 183–237. Cambridge University Press, New York

Price GD, von Caemmerer S, Evans JR, Siebke K, Anderson JM and Badger MR (1998) Photosynthesis is strongly reduced by antisense suppression of chloroplastic cytochrome *bf* complex in transgenic tobacco. Aust J Plant Physiol 25: 445–452

Riley JJ and Hodges CN (1969) Plant responses to CO_2 enrichment: a function of the micro-environment. Paper presented at meeting of Am Assoc Adv Sci, Colorado Springs, CO, American Association for the Advancement of Science, Washington DC (1969) 7–10 May 1969

Roelfsema MRG, Hanstein S, Felle HH and Hedrich R (2002) CO_2 provides an intermediate link in the red light response of guard cells. Plant J 32: 65–75

Rogers A and Humphries S (2000) A mechanistic evaluation of photosynthetic acclimation at elevated CO_2. Glob Change Biol 6: 1005–1011

Rogers A, Fischer BU, Bryant J, Frehner M, Blum H, Raines CA and Long SP (1998) Acclimation of photosynthesis to elevated CO_2 under low- nitrogen nutrition is affected by the capacity for assimilate utilization. Perennial ryegrass under free-air CO_2 enrichment. Plant Physiol 118: 683–689

Rogers A, Allen DJ, Davey PA, Morgan PB, Ainsworth EA, Bernacchi CJ, Cornic G, Dermody O, Dohleman FG, Heaton EA, Mahoney J, Zhu XG, Delucia EH, Ort DR and Long SP (2004) Leaf photosynthesis and carbohydrate dynamics of soybeans grown throughout their life-cycle under free-air carbon dioxide enrichment. Plant Cell Environ 27: 449–458

Rogers A, Ainsworth EA and Kammann C (2006) FACE value. Perspectives on the future of Free Air CO_2 Enrichment studies. In: Nösberger J, Long SP, Norby RJ, Stitt M, Hendrey GR and Blum H (eds) Managed Ecosystems and CO_2: Case Studies, Processes and Perspectives Ecological Studies Series, Vol. 187, pp 431–450, Springer

Rogers H, Sionit N, Cure J, Smith J and Bingham G (1984) Influence of elevated carbon-dioxide on water relations of soybeans. Plant Physiol 74: 233–238

Rolland F, Winderickx J and Thevelein JM (2002) Glucose-sensing and -signaling mechanisms in yeast. FEMS Yeast Res 2: 183–201

Sage RF (2002) Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot 53: 609–620

Sage RF and Kubien DS (2003) Quo vadis C_4? An ecophysiological perspective on global change and the future of C_4 plants. Photosynth Res 77: 209–225

Saliendra NZ, Meinzer FC, Perry M and Thom M (1996) Associations between partitioning of carboxylase activity and bundle sheath leakiness to CO_2, carbon isotope discrimination, photosynthesis, and growth in sugarcane. J Exp Bot 47: 907–914

Samarakoon AB and Gifford RM (1996) Elevated CO_2 effects on water use and growth of maize in wet and drying soil. Aust J Plant Phys 23: 53–62

Saralabai VC, Vivekanandan M and Babu RS (1997) Plant responses to high CO_2 concentration in the atmosphere. Photosynthetica 33: 7–37

Saxe H, Ellsworth DS and Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. New Phytol 139: 395–436

Schimel D, Melillo J, Tian HQ, McGuire AD, Kicklighter D, Kittel T, Rosenbloom N, Running S, Thornton P, Ojima D, Parton W, Kelly R, Sykes M, Neilson R and Rizzo B (2000) Contribution of increasing CO_2 and climate to carbon storage by ecosystems in the United States. Science 287: 2004–2006

Schimel DS, House JI, Hibbard KA, Bousquet P, Ciais P, Peylin P, Braswell BH, Apps MJ, Baker D, Bondeau A, Canadell J, Churkina G, Cramer W, Denning AS, Field CB, Friedlingstein P, Goodale C, Heimann M, Houghton RA, Melillo JM, Moore B, Murdiyarso D, Noble I, Pacala SW, Prentice IC, Raupach MR, Rayner PJ, Scholes RJ, Steffen WL and Wirth C (2001) Recent patterns and mechanisms of carbon exchange by terrestrial ecosystems. Nature 414: 169–172

Schlesinger WH and Lichter J (2001) Limited carbon storage in soil and litter of experimental forest plots under increased atmospheric CO_2. Nature 411: 466–469

Schleucher J, Vanderveer PJ and Sharkey TD (1998) Export of carbon from chloroplasts at night. Plant Physiol 118: 1439–1445

Schwartz A, Ilan N and Grantz DA (1988) Calcium effects on stomatal movement in *Commelina-communis* L - Use of EGTA to modulate stomatal response to light, KCl and CO_2. Plant Physiol 87: 583–587

Seeman JR, Badger MB, Berry JA (1984) Variations in the specific activity of Ribulose-1,5-Bisphosphate Carboxylase between species utilizing differing photosynthetic pathways. Plant Physiol 74: 791–794

Sellers PJ, Dickinson RE, Randall DA, Betts AK, Hall FG, Berry JA, Collatz GJ, Denning AS, Mooney HA, Nobre CA, Sato N, Field CB and Henderson-Sellers (1997) Modeling the exchanges of energy, water, and carbon between continents and the atmosphere. Science 275: 502–509

Seneweera SP, Ghannoum O and Conroy J (1998) High vapour pressure deficit and low soil water availability enhance shoot growth responses of a C_4 grass (*Panicum coloratum* cv. Bambatsi) to CO_2 enrichment. Aust J Plant Physiol 25: 287–292

Servaites JC and Geiger DR (2002) Kinetic characteristics of chloroplast glucose transport. J Exp Bot 53: 1581–1591

Sheen J (1990) Metabolic repression of transcription in higher-plants. Plant Cell 2: 1027–1038

Siegenthaler U, Friedli H, Loetscher H, Moor E, Neftel A, Oeschger O and Stauffer B (1988) Stable-isotope ratios and concentration of CO_2 in air from polar ice cores. Ann Glaciol 10: 1–6

Singsaas EL, Ort DR and Delucia EH (2004) Elevated CO_2 effects on mesophyll conductance and its consequences for interpreting photosynthetic physiology. Plant Cell Environ 27: 41–50

Smeekens S (2000) Sugar-induced signal transduction in plants. Annu Rev Plant Phys Plant Mol Biol 51: 49–81

Spreitzer RJ and Salvucci ME (2002) Rubisco: Structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol 53: 449–475

Springer CJ, DeLucia EH and Thomas RB (2005) Relationships between net photosynthesis and foliar nitrogen concentrations in a loblolly pine forest ecosystem grown in elevated atmospheric carbon dioxide. Tree Physiol 25: 385–394

Stitt M (1991) Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant Cell Environ 14: 741–762

Stitt M and Krapp A (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ 22: 583–621

Thompson S, Govindasamy B, Mirin A, Caldeira K, Delire C, Milovich J, Wickett M and Erickson D (2004) Quantifying the effects of CO_2-fertilized vegetation on future global climate and carbon dynamics. Geophys Res Lett 31: L23211

Tibbitts T and Langhans R (1993) Controlled-environment studies. In: Hall D, Scurlock J, Bolhar-Nordenkampf H, Leegood R and Long S (eds) Photosynthesis and Production in a Changing Environment. A Field and Laboratory Manual, pp 65–78. Chapman & Hall, London

Tissue DT and Oechel WC (1987) Response of *Eriophorum-vaginatum* to elevated CO_2 and temperature in the Alaskan tussock tundra. Ecology 68: 401–410

Travis A and Mansfield T (1979a) Stomatal responses to light and CO_2 are dependent on KCl concentration. Plant Cell Environ 2: 319–323

Travis A and Mansfield T (1979b) Reversal of the CO_2-responses of stomata by fusicoccin. New Phytol 83: 607–614

Triggs JM, Kimball BA, Pinter PJ, Wall GW, Conley MM, Brooks TJ, LaMorte RL, Adam NR, Ottman MJ, Matthias AD, Leavitt SW and Cerveny RS (2004) Free-air CO_2 enrichment effects on the energy balance and evapotranspiration of sorghum. Ag Ecosyst Environ 124: 63–79

USDA (2005) In: World Agricultural Production - World Grains by Commodity for December 2004, p 8. Production Estimates and Crop Assessment Division, Foreign Agricultural Service, United States Department of Agriculture, Washington, DC

Van Groenigen K, Harris D, Horwath W, Hartwig U and Van Kessel C (2002) Linking sequestration of ^{13}C and ^{15}N in aggregates in a pasture soil following 8 years of elevated atmospheric CO_2. Glob Change Biol 8: 1094–1108

Van Kessel C, Horwath W, Hartwig U, Harris D and Luscher A (2000) Net soil carbon input under ambient and elevated CO_2 concentrations: isotopic evidence after 4 years. Glob Change Biol: 435–444

Vavasseur A and Raghavendra AS (2005) Guard cell metabolism and CO_2 sensing. New Phytol 165: 665–682

von Caemmerer S and Evans J (1991) Determination of the average partial-pressure of CO_2 in chloroplasts from leaves of several C_3 plants. Aust J Plant Physiol 18: 287–305

von Caemmerer S, Evans JR, Hudson GS and Andrews TJ (1994) The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in-vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. Planta 195: 88–97

von Caemmerer S, Ghannoum O, Conroy JP, Clark H and Newton PCD (2001) Photosynthetic responses of temperate species to free air CO_2 enrichment (FACE) in a grazed New Zealand pasture. Aust J Plant Physiol 28: 439–450

von Caemmerer S, Lawson T, Oxborough K, Baker NR, Andrews TJ and Raines CA (2004) Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. J Exp Bot 55: 1157–1166

Wall GW, Adam NR, Brooks TJ, Kimball BA, Pinter PJ, LaMorte RL, Adamsen FJ, Hunsaker DJ, Wechsung G, Wechsung F, Grossman-Clarke S, Leavitt SW, Matthias AD and Webber AN (2000) Acclimation response of spring wheat in a free-air CO_2 enrichment (FACE) atmosphere with variable soil nitrogen regimes. 2. Net assimilation and stomatal conductance of leaves. Photosynth Res 66: 79–95

Wall GW, Brooks TJ, Adam R, Cousins AB, Kimball BA, Pinter PJ, LaMorte RL, Triggs L, Ottman MJ, Leavitt SW, Matthias AD, Williams DG and Webber AN (2001) Elevated atmospheric CO_2 improved Sorghum plant water status by ameliorating the adverse effects of drought. New Phytol 152: 231–248

Wand SJE, Midgley GF, Jones MH and Curtis PS (1999) Responses of wild C_4 and C_3 grass (Poaceae) species to elevated atmospheric CO_2 concentration: a meta-analytic test of current theories and perceptions. Glob Change Biol 5: 723–741

Wand SJE, Midgley GF and Stock WD (2001) Growth responses to elevated CO_2 in NADP-ME, NAD-ME and PCK C_4 grasses and a C_3 grass from South Africa. Aust J Plant Physiol 28: 13–25

Watling JR and Press MC (1997) How is the relationship between the C_4 cereal *Sorghum bicolor* and the C_3 root hemiparasites *Striga hermonthica* and *Striga asiatica* affected by elevated CO_2? Plant Cell Environ 20: 1292–1300

Watling JR, Press MC and Quick WP (2000) Elevated CO_2 induces biochemical and ultrastructural changes in leaves of the C_4 cereal sorghum. Plant Physiol 123: 1143–1152

Watson R, Albritton D, Barker T, Bashmakov I, Canziani O, Christ R, Cubasch U, Davidson O, Gitay H, Griggs D, Halsnaes K, Houghton J, House J, Kundzewicz Z, Lal M, Leary N, Magadza C, McCarthy J, Mitchell J, Moreira J, Munasinghe M, Noble I, Pachauri R, Pittock B, Prather M, Richels R, Robinson J, Sathaye J, Schneider S, Scholes R, Stocker T, Sundarararman N, Swart R, Taniguchi T and Zhou D (2001) Climate Change 2001: Synthesis report. Summary for Policymakers. Intergovernmental Panel for Climate Change, Geneva

Whitney SM, Baldett P, Hudson GS, and Andrews TJ (2001) Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. Plant J 26:535–547

Whitehead D, Hogan KP, Rogers GND, Byers JN, Hunt JE, McSeveny TM, Hollinger DY, Dungan RJ, Earl WB and Bourke MP (1995) Performance of large open-top chambers for long-term field investigations of tree response to elevated carbon dioxide concentration. J Biogeogr 22: 307–313

Widodo W, Vu J, Boote K, Baker J and Allen L (2003) Elevated growth CO_2 delays drought stress and accelerates recovery of rice leaf photosynthesis. Environ Exp Bot 49: 259–272

Wiese A, Groner F, Sonnewald U, Deppner H, Lerchl J, Hebbeker U, Flugge UI and Weber A (1999) Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Lett 461: 13–18

Willmer C and Fricker M (1996) Stomata. Chapman & Hall, London

Wong SC (1979) Elevated atmospheric partial pressure of CO_2 and plant growth. I. Interactions of nitrogen nutrition and photosynthetic capacity in C_3 and C_4 plants. Oecologia 44: 68–74

Woodrow IE (1994) Optimal acclimation of the C_3 photosynthetic system under enhanced CO_2. Photosynth Res 39: 401–412

Zak DR, Pregitzer KS, Curtis PS and Holmes WE (2000) Atmospheric CO_2 and the composition and function of soil microbial communities. Ecol Appl 10: 47–59

Zelitch I (1973) Plant productivity and the control of photorespiration. Proc Natl Acad Sci USA 70: 579–784

Zhu X-G, Portis Jr. AR and Long SP (2004) Would transformation of C_3 crop plants with foreign Rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. Plant Cell Environ 27: 155–165

Ziska LH and Bunce JA (1997) Influence of increasing carbon dioxide concentration on the photosynthetic and growth stimulation of selected C_4 crops and weeds. Photosynth Res 54: 199–208

Ziska LH, Hogan KP, Smith AP and Drake BG (1991) Growth and photosynthetic response of 9 tropical species with long-term exposure to elevated carbon-dioxide. Oecologia 86: 383–389

Ziska LH, Sicher RC, Bunce JA (1999) The impact of elevated carbon dioxide on the growth and gas exchange of three C_4 species differing in CO_2 leak rates. Physiol Plant 105: 74–80

Part VII

Historical Perspective

Chapter 30

Early Pioneers of Photosynthesis Research

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Summary

Before the early researchers on plant nutrition worked out the fundamentals of photosynthesis, plants were believed to obtain their nutrients from humus in the soil. Then, over a period of about two hundred years, a succession of investigators supplied pieces of the puzzle that eventually allowed formulation of the overall equation for photosynthesis. Jan van Helmont, in the early seventeenth century, concluded that plants are composed not of humus but of water that has been transmuted into plant substance. In the early eighteenth century, Stephen Hales found evidence that "air" was a component of the plant body. The involvement of water and air remained conjectural, however, until the late eighteenth century, when the new chemical theories of Antoine Lavoisier allowed the understanding of plant nutrition to advance. Joseph Priestley found that plants and animals are interdependent through their complementary effects on the atmosphere, and his discovery of oxygen was important for Lavoisier's formulation of the new chemistry. Jan Ingen-Housz discovered that plants need light in order to release oxygen and that the green color of plants is important. Jean Senebier showed that carbon dioxide is also essential. Nicholas de Saussure, with his embrace of the new chemical concepts and his well-targeted experiments, was able to assemble a more comprehensive picture of plant nutrition, encompassing carbon dioxide from the atmosphere, water and mineral nutrients from the soil, and light from the sun. In the mid-nineteenth century, Robert Mayer completed the outline of the overall scheme with his insight that photosynthesizing

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plants convert light energy into chemical energy. Some of the important eighteenth-century photosynthesis pioneers were overshadowed by the drama of the profound changes shaking chemistry and are less well known than they should be. Some of their discoveries are misattributed or misstated in the literature, even down to the present day.

I. Introduction

Over a period of about 200 years, from the early seventeenth to the early nineteenth centuries, a succession of researchers unraveled the basic scheme by which plants nourish themselves. The story of this gradual assembling of fundamental facts is closely interwoven with the story of progress in chemistry. The earliest of the photosynthesis pioneers struggled with the limited scope of chemical knowledge, while the later ones had to adapt to, but ultimately benefited from, the rapid changes in chemical perceptions that occurred during the "Chemical Revolution" of the late eighteenth century. Collectively, the discoveries of the photosynthesis pioneers revealed how plants take simple, inorganic, raw materials – water and mineral salts from the soil, and carbon dioxide from the atmosphere – and, in the presence of sunlight, convert them into organic substances.

Until these fundamental facts of photosynthesis were established, the prevailing belief about plant nutrition was that held by Empedocles (ca. 490–430 B.C.), and developed more fully by Aristotle (384–322 B.C.). According to this view, plants derive their nutrition from humus, or organic material, in the soil, in a manner analogous to the ingestion of food by animals. The root system was thought to function as a "kind of diffuse mouth sucking nutrition from the Earth's breast" (Galston, 1994), or, as Hill (1970) describes it, "…a plant would have seemed to resemble a foetus drawing its nourishment from Mother-Earth." This concept persisted even long after the true sources of plant nutrition had been discovered.

Another idea of Aristotle's that is often regarded as having impeded progress, in both chemistry and plant physiology, was his theory that there are only four elements – earth, air, fire, and water. An aspect of that scheme that was particularly significant during the Middle Ages was the idea that elements could be "transmuted" into one another. In addition, there was the sixteenth-century, Paracelsian notion of three chemical principles – salt, sulfur, and mercury. Advances in chemistry during the eighteenth century swept all these ideas away. With these changes in concepts came a change in terminology. For a chart of older terms and their meanings, see Table 30.1.

Of special importance for this advancement of chemistry was the dawning understanding of gases. The atmosphere came to be seen not as one of four fundamental "elements," but as a mixture of gases. In order to study gases, however, better techniques had to be developed for preparing, collecting, transferring, separating, mixing, and identifying them. Progress also came from the realization that gases participated in chemical reactions: They reacted with one another, and also could be "fixed" in solid substances and then released from a solid back into a gaseous form.

Before chemistry could advance significantly, however, it also had to abandon the theory of phlogiston, developed by Johann Joachim Becher (1635–1682) and Georg Ernst Stahl (1660–1734). During much of the eighteenth century, this theory was the prevailing explanation for combustion and many other chemical phenomena. Phlogiston was believed to exist as a component of all combustible materials and of all metals that could be calcined (turned into calxes, or what we call oxides; see Table 30.1). When materials burned or were calcined, phlogiston was believed to be lost to the surrounding air. Stahl posited a cycle in nature, such that phlogiston was exchanged among the plant, animal, and mineral realms. Plants recombined the phlogiston that was given off in burning or calcination. Animals derived phlogiston from the plants or plant-eating animals that they consumed. Plants also provided phlogiston to the mineral realm, in the form of the charcoal (or other organic substance) that was commonly used to reduce calxes to metals. Thus the metal regained the phlogiston it had lost when it had originally formed a calx (Brock, 1993).

The nutritional interactions of a plant with its environment are not obvious, and could not be understood until gases had been discovered and studied. Rabinowitch (1945, p. 13) noted that

the growing knowledge of the chemistry of gases in the mid- to late-eighteenth century "made the discovery of photosynthesis possible, yes, almost inevitable." In the end, the overall scheme of photosynthesis proved to be relatively simple, but, of course, that simplicity masks a complexity of processes still being unraveled today.

The photosynthesis pioneers whose discoveries helped establish the basic biochemical scheme were: Jan van Helmont in the early seventeenth century; Stephen Hales in the early eighteenth century; Charles Bonnet, Joseph Priestley, Carl Wilhelm Scheele, Antoine-Laurent Lavoisier, Jan Ingen-Housz (who also demonstrated the importance of light), and Jean Senebier in the mid- to late-eighteenth century; and Nicholas de Saussure in the late eighteenth to early nineteenth centuries. Those pioneers who worked during the period of rapid progress in chemical theory both benefited from, and contributed to, that progress. After the rudimentary biochemistry of photosynthesis had been unraveled, Robert Mayer, in the mid-nineteenth century, contributed the important insight that energy plays a role in the process. (Ingen-Housz, with his insight that light is important, had anticipated Mayer, but had been unable to interpret his finding.) An examination of the birth and death dates of the pioneers and others important in laying the groundwork for discoveries in photosynthesis reveals how brief was the period in which the essentials of the process were uncovered (Fig. 30.1).

Table 30.1. Terms, old and new

Gases:	
Azote	Nitrogen (Azote is Lavoisier's term; from the Greek word meaning "not sustaining life")
Dephlogisticated (pure or vital) air	Oxygen
Fixed air	Carbon dioxide
Inflammable air	Hydrogen
Mephitic air	Carbon dioxide
Nitrous air	Nitric oxide
Phlogisticated air	Nitrogen (or sometimes carbon monoxide, which wasn't identified as a separate gas until about 1800)
Vitiated air	Air from which oxygen has been removed, hence, mainly nitrogen
Other terms:	
Calcination	Formation of a calx by the action of heat on a metal
Calx:	A powder obtained by strongly heating a metal in air. Almost always an oxide
Eudiometer	An apparatus for measuring the changes in volume that occur when gases combine, and thus useful for determining the constituents of a gaseous mixture or to ascertain the purity of the air
Natural philosophy	The investigation of, or theorization about, the natural world; similar to what we today would call "science"
Phlogiston	A theoretical substance believed in the eighteenth century to be the inflammable part of a material
Pneumatic trough	An apparatus for collecting gases over water or mercury, enabling them to be studied and analyzed
Transmutation	The alchemical goal of changing one element into another
Vegetate, vegetation	Formation, growth, and development of plants, which are sometimes called "vegetables"

Sources: Johnson (1756, 1785), Diderot et al. (1765), and Eklund (1975)

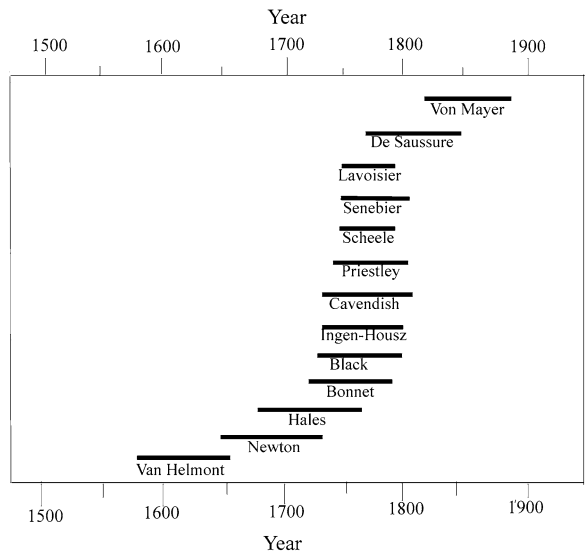

Fig. 30.1. Lifespans of the pioneers and some of the other researchers whose work contributed to the early discoveries in photosynthesis. The chart is constructed after the manner of Joseph Priestley's *Chart of Biography* (1765) and similar charts in his works on electricity (1767) and optics (1772a). Note the clustering of photosynthesis pioneers in the late eighteenth century.

Natural philosophers (as eighteenth-century investigators of nature were called; see Table 30.1) pursued their interests more as a passion than a profession. The early workers in photosynthesis came mainly from the ministry, law, and medicine. Their rivalry with their fellow researchers could be as fierce as that among today's professional scientists, however, and was a strong motivating force in their accomplishments (Djerassi and Hoffman, 2001). In addition, many of the pioneers, like many of their contemporaries, viewed the study of nature as a means of apprehending the creator (Kottler, 1973).

Previous writing about the early pioneers has covered them both individually and collectively. Early issues of the journal Plant Physiology paid tribute to a number of them, with articles on Van Helmont, Hales, Ingen-Housz, Senebier, and de Saussure. (The portraits included in these articles are reproduced here as Fig. 30.2; the figure caption provides article citations.)

Nineteenth-century German plant physiologist Julius von Sachs (1832–1897), who laid the foundations of modern plant physiology (Pringsheim, 1932; Gest, 1988), provided an overview of the early pioneers in his *History of Botany* (Sachs, 1875; 1890 translation). In addition, Eugene Rabinowitch (1945, Chapter 2) wrote a useful summary of the early workers' contributions. Leonard K. Nash, in his 122-page booklet *Plants and the Atmosphere* (1952), gave a detailed, scholarly account, drawing solely on primary source material.

Briefer, more recent, overviews of the pioneers include those by Rabinowitch and Govindjee (1969, Chapter 1), Hill (1970), and Rabinowitch (1971). Recent issues of Photosynthesis Research have published accounts of the entire history of photosynthesis research: Huzisige and Ke (1993), the "Celebrating the Millennium" series (Govindjee and Gest, 2002; Govindjee et al., 2003; Govindjee et al., 2004), and Govindjee and

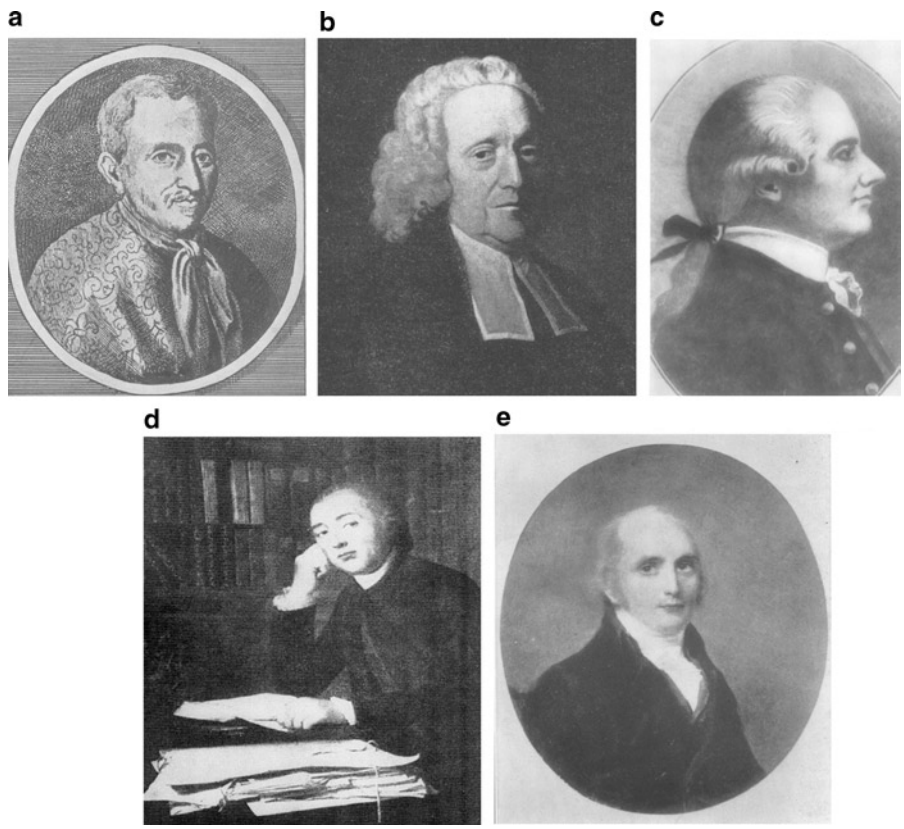

Fig. 30.2. Portraits of photosynthesis pioneers, taken from papers published in early issues of Plant Physiology. Artists mostly unknown. (**a**) Jan van Helmont (From Harvey, 1929); (**b**) Stephen Hales (From White, 1942); (**c**) Jan Ingen-Housz (From Harvey and Harvey, 1930); (**d**) Jean Senebier (From Bay, 1931); (**e**) Nicholas de Saussure (From Hart, 1930).

Krogmann (2004). These histories include brief surveys of the work of the early pioneers. The book *Discoveries in Photosynthesis* (Govindjee et al., 2006) incorporates these articles. Höxtermann (1992) also covered the full history of photosynthesis research. Morton (2008) discusses the early pioneers as well as some more recent figures. The present discussion, the first in many years devoted solely to an overview of the work of the early pioneers, provides references to recent secondary literature. One of the goals is to address the persistent confusion surrounding the attribution of the discoveries of several of these workers.

II. Jan van Helmont (1579–1644): A Possible Role for Water

The first unmistakable quantitative experiment of the Renaissance was Flemish physician Jan van Helmont's study of the growth of a willow tree planted in a pot of soil of known weight (Hoff, 1964). Van Helmont's son, Francisco Mercurio van Helmont, posthumously published Jan van Helmont's collected works as *Ortus medicinae* (Van Helmont, 1648). In the English translation of that work, titled *Oriatrike* (Van Helmont, 1662), the following account of the willow experiment is given (p. 109):

> But I have learned by this handicraft-operation, that all Vegetables do immediately, and materially proceed out of the Element of water onely. For I took an Earthen Vessel, in which I put 200 pounds of Earth that had been dried in a Furnace, which I moystened with Rain-water, and I implanted therein the Trunk or Stem of a Willow Tree, weighing five pounds; and at length, five years being finished, the Tree sprung from thence, did weigh 169 pounds, and about three ounces: But I moystened the Earthen Vessel with Rain-water, or distilled water (alwayes when there was need) and it was large, and implanted into the Earth, and least the dust that flew about should be comingled with the Earth, I covered the lip or mouth of the Vessel, with an Iron-Plate covered with Tin, and easily passable with many holes. I computed not the weight of the leaves that fell off in the four Autumnes. At length, I again dried the Earth of the Vessel, and there were found the same 200 pounds, wanting about two ounces. Therefore 164 pounds of Wood, Barks, and Roots, arose out of water onely.

Van Helmont (Fig. 30.2a) combined genuine scientific research with nonscientific, somewhat mystical pursuits (Pagel, 1972), and represents the transition from alchemy to chemistry (Partington, 1957). He studied gases, coined the term "gas," and isolated two of them (including carbon dioxide, which he called *gas sylvestre*). He thought that these were just varieties of common air, however.

Van Helmont was a quiet, scholarly man (Harvey, 1929), who rebelled against the traditional medical training of his time. That training, however, instilled in him an interest in the physiology of both animals and plants. He was financially secure and could work on experiments uninterruptedly in his own private laboratory (Harvey, 1929). For biographical details, see Redgrove and Redgrove (1922), Harvey (1929), de Waele (1947), and Pagel (1972).

Van Helmont was spurred to conduct the willow experiment by his conviction that water is the "first matter," or material cause, of all things and thus the chief constituent of organisms. The willow experiment was his way to test the Aristotelian notion that water could be transmuted into earth – in this case, the "earth" of plant substance (Brock, 1993).

The idea for the willow experiment may not have originated with Van Helmont. About 200 years earlier, a German, Nicolaus of Cusa (1401–1464), proposed an experiment (but presented no experimental data) much like Van Helmont's willow experiment (Hoff, 1964). On the basis of his highly detailed proposal, Krikorian and Steward (1968) speculated that Cusa either performed the experiment himself or drew on earlier sources that may subsequently have been lost (see Howe, 1965).

Van Helmont's willow experiment demonstrated that the major part of the weight gained by a growing plant does not come from the soil, as had been thought. Van Helmont believed that he had excluded all sources of nutrition save water, but, of course, he was unaware of the large, important contribution made by atmospheric carbon dioxide–a gas that, ironically, he himself had discovered. His conclusion, however, was logical based on the information available at the time (Harvey and Harvey, 1930).

Some years after Van Helmont carried out his experiment, chemist and physicist Robert Boyle (1627–1691) had his gardener perform a similar experiment, using squash and cucumber. Boyle concluded (1661, p. 109), "it appears that… the Main Body of the Plant consisted of Transmuted Water."

III. Stephen Hales (1677–1761): The Presence of Air in Plants

According to Guerlac (1972), Stephen Hales (Fig. 30.2b) was "the leading English scientist during the second third of the eighteenth century" and "the acknowledged founder of plant physiology." Hales made important discoveries regarding the movement of the sap in plants. Of special significance to the understanding of photosynthesis, however, was his demonstration that plants interact with the atmosphere. His experiments indicating that some of a plant's nutrition was derived from "air" suggested that Van Helmont's conception of the plant body as a structure built entirely of transmuted water was incomplete. Hales also speculated that light is involved in plant nutrition.

Hales was born at Bekesbourne, Kent. He studied Divinity at Cambridge University, where he was influenced by the ideas of Isaac Newton (1642–1727). In 1709, Hales was ordained and appointed curate at Teddington, near London, where he remained the rest of his life (Guerlac, 1972). Further biographical details about Hales can be found in Hoskin (1961) and Guerlac (1972).

Hales subscribed to Newton's concept that most changes in nature can be explained by the action of gravitational attraction and other attractive and repulsive forces (Hoskin, 1961). Among these phenomena Hales included the physiological activities of animals and plants.

Physician William Harvey (1578–1657), a century earlier, had demonstrated the circulation of the blood (Harvey, 1628), and Hales followed up by conducting experiments, many by horrific means, on horses and other animals to obtain the first measurements of blood pressure. These investigations of fluid movement sparked his interest in the movement of the sap in plants.

Hales began to experiment on plants about 1718 (Hoskin, 1961), and published the results as *Vegetable Staticks* (Hales, 1727, reprinted 1961). His early experiments were measurements of the quantities of moisture taken in by the roots and transpired by the leaves. Using rudimentary laboratory apparatus and hydrostatic principles, he made many attempts to calculate the force with which the sap moves. In his day, the sap was thought to circulate in a manner analogous to the circulation of the blood, moving upward in the inner part of the stem and downward in the outer part (Hoskin, 1961). Hales concluded that the sap probably does not circulate, "…it probably having only a progressive and not a circulating motion, as in animals" (1961 reprint of Hales (1727), p. 6). Hales also found that the rate of transpiration depends in part on the amount of leaf surface area available (1961 reprint of Hales (1727), p. 185). The importance that he attached to leaves is evident in his statement that leaves contain the "main excretory ducts in vegetables," and that these ducts "separate and carry off the redundant watry fluid" (1961 reprint of Hales (1727), p. 185).

While pursuing his research on sap movement, Hales noticed bubbles of air in the sap. He then showed experimentally that stems draw up air (1961 reprint of Hales (1727), pp. 85–88). Having discovered a role for leaves in transpiration, he was prepared to believe that they might also function in bringing air into the plant. He wrote (1961 reprint of Hales (1727), p. 87) "…it is very probable, that the air freely enters plants, not only with the principal fund of nourishment by the roots, but also thro' the surface of their trunks and leaves, especially at night, when they are changed from a perspiring to a strongly imbibing state."

Hales then began to investigate air as a component of a wide variety of plant, animal, and mineral substances. He tested for it by heating materials and capturing the ejected air, using an apparatus of his own devising, called a pneumatic trough (Fig. 30.3). (Later workers modified this device and used it to great advantage in the study of gases. For a history of its development, see Parascandola and Ihde, 1969.) With this apparatus, Hales demonstrated that many substances contained, or held within their pores, large quantities of air. He wrote (1961 reprint of Hales (1727), p. 166), "…we have from these Experiments many manifest proofs of considerable quantities of true permanent air, which are by means of fire and fermentation raised from, and absorbed by animal, vegetable and mineral substances."

Hales had little idea how to explain his observation that air in its "elastick" (gaseous) state was interconvertible with air that was "fixt" in substances. He wrote (1961 reprint of Hales (1727), p.179), "…they [air particles] are, we see, easily changed from an elastick to a fixt state, by the strong attraction of the acid, sulphureous and

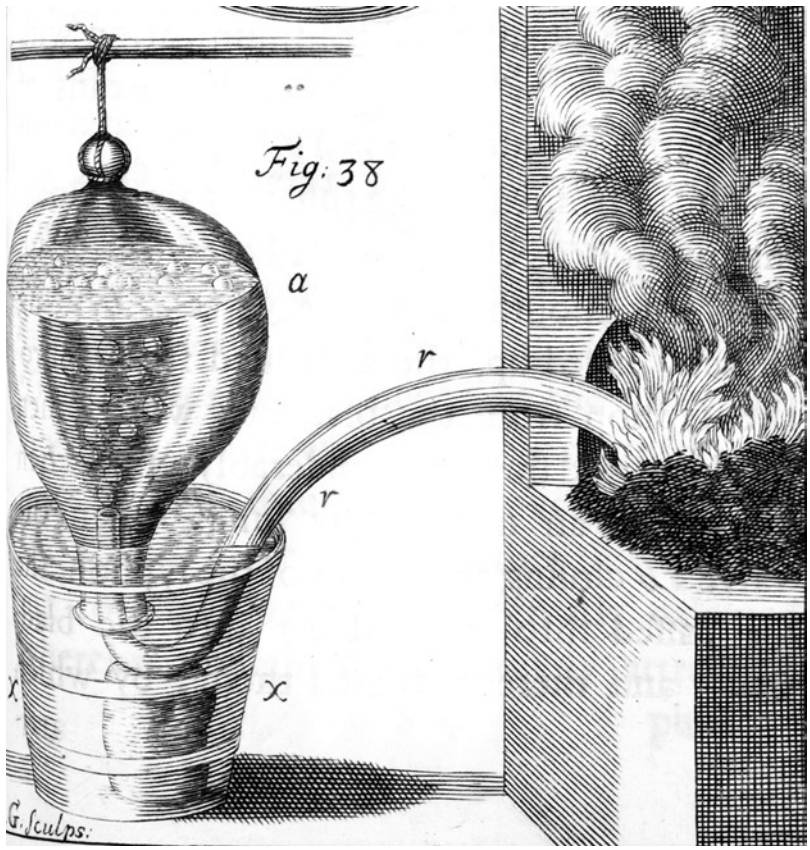

Fig. 30.3. Stephen Hales's pneumatic trough (Hales, 1727, Plate 17, Fig. 38). Hales heated materials, by fire, in a bent gun barrel (*rr*) and collected the "air" that was given off in a large glass globe (*a*) filled with water and suspended by a rope in a tub of water (*xx*).

saline particles which abound in the air." By means of fire or fermentation, air that is fixed may, in turn, resume "its former elastick state." Air to Hales was (1961 reprint of Hales (1727), p.180) "this now fixt, now volatile *Proteus* among the chymical principles…this much neglected volatile *Hermes*, who has so often escaped thro'… burst receivers."

Hales considered air to be a source of nourishment for plants, writing (1961 reprint of Hales (1727), p. xxiv):

> The admirable provision she [nature] has made for them [plants], not only vigorously to draw to great heights plenty of nourishment from the earth; but also more sublimed and exalted food from the air, that wonderful fluid, which is of such importance to the life of Vegetables and Animals: And which by infinite combinations with natural bodies, produces innumerable surprizing effects; many instances of which I have here shewn.

Hales says further (1961 reprint of Hales (1727), pp.185–186) "We may therefore reasonably conclude, that one great use of leaves is what has been long suspected by many, *viz.*, to perform in some measure the same office for the support of the vegetable life, that the lungs of animals do, for the support of the animal life; Plants very probably drawing thro' their leaves some part of their nourishment from the air."

Sachs wrote (1890 translation of Sachs (1875), pp. 476–477): Hales "was the first who proved, that air co-operates in the building up the body of the plant, in the formation of its solid substance, and that gaseous constituents contribute largely to the nourishment of the plant." Nevertheless, Hales's views had little immediate impact on general conceptions of plant nutrition because of the lingering conviction that plants derived their nourishment only from the soil or,

as Van Helmont's willow experiment seemed to demonstrate, water.

In Hales's day, air was still not understood to consist of distinct gases, and Hales did not look for or discover any. Further, like his contemporaries, Hales believed that air was without chemical properties (Hoskin, 1961). Nonetheless, the discovery that air could be "fixed" was important for pneumatic chemistry and for the development of chemistry in general in the eighteenth century (Brock, 1993).

In addition to his insights into air in plants, Hales speculated, in 1725, about the role of light. He seemed to have sensed that sunlight might have some kind of direct effect on plants, apart from its provision of heat (Gest, 1988). Hales asked (1961 reprint of Hales (1727), pp.186–187):

> And may not light also, by freely entring the expanded surfaces of leaves and flowers, contribute much to the ennobling the principles of vegetables? for Sir *Isaac Newton* puts it as a very probable query, 'Are not gross bodies and light convertible into one another? and may not bodies receive much of their activity from the particles of light, which enter their composition? The change of bodies into light, and of light into bodies, is very conformable to the course of nature, which seems delighted with transmutations.'

After publishing *Vegetable Staticks* (1727), Hales turned to medical investigations. The second edition of *Vegetable Staticks* appeared in 1731, and 2 years later his (fruitless) work on kidney stones, together with his experiments on blood pressure, were combined into *Haemastaticks,* which was united with *Vegetable Staticks* to form a volume titled *Statical Essays* (Hales, 1733). A second edition of *Statical Essays* appeared in 1738, and by the time the fourth edition was published, in 1769, *Vegetable Staticks* had been translated into Dutch, French, German, and Italian (Hoskin, 1961).

IV. Charles Bonnet (1720–1793): A Useful Observation: Bubbles on Submerged Leaves

Following the publication of Hales's *Vegetable Staticks* (1727), there was a virtual eclipse – for half a century – in progress towards understanding the biochemistry of plant life (Gest, 1988). During this period, however, Charles Bonnet (Fig. 30.4), in 1747, made an observation that, although unimportant in itself, was to prove useful as an experimental tool for later investigations, notably by Ingen-Housz and Senebier.

Fig. 30.4. A portrait of Charles Bonnet.

Bonnet was born into a wealthy family in Geneva. He was a mentor and close friend of photosynthesis pioneer Jean Senebier. A lawyer by profession, Bonnet was drawn to natural history (Pilet, 1970). He became interested in the structure and function of leaves and the movement and exchanges of moisture in plants (Kottler, 1973).

In his book *Recherches sur l'usage des feuilles dans les plantes* (1754), Bonnet reported finding that air bubbles form on submerged, illuminated leaves. Because he observed bubbles on submerged dead as well as living leaves, he dismissed the possibility that the bubbles arose from a process going on within the leaves, despite the larger size of the bubbles on the living leaves. His (incorrect) explanation for bubble formation was that the sun's heat dilates initially small bubbles that adhere to the plant surfaces (Bonnet, 1754). He did not analyze the air in the bubbles.

V. Joseph Priestley (1733–1804): The Role of Plants in "Purifying" Air, and the Discovery of Oxygen

Each of three men – Joseph Priestley, Carl Wilhelm Scheele, and Antoine Lavoisier (see portraits, Fig. 30.5) – has some claim to the discovery of oxygen. Of the three, Englishman Joseph Priestley (Fig. 30.5a) is most often given the accolade. Priestley also discovered the reciprocal relationship between plant and animal life, mediated by gases, thereby providing support for Hales's view that air is important for plants. Priestley's discovery of oxygen, which he achieved independently of his finding of the mutual interdependence of plant and animal life, was pivotal in Lavoisier's formulation of the "new chemistry."

Much has been written about Priestley, but Robert Schofield's two-volume biography, which takes a detailed look at the life and achievements of this versatile man, stands out. Volume I is *The Enlightenment of Joseph Priestley: A Study of his Life and Work from 1733 to 1773* (Schofield, 1997); and Volume II is *The Enlightened Joseph Priestley: A Study of his Life and Work from 1733 to 1804* (Schofield, 2004). The publication of the second volume, in 2004, marked the bicentennial of Priestley's death, in 1804. Schofield (1975) provides a shorter biographical account of Priestley.

Priestley was born in Birstal Fieldhead, a small village near Leeds, England. He was trained as a minister, but his career and interests were wide ranging. In addition to being a nonconformist (Unitarian) theologian, he was a teacher, radical political theorist, and chemist (Schofield, 1975). He first ventured into natural philosophy by writing a book on electricity (Priestley, 1767), in which he included some experiments of his own.

By this time, gases had begun to be studied and identified: Van Helmont's *gas sylvestre* (carbon dioxide) had been rediscovered by Joseph Black in 1754 and renamed "fixed air" by him (Partington, 1957); and Henry Cavendish (1766) had studied both fixed air and hydrogen, which he called "inflammable air." Priestley's interest in gases was kindled when, having taken a job as a minister in Leeds in 1767, he settled next to a brewery, where vats of fermenting beer were producing abundant fixed air. While experimenting on this air, he developed a technique for impregnating water with fixed air, thus artificially preparing soda water (Priestley, 1772b).

Priestley made full use of the pneumatic trough to isolate gases for qualitative study, in contrast to Hales, who had studied "air" only quantitatively (Parascandola and Ihde, 1969). Priestley's pneumatic trough, like one devised by English physician William Brownrigg (1712–1800) in 1765, included a shelf for collecting gases over water (Badash, 1964; Parascandola and Ihde, 1969; see Fig. 30.6). (Cavendish (1766) had also improved the pneumatic trough and was the first to collect water-soluble gases over mercury; this allowed gases to be collected without loss due to solution in water.) By 1772, Priestley had identified a number of new gases, including nitric oxide (Priestley, 1772b).

Priestley also experimented on the effects of different "airs" on the survival of small animals. Because animals could live only a limited time in an enclosed air space, he concluded that they converted "pure" into "impure" air, and that they could not live in impure air. Similarly, candles

Fig. 30.5. Three researchers having roles in the discovery of oxygen: (**a**) Joseph Priestley; (**b**) Carl Wilhelm Scheele; (**c**) Antoine-Laurent Lavoisier.

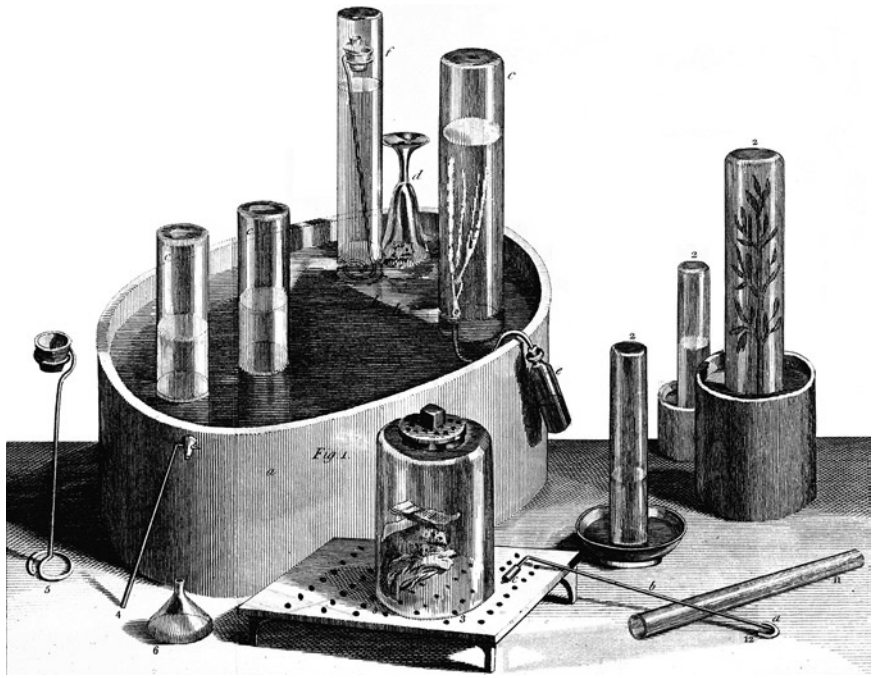

Fig. 30.6. Laboratory apparatus of Joseph Priestley (Priestley, 1775c, plate opposite title page). Pneumatic trough (*a*) with shelf; trough contains water in which stand jars (*cc*) containing gases; another jar (*c*), on the shelf, is receiving gas from a generating bottle (*e*). The inverted glass (*d*) contains a mouse; mice are kept for use as in *3*. A plant is growing in a gas in *2*. Other apparatus for the manipulation of gases are also depicted (Explanation based on Partington, 1957).

were extinguished after a time in an enclosed space. Because he believed strongly in the unity of living things, Priestley expected that a sprig of mint placed in an enclosed space would, like a mouse, render the air impure and that the plant would die. To his surprise, the plant flourished. To his even greater surprise, a growing plant restored air that had been "vitiated" by the burning of a candle. Priestley wrote of his momentous finding (Priestley, 1772b, pp. 166–168):

> …I flatter myself that ...I have accidentally hit upon a method of restoring air which has been injured by the burning of candles, and that I have discovered at least one of the restoratives which Nature employs for this purpose. It is vegetation.
>
> …One might have imagined that, since common air is necessary to vegetable, as well as to animal life, both plants and animals had affected it in the same manner, and I own I had that expectation, when I first put a sprig of mint into a glass jar, standing inverted in a vessel of water; but when it had continued growing there for some months, I found that the air would neither extinguish a candle, nor was it at all inconvenient to a mouse, which I put into it.
>
> …Finding that candles burn very well in air in which plants had grown a long time, and having had some reason to think, that there was something attending vegetation, which restored air that had been injured by respiration, I thought it was possible that the same process might also restore the air that had been injured by the burning of candles.
>
> Accordingly, on the 17th of August, 1771, I put a sprig of mint into a quantity of air, in which a wax candle had burned out, and found that, on the 27th of the same month, another candle burned perfectly well in it. This experiment I repeated, without the least variation in the event, not less than eight or ten times in the remainder of the summer.

(Note that, in the eighteenth century, the term "vegetation" meant the active process of plant growth and development, rather than today's meaning of plant life in general; see Table 30.1.)

Priestley obtained the same results using groundsel and spinach in place of mint (Priestley, 1772b, pp. 169–170). He also showed that plants thrive in air made noxious by the respiration of animals or by animals putrefying (Priestley, 1772b). In Volume I of his three-volume work,

Experiments and Observations on Different Kinds of Air (Priestley, 1774, 1775a, 1777), Priestley concluded (1774, pp. 86–87), "plants, instead of affecting the air in the same manner with animal respiration, reverse the effects of breathing, and tend to keep the atmosphere sweet and wholesome, when it is become noxious, in consequence of animals either living and breathing, or dying and putrefying in it."

Priestley was on the trail of something new (oxygen), but at that time he had no idea what he had. He thought that plants, rather than producing a new air, removed from "vitiated air" a "putrid effluvium," which he equated with phlogiston. He wrote (1772b, pp. 231–232), "May not plants … restore air diminished by putrefaction, by absorbing part of the phlogiston with which it is loaded?… May not this phlogistic matter be even the most essential part of the food and support of both vegetable and animal bodies?"

Even before he isolated oxygen gas, Priestley had developed a chemical test for the "goodness" of air, that is, its concentration of "pure" air (oxygen) (Priestley, 1772b, pp. 210–216). This "nitrous air test," as he called it, gave him a quantitative tool to use instead of having to rely on the responses of small animals confined to chambers containing the air in question. The nitrous air test was based on the reaction between pure air and nitric oxide (NO), a colorless, insoluble gas: The reaction product, nitrogen dioxide (NO_2), dissolved in water, causing a decrease in the volume of the gas phase. This decrease was a measure of the amount of pure air in the original sample. The nitrous air test was widely used in the 1770s and 1780s and led to the construction of an apparatus that came to be called the eudiometer. (See Golinski (1992) for discussion of this instrument.)

In 1773, the Royal Society of London awarded Priestley its highest honor, the Copley Medal, in recognition of his work in electricity and on the "different kinds of air" (Partington, 1962). Physician John Pringle, in his presidential address to the Society on the occasion of the award, noted both Priestley's invention of soda water and his discovery of the balance of nature, mediated by gases. A printed form of his address was widely circulated, arousing great interest in Priestley's work (Nash, 1952). One of those inspired by the finding of complementarity in nature was Jan Ingen-Housz.

The same year in which he received the award, Priestley left Leeds to become librarian and literary companion to William Petty, Second Earl of Shelburne (1737–1805). Priestley and his family settled near Shelburne's country estate, Bowood House, in Calne, Wiltshire, where they remained for 7 years. Lord Shelburne provided Priestley with laboratory facilities at Bowood House. This post allowed Priestley the leisure to pursue his experiments, and he did his most productive work in the experimental chemistry of gases while there. He discovered many new gases, including oxygen, and published his findings prolifically.

One method Priestley used to study airs was to heat chemicals, such as calxes (metal oxides), by means of a large convex lens, or "burning glass" (Fig. 30.7). With the lens, which was a time-honored instrument for heating chemicals, he focused the sun's rays onto substances he had placed in

Fig. 30.7. A compound burning-glass used by Joseph Priestley. The lenses are 16 and 7 in. in diameter, 16 in. apart. The glass is housed in the Archives and Special Collections, Dickinson College, Carlisle, Pennsylvania (Photo by the author).

the receiver of a pneumatic trough, and heated the materials hot enough that "air" could be extracted from them. He then collected the air over water or mercury and analyzed it. The chemical that led Priestley to oxygen was mercuric oxide (HgO), also known as red precipitate or *mercurius calcinatus per se*. The behavior of this compound had long puzzled chemists. Most metal oxides can be reduced to the metal only by heating them with a reducing agent, such as charcoal. In the process, the oxygen in the oxide combines with the carbon of the charcoal, thus yielding carbon dioxide and the metal (Brock, 1993). Mercuric oxide, in contrast, can be converted into the metal mercury when heated quite hot without charcoal. Oxygen is released in the process (Conant, 1950).

Priestley describes his experiment with red oxide of mercury and his surprise at the results (Priestley, 1776, pp. 33–34):

> Having … procured a lens of twelve inches diameter, and twenty inches focal distance, I proceeded with great alacrity to examine, by the help of it, what kind of air a great variety of substances, natural and factitious, would yield, putting them into the vessels … which I filled with quick-silver, and kept inverted in a bason of the same….
>
> With this apparatus, after a variety of other experiments, … on the 1st of August, 1774, I endeavoured to extract air from *mercurius calcinatus per se*; and I presently found that, by means of this lens, air was expelled from it very readily. Having got about three or four times as much as the bulk of my materials, I admitted water to it, and found that it was not imbibed by it. But what surprised me more than I can well express, was, that a candle burned in this air with a remarkably vigorous flame… I was utterly at a loss how to account for it.

Priestley at first believed that he had obtained "modified nitrous air" (N_2O, or nitrous oxide) (Priestley, 1776, p. 39), an air that he knew supported combustion, as did this gas. Then, on a trip to France with Lord Shelburne in October 1774, Priestley told Lavoisier and others of the air he had obtained with a burning glass, and how it supported combustion. Clearly this was not fixed air, which would have been formed by the usual method of reducing a calx. Although Priestley had not grasped the importance of this air, Lavoisier saw its likely significance for the new system of chemistry he was developing.

Early in 1775, after discovering that mice lived longer in the air derived from mercuric oxide than they did in an equal volume of ordinary air, Priestley finally concluded that he had discovered something new. He called it *dephlogisticated air* (Priestley, 1775b), because he thought that it was air from which phlogiston had been removed and that could therefore readily absorb more of that substance. Ironically, although his discovery of oxygen was a central event in the ultimate demise of the phlogiston theory, Priestley himself persisted in explaining his oxygen experiments in terms of phlogiston. He is generally credited with the discovery of oxygen but faulted for not recognizing its significance.

In 1777, Priestley resumed his experimentation on plants upon learning that several investigators, notably Carl Wilhelm Scheele, had been unable to duplicate Priestley's earlier finding that plants "improve" the air. During this new round of plant studies, Priestley obtained mostly negative results, in marked contrast to his initial successes. He did not understand that his failures were due to poor experimental conditions, and, at this point, he more or less lost his way in plant research. Priestley and others were unable to demonstrate consistent oxygen production by plants because they were unaware of the requirement for light (Gest, 2000).

Priestley published his new results in the first volume of his second three-volume physicochemical work, *Experiments and Observations Relating to Various Branches of Natural Philosophy* (Priestley, 1779, 1781, 1786). Sounding less confident than previously, he nonetheless maintained (1779, p. 302), "Upon the whole, I still think it *probable* that the vegetation of healthy plants, growing in situations natural to them, has a salutary effect on the air in which they grow."

Priestley also had difficulty interpreting what he called the "*green matter*" (actually, microscopic green algae) that developed in the jars of pump water in which he grew his experimental plants. He at first did not identify this matter as living (Priestley 1779, p. 342). He also had difficulty recognizing that the green matter was a source of dephlogisticated air. At one point, he even thought that water under the influence of light produced this air (Priestley, 1779, p. 349). He did not satisfy himself until late in 1779 that the green matter is a plant, that it converts

Fig. 30.8. Joseph Priestley House, Northumberland, Pennsylvania, where Priestley lived from 1794 until his death in 1804; now a Pennsylvania State Museum (Photo by the author).

impure air contained in the water into pure, or "dephlogisticated," air, and that light is necessary for this process. Before he reported these findings (Priestley, 1781, pp. 16–17, 21–25), however, Jan Ingen-Housz (1779) beat him to publication on the light requirement.

In 1780 Priestley left Calne for a ministerial post in Birmingham, England, where he resumed his experiments on gases. He also completed his second series of *Experiments and Observations* and published new editions of earlier works. In 1790, he published a revised, abridged edition of his two, three-volume works as *Experiments and Observations on Different Kinds of Air and other Branches of Natural Philosophy*.

Then, in 1791, Priestley's life took a difficult turn. An ardent religious and political nonconformist, he was often in conflict with the authorities. Because of his support for the French Revolution, his house and laboratory in Birmingham were burned by an anti-radical, anti-republican, "church and king" mob. In 1794, he emigrated to the United States of America. He settled at Northumberland, Pennsylvania, where he lived until his death, in 1804 (Fig. 30.8). He resumed his chemical experimentation and wrote prolifically in his new land, especially in defense of the phlogiston theory, but he had little new to contribute, other than the discovery of carbon monoxide. His work was largely ignored (Schofield, 2004).

VI. Carl Wilhelm Scheele (1742–1786): Early Identifier of Oxygen

There is little doubt that the Swedish apothecary Carl Wilhelm Scheele (Fig. 30.5b) discovered oxygen about 1772, 2 years before Priestley did. He did not publish his discovery for several years, however.

Scheele was born in Stralsund, Pomerania, Germany, a city that was in Swedish hands at the time (Hoffman and Torrence, 1993). He was a modest man whose circumstances were often poor, and he used simple laboratory equipment (Partington, 1957), yet he was an outstanding experimental chemist (Hoffman and Torrence, 1993). Scheele discovered many acids and several elements. Biographical information is available in Boklund (1975).

Scheele achieved the isolation of oxygen through the decomposition of various compounds, including nitric acid, saltpeter (potassium nitrate), manganese dioxide–and mercuric oxide, the compound that later led Priestley to oxygen (Partington, 1957). Due to delays in publication, however, his book, *Chemische Abhandlung von der Luft und dem Feuer* (Scheele and Bergman, 1777), did not appear until at least 4 years after he had made the discovery, and 2 years after Priestley had published his own (Priestley, 1775b). Thus Scheele did not receive credit for the discovery.

Scheele states in his book that the atmosphere is composed of two gases: "fire air," which supports combustion, and "foul air" (nitrogen), which prevents it. Unlike Priestley and Lavoisier, however, Scheele did not demonstrate the importance of the new gas in respiration and combustion. Further, his attempts to confirm its production by plants failed.

Nearly 3 years before his book was published, Scheele communicated his finding of the new gas to Lavoisier, in a letter written on September 30, 1774. Lavoisier received the letter in mid-October 1774. No reply from Lavoisier is known (Partington, 1962). Swedish historians of science have not forgiven Lavoisier for his failure to respond (Poirier, 1996). Lavoisier gave neither Scheele nor Priestley credit for the discovery.

VII. Antoine-Laurent Lavoisier (1743–1794): The "New Chemistry"

During the 1770s and 1780s, the new, more systematic and quantitative chemistry developed by Antoine-Laurent Lavoisier (Fig. 30.5c) and his colleagues replaced the older concepts, including the notions of the four Aristotelian "elements," the Paracelsian chemical "principles," and the phlogiston theory. Lavoisier's quantitative approach did not represent a complete break with the past, however, but rather a difference of degree and of technical expertise (Newman and Principe, 2002).

Lavoisier was born in Paris, the son of a wealthy French magistrate. He attended the College Mazarin, in Paris, and then was trained as a lawyer. Like Priestley, he had wide-ranging careers: He was a geologist, tax collector, administrator, and chemist. His life and work are the subject of two in-depth biographies published during the 1990s, approximately coinciding with the bicentenary of Lavoisier's death, in 1794. These works are: *Antoine Lavoisier: Science, Administration and Revolution*, by Arthur Donovan (1993); and *Lavoisier: Chemist, Biologist, Economist,* by Jean-Pierre Poirier (1996).

Lavoisier himself did no experimental work with plants. His chemical ideas, however, were gaining acceptance at the time that photosynthesis pioneers Jan Ingen-Housz and Jean Senebier were carrying out their experiments, and the new concepts influenced their work. Nicholas de Saussure, who came later, had the full benefit of the new ideas in chemistry and was able to use them skillfully.

Early in his career in chemistry, Lavoisier recognized the need to test the long-held notion that the four Aristotelian elements could be transmuted into one another through such processes as heating, fermentation, and plant growth. He knew that if they could, then these processes could not be used to determine the quantities of the elements present in various compounds. Lavoisier charged that previous investigators had "pretended" to prove that water could be transmuted into earth, by two kinds of experiments: growing plants in water as Van Helmont had done; and repetitive distillation of water in a glass vessel, which led to a slight earthy residue (Lavoisier, 1770). Pointing to Hales's (1727) largely forgotten finding that the solid parts of plants contain a great deal of air, Lavoisier noted that Van Helmont and others had overlooked air as a possible source of vegetative weight gain. Lavoisier concluded, "The experiments on vegetative assimilation of water prove nothing regarding the possibility of changing water into earth" (Lavoisier, 1770, p. 8, as translated by Donovan, 1993, p 93). In addition, Lavoisier demonstrated that the small but significant residue left after repeated distillation of water came from the glass of the vessel, not from a transmutation of water (Lavoisier, 1770).

By the time Lavoisier began his work in chemistry, the atmosphere was coming to be seen as a mixture of different kinds of gases rather than as a single element. Further, these gases were being found to be able to enter into chemical reactions. This knowledge was pivotal in Lavoisier's challenge to the phlogiston theory, even before the discovery of oxygen. He recognized that a major problem with the theory was that, during calcination, metals gained weight rather than losing it as one might expect if phlogiston were being released from the metal during the process. Based on the knowledge that most calxes (oxides) can be reduced to the metal only by heating them with a reducing agent, such as charcoal, thereby producing fixed air, Lavoisier supposed that the calxes might originally have been formed by a combination of the metal with fixed air, but he had been unable to prove it (Conant, 1950; Brock, 1993).

After receiving the tip from Priestley, and presumably having read Scheele's letter claiming discovery of "fire-air," Lavoisier performed experiments showing that heating mercuric oxide with charcoal yielded fixed air, which did not support combustion, whereas heating this oxide by means of a burning glass yielded an air that supported a vigorous flame (Partington, 1962). He concluded that, if other calxes could be reduced without addition of carbon, they, too, would give this kind of air.

At first Lavoisier reported that the weight gained by a metal in forming a calx is due to the reaction of the metal with air in itself, but he later modified this view, saying that the reaction was a result of the addition to the metal of "the purest part of the very air which surrounds us, which we breathe" (Lavoisier, 1775, p. 127, as translated by Conant, 1950, p. 27). In Lavoisier's view, calcination (and combustion, as well) was the combination of substances with oxygen. The remainder of the atmosphere, which he called "azote" and we know as nitrogen, was nonreactive. His findings cast serious doubt on the phlogiston theory because he had shown that a metal could be regenerated from a calx without a source of phlogiston in the form of charcoal (Brock, 1993). Lavoisier has been criticized for not giving Priestley and Scheele due credit for the discovery of oxygen, but Lavoisier understood the meaning of the finding in a way that had eluded both of them (Conant, 1950).

In 1777, Lavoisier renamed the gas that Priestley had called dephlogisticated air "*principe oxygène*" (oxygène meaning "acid former" in Greek; thus, "acid-forming principle") (Partington, 1957). Even though he was wrong that all acids contain oxygen, the misnomer, oxygen, stuck.

Lavoisier built his new chemical system on the law of the conservation of mass, first advanced in 1748 by Mikhail Lomonosov (1711–1765) (Kedrov, 1973). Lavoisier also based his system on the idea of persistent, well-defined elements (Nash, 1952), which were not decomposable by any known means of analysis. Lavoisier and several chemical colleagues published *Méthode de nomenclature chimique* (Morveau et al., 1787), in which they renamed chemical substances so as to bring them into line with the new chemical theory. This helped in the acceptance of the theory, as did Lavoisier's *magnum opus*, *Traité élémentaire de Chimie* (1789) (see Klein and Lefèvre, 2007, on the interplay of theory with classification and nomenclature in the chemical revolution). In the *Traité,* which was highly influential and used as a chemistry textbook for decades, Lavoisier states (p. 149, as translated by Partington, 1957, p. 124):

> …for nothing is created in the operations either of art or of nature, and it can be taken as an axiom that in every operation an equal quantity of matter exists both before and after the operation, that the quality and quantity of the principles remain the same and that only changes and modifications occur. The whole art of making experiments in chemistry is founded on this principle: we must always suppose an exact equality or equation between the principles of the body examined and those of the products of its analysis.

During the late 1770s and the 1780s, Lavoisier and his collaborators made a number of chemical discoveries that were of great importance for the understanding of plant and animal physiology. These included the discovery that fixed air is composed of carbon and oxygen, and that water, rather than being an element, is a compound of hydrogen and oxygen. These investigators also contributed to the understanding of respiration, through studies on humans and guinea pigs (Partington, 1957).

Unfortunately for Lavoisier (and for chemistry), his achievements could not save him from the wrath of the French Revolution's Reign of Terror. In 1794, he was beheaded, on a trumped-up charge related to his activities as a tax collector and a director of the French Gunpowder Administration. Hoffman and Torrence (1993, p. 91) comment that Lavoisier "died at the hands of the perversion of the revolution that Priestley supported, and for which the latter was hounded out of his native country."

Thomas Kuhn, in his influential book *The Structure of Scientific Revolutions* (1996), ranks the oxygen theory of combustion as a "paradigm shift." He says (p. 56), "What the work on oxygen did was to give much additional form and structure to Lavoisier's earlier sense that something was amiss. It told him a thing he was already prepared to discover – the nature of the substance that combustion removes from the atmosphere."

Several recent works of popular literature have focused on subjects related to the new chemistry and to oxygen and its discovery. In *Oxygen: A Play in Two Acts* (2001), chemists Carl Djerassi

and Roald Hoffmann address the question who deserves credit for the discovery of oxygen. In *Oxygen: The Molecule that Made the World* (2002), Nick Lane discusses the role of oxygen in geological and evolutionary history and in aging and disease. Other works include Madison Smartt Bell's biography *Lavoisier in the Year One: The Birth of a New Science in an Age of Revolution* (2005), and Joe Jackson's *A World on Fire: \A Heretic, an Aristocrat, and the Race to Discover Oxygen* (2005).

VIII. Jan Ingen-Housz (1730–1799): The Role of Light, and the Importance of Plants' Green Color

The discovery of the reciprocal nature of plant and animal life, mediated by gases (Priestley, 1772b), seems to have inspired at least three independent researchers in plant nutrition–Jan Ingen-Housz, Jean Senebier, and Nicholas de Saussure (Hill, 1970). Less well known than Priestley and Lavoisier, these men have often had their discoveries overlooked or misstated, or misattributed, either to one another or to Priestley.

The first of the three to make a significant contribution was the Dutch physician Jan Ingen-Housz (Fig. 30.2c). His most important discovery was that plants require light in order to produce dephlogisticated air (oxygen). He also showed that leaves are the primary sites of the formation of dephlogisticated air, and he discovered plant respiration.

Ingen-Housz's life and work are detailed in Julius von Wiesner's biography, *Jan Ingen-Housz. Sein Leben und Sein Wirken als Naturforscher und Arzt* (1905) and Magiels (2010). Shorter accounts include those by Harvey and Harvey (1930), Reed (1949), Van der Pas (1973), Smit (1980), Gest (2000), and Magiels (2007).

Ingen-Housz was born in Breda, The Netherlands. His father was a leather merchant and, after 1755, may have been a pharmacist. British physician John Pringle mentored the young Ingen-Housz, who earned a medical degree from the Catholic University of Louvain, Belgium, in 1753. Ingen-Housz then pursued further studies at the University of Leiden and possibly at the universities of Paris and Edinburgh (Van der Pas, 1973). He practiced medicine in Breda until 1765, when, at the invitation of Pringle, he moved to London to learn the then-new art of smallpox inoculation (Beale and Beale, 2001). The technique involved the use of live smallpox virus. (Although viruses had not been discovered yet, it was known that serum taken from an infected person and injected subcutaneously in an uninfected person provided protection. This method was superseded by the introduction of vaccination with cowpox virus, by English physician Edward Jenner, in 1798.) In 1768, British King George III sent Ingen-Housz to Vienna to inoculate the Habsburg royal family. Ingen-Housz's success at this endeavor so impressed Empress Maria Theresa that she appointed him court physician and endowed him with a life-long annual income (Van der Pas, 1973).

Upon reading Pringle's address to the Royal Society on the occasion of the awarding of the Copley Medal to Joseph Priestley, in 1773, Ingen-Housz became interested in the ability of plants to purify the air. He later wrote (Ingen-Housz, 1779, p. xv) of being impressed at Priestley's discovery that "plants wonderfully thrive in putrid air; and that the vegetation of a plant could correct air fouled by the burning of a candle." In the summer of 1779, having obtained a leave of absence from the Austrian court, Ingen-Housz went to England. There, at a country house at Southall Green, near London, he performed more than 500 experiments in less than 3 months' time (Ingen-Housz, 1779, p. xlii).

Ingen-Housz reported his discoveries almost immediately, in the book *Experiments Upon Vegetables, Discovering Their Great Power of Purifying the Common Air in the Sun-shine, and of Injuring it in the Shade and at Night* (1779). The book was an immediate success. He himself translated it into French (Ingen-Housz, 1780). He later published a second French edition, in two volumes (Vol. I in 1787, Vol. II in 1789). In 1949, Howard S. Reed republished the text of Ingen-Housz (1779) in combined issues of Chronica Botanica, with a few interpolations from the French edition of 1787. Reed omitted Ingen-Housz's experimental protocols but added extensive commentaries of his own.

Ingen-Housz made an important innovation in experimental technique. Based on Charles Bonnet's (1754) observation that bubbles form on submerged, illuminated, green leaves, Ingen-Housz

suspected that detached leaves might perform the function that Priestley had regarded as an activity of the plant as a whole. Using eudiometry, Ingen-Housz determined that the "air" produced by submerged leaves was dephlogisticated air. He then proceeded to do most of his experimental work with such leaves. This substitution, because of its simplicity, enabled him to make observations that had eluded Priestley (Nash, 1952).

Ingen-Housz (1779, pp. 14–16), in explaining his techniques and the resulting formation of bubbles, wrote that leaves:

> …are to be put in a very transparent glass vessel, or jar, filled with fresh pump water;… which, being inverted in a tub full of the same water, is to be immediately exposed to the open air, or rather to the sun-shine: thus the leaves continuing to live, continue also to perform the office they performed out of the water, as far as the water does not obstruct it. The water prevents only new atmospheric air being absorbed by the leaves, but does not prevent that air, which already existed in the leaves, from oozing out. This air, prepared in the leaves by the influence of the light of the sun, appears soon upon the surface of the leaves in different forms, most generally in the form of round bubbles, which … rise up and settle at the inverted bottom of the jar: they are succeeded by new bubbles, till the leaves, not being in the way of supplying themselves with new atmospheric air, become exhausted. This air, gathered in this manner, is really dephlogisticated air.…

Ingen-Housz used Priestley's pneumatic trough and nitrous air test. He controlled the illumination more carefully than had his predecessors, however. By using more intense illumination, he was able to carry out the experiments more rapidly, before green matter could develop and improve the air, or putrefaction could vitiate it (Nash, 1952). He said that Priestley's and Scheele's failures with plants (Ingen-Housz, 1779, p. 45) occurred because "These gentlemen expected the good effects from the vegetation of the plants, as such. By making a plant grow night and day in ordinary air kept in a phial with the plant, the effect will depend upon the greater or less exposure of the plant to the light." Ingen-Housz stated his belief (1779, p. 44) that "the faculty which plants possess of yielding dephlogisticated air, of correcting foul air, and improving ordinary air" is attributable to the influence of sunlight on a chemical process within the leaf. He pointed out that if the process of vegetation were responsible, as Priestley claimed, then dephlogisticated air would continue to be produced even in the dark, since plants are known to grow in the absence of light (as in etiolation). Plants do not produce dephlogisticated air in the dark, however.

For many years, Ingen-Housz did not understand that fixed air was important in plant physiology. He nowhere stated in his 1779 book that fixed air gives rise to dephlogisticated air, and he quarreled over this point with Senebier, who strongly believed that it did. Further, even though sunlight was presumably operating both for plants immersed in water and for those growing naturally in the atmosphere, Ingen-Housz did not regard the two situations as comparable. He thought that plants not growing under water purify the atmosphere by removing its phlogiston, but for plants growing under water, the mechanism was a transmutation brought about by light, with no involvement of phlogiston. He seems to have believed that the dephlogisticated air produced by plants submerged in water came from a transmutation of the water itself rather than from a gas in the water (Kottler, 1973).

Ingen-Housz thought that the phlogiston that plants obtained from the atmosphere served them nutritionally. He wrote (1779, pp. 74–75):

> Vegetables seem to draw the most part of their juices from the earth, by their spreading roots; and their phlogistic matter chiefly from the atmosphere, from which they absorb the air as it exists. They elaborate this air in the substance of their leaves, separating from it what is wanted for their own nourishment, viz., the phlogiston, and throwing out the remainder, thus deprived of its inflammable principle, as an excrementous fluid….

Ingen-Housz made another misinterpretation in assuming that dephlogisticated air is highly soluble in water. He did not test this assumption (though Senebier later did). Instead, Ingen-Housz thought that bubbles appeared on leaves if the water was saturated with air of any kind, but if the water was unsaturated, the dephlogisticated air simply dissolved in the water without forming bubbles (Nash, 1952).

Despite such interpretive errors, Ingen-Housz refined the knowledge of plant nutrition in several ways, in addition to his discovery of the light requirement. He wrote (1779, p. xxxiv–xxxv): "I found …that this office [the production of dephlogisticated air] is not performed by the whole

plant, but only by the leaves and the green stalks that support them." He also recognized the green matter as a plant before Priestley did, and he demonstrated that, as the intensity of the illumination increased, plants produced more dephlogisticated air. Further, he found that, contrary to Bonnet's (1754) hypothesis, it is the sun's light, not its heat, that leads to the liberation of gas bubbles from submerged leaves. He thereby confirmed Hales's suspicion that sunlight has some effect on plants other than through warming them. Ingen-Housz wrote (1779, pp. 29–30):

> I placed some leaves in pump water, inverted the jar, and kept it as near the fire as was required to receive a moderate warmth, near as much as a similar jar, filled with leaves of the same plant, and placed in the open air, at the same time received from the sun. The result was, that the air obtained by the fire was very bad, and that obtained in the sun was dephlogisticated air.

Ingen-Housz also discovered that plants perform respiration, as Lavoisier (1777) had demonstrated for animals. Ingen-Housz (1779) showed not only that all parts of plants vitiate the air in the absence of light, but also that the non-green parts, such as the stems and roots, vitiate it in the daylight as well. Senebier did not accept Ingen-Housz's finding of plant respiration, and this caused further quarreling between them.

Ingen-Housz considered vitiated air to be highly toxic, and greatly exaggerated its dangers. Höxtermann (2007, p. 147, as translated from the German by K. Nickelsen) quotes Ingen-Housz (1786, p. Lvf.):

> I observed … that in general fruit at any time keep this noxious property, especially in the dark, and that this poisonous property goes so far that even the most delicious fruit, such as peaches, are able to contaminate the air in a single night, so that one would be in mortal danger if one were locked up in a small room with a great many of these fruit.

He discovered, however, that the production of pure air normally more than compensates for the production of this poisonous air. He says (1779, p. 47), "The plants evaporate by night bad air, and foul the common air which surrounds them; yet this is far over-balanced by their beneficial operation during the day." Had Ingen-Housz used whole plants in his experiments, as had Priestley, he would have had a much more difficult time discovering this opposition of activities (Nash, 1952).

In 1780, Ingen-Housz returned to the Viennese court, where he pursued plant research as time permitted. He incorporated some of his new findings in the two-volume, French edition of his book (Ingen-Housz, 1787, 1789). In this edition, he still did not acknowledge that fixed air is the ultimate source of the dephlogisticated air produced by plants.

In 1788 Ingen-Housz went back to England for good. According to Harvey and Harvey (1930), he wished throughout this period to return to Vienna. Conley and Brewer-Anderson (1997), however, point to letters he wrote to his friend Benjamin Franklin in which he confided that he had chafed at his confinement at the court, which he was permitted to leave only with permission of the emperor. His correspondence with Franklin reveals further that, although he received a good income from the court, he suffered financial difficulties due to unwise investments, and as a result was unable to fulfill his wish to emigrate to the United States in his later years to be near Franklin (Conley and Brewer-Anderson, 1997). During his final years in England, Ingen-Housz spent some of his time at Bowood House, the estate of Priestley's former patron, the Earl of Shelburne (who in 1784 had become the First Marquess of Lansdowne). In a laboratory that Lord Lansdowne equipped for him there, which included some of Priestley's apparatus (Gest, 2000), Ingen-Housz resumed his research on plant nutrition (Harvey and Harvey, 1930).

It was during these years in England that Ingen-Housz wrote his second, and last, important work in plant physiology: *An Essay on the Food of Plants and the Renovation of Soils* (1796). In this short work, which appeared as an appendix to an obscure publication by the English Board of Agriculture, Ingen-Housz re-interpreted his earlier results in conformity with the new chemistry developed by Lavoisier. Fixed air became carbon dioxide, and pure (vital or dephlogisticated) air became oxygen. This was the first publication to describe clearly the importance of the process of gas exchange for plant nutrition.

Oddly, Ingen-Housz (1796, p. 16) claimed to have originated the idea that carbon dioxide is a

plant nutrient. Nash (1952, pp. 104–105) quotes that passage, adding his own sarcastic, parenthetical comment:

> I inferred from these, and some other facts quoted before, that the plants in the common course of nature draw from the air, in a great measure, what is necessary for their subsistence; and that being thus incessantly occupied in decomposing the common air, they render a part of it miscible with the ground, or with substances inherent in the earth, such as moisture, salts, &c.; that the carbonic acid, which is now admitted (according to my original idea) [this is rather cool: Senebier's priority is completely ignored] as a nourishing substance for plants, is prepared without intermission, day and night by the roots and flowers, and in the night by the leaves and the rest of the whole plant, must have been destined by nature to some important use for the plants themselves….

Further arguing that plants obtain their carbon mainly from carbon dioxide in the atmosphere rather than from the soil solution, Ingen-Housz wrote (1796, p. 9):

> …there is no difficulty in ... conceiving how the largest tree finds, during centuries, that immense quantity of food it requires for its maintenance, growth, and abundant production of fruit or seed, all which is certainly derived in part from the soil; but I still believe chiefly from the atmosphere, by means of the leaves absorbing and decomposing the air in contact with them.

Ingen-Housz mistakenly thought, however, that plants take up nitrogen from the atmosphere (1796, p. 10).

Like his 1779 book, which was reprinted by Reed (1949), Ingen-Housz (1796) saw its way into a twentieth-century reprinting: J. Christian Bay (1933) printed 100 copies for private distribution. Gest (1997) points out that the reprint omits the marginal notes and comments that Ingen-Housz had included in his original publication.

Ingen-Housz's achievements in plant physiology have, according to some authors, been overlooked and neglected. Gest (1991) ventures that this was "in no small measure because Priestley's prolific writings obfuscated the great advances made by the Dutch physician." Magiels (2007) also maintains that Ingen-Housz did not receive due credit. Gest (2000), after analyzing letters and other writings of Priestley, concluded that Priestley could refer only to his letters, not to any "publication" in the generally accepted sense, indicating a discovery by him of the light requirement, predating Ingen-Housz (1779).

Ingen-Housz (1796, pp. 2–3) asserted his priority in this discovery:

> I was fortunate enough to discover the true reason, why plants did sometimes correct bad air, and sometimes made it worse, which reason was never so much as even suspected either by Dr. Priestley or by Scheele; and indeed if either of them had had the least suspicion of it, their known eagerness for fame would not have allowed them to keep the discovery from the public eye, and Dr. Priestley would not have gone much farther than Mr. Scheele did; *viz*. to acknowledge openly, (even in his book printed 1779,) that he had been mistaken, and that he was entirely ignorant of the reason why vegetables are so inconstant in their effects on the air in contact with them.

With all his quarreling with colleagues, Ingen-Housz's personality has been a subject of discussion. There is considerable disagreement. Van der Pas (1973) characterizes him as a "shy, kind man." Schofield (1966, p. 360) comments that he was a conformist socially and religiously and had "the genial manners and social graces that Priestley lacked." Smit (1980) claims that Ingen-Housz "did not have the character of a fighter" so did not defend his priority in his discoveries.

In contrast, Beale and Beale (2005) state that, although Ingen-Housz may have been mild-mannered in social affairs, he was also called pompous and dictatorial, including in his relations with Edward Jenner. There is evidence, Beale and Beale say (p. 97), that Ingen-Housz could be "stubborn and persistent in scientific disputes. For example, he doggedly fought his corner after discoveries of his own had been claimed by others such as Joseph Priestley and Jean Senebier." Rabinowitch (1945, p. 18) claims that Ingen-Housz realized the importance of his discovery of the light requirement "and was decided not to let anybody deprive him of it or even as much as share in it." In the view of Partington (1962, p. 282), Ingen-Housz's defensiveness may have been justified: "Priestley and Senebier, both ministers of religion, seem to have been anxious to give Ingen-Housz as little credit for his work as they possibly could."

Although Ingen-Housz published before Priestley on the light requirement, Schofield

(2004, pp. 155–156) notes that Priestley could have discovered this requirement on his own during the interval between the completion of Ingen-Housz's book in October 1779 and the appearance of the printed volume later that year. Schofield (2004) points out that Priestley may not have seen that book when he wrote, on December 12, 1779, to a friend, "I soon discovered that the 'green matter' …is a vegetable substance, and that all other water plants do the same, converting the impure air contained in water into pure air, and therefore I conclude that all plants do the same in the light." Nash (1952) offers a similar opinion. Priestley never acknowledged Ingen-Housz's priority in the discovery of the light requirement.

Because Ingen-Housz had priority in publication, some scholars credit him with the discovery of photosynthesis. Others give the credit to Priestley–for example, Hill (1972)–or to Senebier. In reality, however, a number of individuals contributed, supplying various pieces of the puzzle, and no single person is responsible for the "discovery" of photosynthesis as such.

Ingen-Housz died at Bowood House, Calne, England. In 1999, a ceremony there marked the bicentennial of his death. Gest (2000) reported on the event.

IX. Jean Senebier (1742–1809): The Role of Carbon Dioxide

Swiss clergyman and naturalist Jean Senebier (Fig. 30.2d) provided evidence that plants must have access to fixed air (carbon dioxide) in order to produce dephlogisticated air (oxygen). He demonstrated further that the amount of dephlogisticated air that a plant produces is related to the amount of fixed air available (Senebier, 1782). He thus corrected the interpretation of Ingen-Housz (1779), who did not concede the importance of carbon dioxide until 1796, 14 years after Senebier had published on it.

Biographical information about Senebier is sparse (Kottler, 1973). Bay (1931), Kottler (1973), and Pilet (1975a) provide some background. Senebier was born in Geneva, the son of a wealthy merchant. Although he was interested in natural philosophy, his family directed him into the ministry. He was ordained a minister in Geneva in 1765. In 1769 he became pastor of a church in Chancy, near Geneva. In 1773, he left that post to become librarian for the Republic of Geneva, a position he held for the rest of his life (Bay, 1931; Pilet, 1975a).

The young Senebier was attracted to the circle inspired and guided by Charles Bonnet (Bay, 1931). Bonnet enabled Senebier to perform his first experiments in plant physiology (Pilet, 1975a). Following Bonnet's advice, in 1769 Senebier answered a question on the art of observing, posed by the Haarlem Academy of Sciences (Kottler, 1973). He published an expanded version of his essay as *L'Art d'observer* (Senebier, 1775), and subsequently a lengthier edition (Senebier, 1802). According to Bay (1931), Senebier's book was the first systematic attempt at a philosophy of the art of experimentation.

In the late 1770s, Senebier wrote several memoirs on phlogiston in the economy of nature. Then he turned to the nature of light and its interactions with natural objects, including plants, and began experimenting on etiolation (Kottler, 1973). Upon reading the French translation of *Experiments Upon Vegetables* (Ingen-Housz, 1780), Senebier's experimental focus veered to gas exchange in plants. He repeated nearly all of Ingen-Housz's 1779 experiments (Nash, 1952). Senebier claimed to have written of his ideas to Bonnet before he saw Ingen-Housz's book, although he admits he made use of the book. His publications mention Ingen-Housz only in passing (Partington, 1962).

To study air production by plants, Senebier used an experimental setup similar to Ingen-Housz's: leaves submerged in water (Fig. 30.9). He repeated the same experiments hundreds and hundreds of times, "now with reference to Ingen-Housz, then testing the exactness of Priestley's work, then again striking a new track" (Bay, 1931). Senebier published voluminously on his results. Most of these results were reported in four works (Senebier, 1782, 1783, 1788, 1800). His other works in plant physiology include Senebier (1791, 1792).

Senebier's important findings on the exchange of gases between plants and the atmosphere are contained mostly in Volume I of *Mémoires physico-chimiques sur l'influence de la lumière solaire pour modifier les êtres des trois règnes de la nature, & sur-tout ceux du règne vegetal* (1782). Volumes II and III of that work present his views

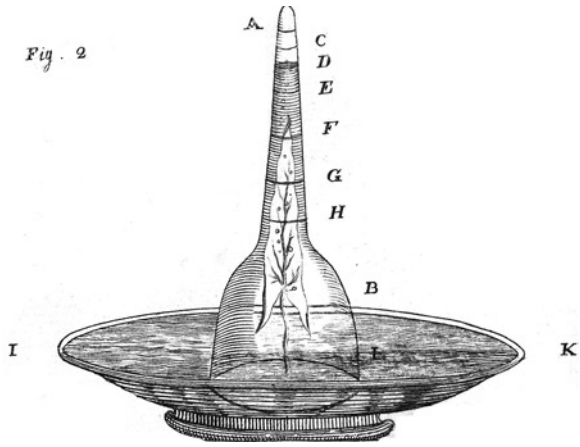

Fig. 30.9. Jean Senebier's laboratory apparatus for studying production of dephlogisticated air by submerged leaves (Senebier, 1782, Plate I, Fig. 2). AB, receptacle with glass tube, closed at A and filled with water to D; CDEFGH, divisions on tube; IK, saucer filled with water, on which the receptacle containing water and the leaf is placed.

on the influence of light on plants (Kottler, 1973). With characteristic prolixity, Senebier devoted more than 400 pages of his books of 1782 and 1783 to his finding of proportionality between the amount of air present in the water at the outset and the amount given off by the leaves (Nash, 1952). He states (1782, Vol. I, pp. 178–179):

> It was presently a matter of knowing, if really the waters the most charged with fixed air were also those that made leaves, that were plunged into them and then exposed to the sun, render the largest quantity of pure air. To discover this, I filled several recipients with boiled water: I introduced in some one measure of fixed air…; I introduced into others two measures of this air, into others three, to others four, etc. When this air was absorbed, I passed leaves under each of them. I then exposed them to the action of the sun; and I found that … the quantity of air, produced by the leaves, had accrued in proportion to the numbers of measures of fixed air previously absorbed by the water…

Fixed air is present in significant concentrations in water that has been exposed to the atmosphere for some time because, even though fixed air composes only a tiny percentage of the atmosphere, it is highly soluble in water. Thus some dephlogisticated air can always be produced by the illumination of leaves immersed in water that has been in contact with the atmosphere (Nash, 1952). Fixed air is especially abundant in pump water, which Senebier and Ingen-Housz used in many of their experiments.

When his experimental plants ceased to produce dephlogisticated air, a change of the water led to renewed production but a change of the leaves did not. He wrote (Senebier, 1783, p. 326), "I am convinced that the quantity of fixed air contained in the water was strongly diminished, when the leaves that I exposed to the sun had furnished their air." He also wrote (Senebier, 1783, pp. 15–16), "...fresh leaves, exposed anew to the sun in water where this has already been going on, while sunlight acted on them, furnished much less air than the first."

Because Senebier repeated most of Ingen-Housz's experiments, it is not surprising that many of the findings of these two researchers were similar. Both men found that abundant dephlogisticated air is produced by leaves submerged in pump water, but none is formed by leaves submerged in water that has been successively distilled and boiled (thus containing no fixed air) (Nash, 1952). The similarity of Senebier's experiments to Ingen-Housz's, and Senebier's failure to acknowledge the similarities and differences (Magiels, 2007), irked Ingen-Housz. Rabinowitch (1945, p. 20) observed, "Ever after he [Senebier] found himself exposed to the merciless irony and clever insinuations of Ingen-Housz, whose wrath would not be assuaged by the long-winded explanations of the Swiss pastor. The subsequent publications of both adversaries… are filled with acid polemics, and make sad reading."

In contrast to Ingen-Housz's denial of the role of fixed air, Senebier concluded that both submerged leaves and plants growing in their natural environment require fixed air in order to produce dephlogisticated air (Kottler, 1973). This led to further quarreling. They also argued over plant respiration. Senebier (1782) maintained that the gas produced in the dark by submerged, experimental plants must be a result of a diseased condition arising from the unnatural conditions, and that plants growing in the atmosphere did not normally produce fixed air. In this heated dispute, Ingen-Housz successfully maintained his position that plants respire (Nash, 1952).

Their views differed in other ways, as well. Senebier showed that dephlogisticated air tends to dissolve in water only if it is agitated with it, thus disproving Ingen-Housz's contention that dephlogisticated air is very soluble in water.

Senebier wrote (1782, Vol. I, pp. 35–36), "This conclusion…assures us that, in all the products of air furnished by the leaves, one has almost entirely the air that they have really filtered, and that the water has absorbed little of it…."

Senebier also contributed the insight that dephlogisticated air originates in the green parenchyma, not in leaf ribs or epidermal structures. Further, in comparing the effects of different colors of light on plants' production of dephlogisticated air, he anticipated an important approach to the study of photosynthesis in the nineteenth and early twentieth centuries (Kottler, 1973). He was wrong, however, in stating (Senebier, 1782, Vol. I, pp. 255–258) that plants growing normally in the atmosphere absorb fixed air in solution through their leaves and roots. In contrast, Ingen-Housz ultimately concluded (1796) that the source of the carbonic acid is chiefly the atmosphere.

In his several treatises on vegetation written during the 1780s, Senebier showed a gradual conversion from the phlogiston theory to oxygen chemistry (Kottler, 1973). (For a detailed discussion of the evolution of Senebier's theoretical views during the period 1782–1792, see Nash, 1952, pp. 77–95.)

Senebier's discovery of the importance of fixed air is another of those early, major advances in the understanding of photosynthesis that has at times been overlooked, misstated, or misattributed. Strangely, confusion has persisted regarding which man, Ingen-Housz or Senebier, deserves credit for this important finding, even though Senebier (1782) presented voluminous evidence supporting the idea long before Ingen-Housz accepted its validity. Although none of Senebier's evidence was individually conclusive, the many complementary findings argued strongly in favor of his view (Nash, 1952).

Two relatively recent books in which Senebier's contributions are overlooked, misattributed or misstated will be mentioned here. One is John King's *Reaching for the Sun: How Plants Work* (1997), which, on pages 19–20, credits Ingen-Housz with finding the "first hint that light and carbon dioxide were linked in some way leading to the release of oxygen," thus denying Senebier credit for the carbon dioxide finding. The second example is Maurice McDonald's *Photobiology of Higher Plants* (2003). McDonald (2003, p. 34) states: "In 1782, … Jean Senebier claimed that 'fixed air' (CO_2) produced by animals and by plants in darkness stimulated production of 'purified' air (O_2) by plants in light." Senebier, however, did not believe that healthy plants produced carbon dioxide. A further example is Huzisige and Ke (1993), in which a chart lists both Ingen-Housz and Senebier as discovering the involvement of fixed air in photosynthesis.

The confusion over priority may be attributable partly to the unsupported claim of Ingen-Housz (1796) that he originated the hypothesis regarding fixed air as the source of nourishment for plants. Partington (1962, p. 280, footnote 5) points a finger at Sachs (1875), who, Partington notes, "mistakenly says Ingen-Housz found in his *earlier* work [i.e., 1779] that 'green plants, under the influence of light, take up carbonic acid, separate the oxygen, and so assimilate carbon.'"

Another reason for the confusion may be that, in contrast to Senebier's voluminous writings, Ingen-Housz (1796) presented a concise, largely accurate summary of the fundamentals of plant nutrition in terms of the new chemistry, including Senebier's findings regarding fixed air. The actual achievement of a milestone in research (the discovery of the importance of fixed air) became obscured by a subsequent, persuasive synthesis of a range of findings, including the discovery in question, into a coherent statement.

Senebier may have suffered a somewhat similar fate vis-à-vis Nicholas de Saussure, who was economical both in his experimentation and in his writing. Further, since de Saussure's work included precise measurements of gas exchange, de Saussure sometimes gets the credit.

X. Nicholas-Théodore de Saussure (1767–1845): Confirming the Role of Water, and Developing a Unified Concept of Plant Nutrition

Nicholas-Théodore de Saussure (Fig. 30.2e) clarified and expanded upon the data and interpretations of plant aerial nutrition and the scheme of plant nutrition in general (Nash, 1952). He is particularly known for his conclusive demonstration that water is a reagent directly involved in photosynthesis. De Saussure also provided quantitative evidence that plants obtain all their carbon from

carbon dioxide in the atmosphere rather than from humus in the soil, and that the soil provides plants with minerals. Details about his life can be found in Hart (1930) and Pilet (1975b), and in Freshfield's (1920) biography of Nicholas' father, Horace-Bénédict de Saussure (1740–1799), who was a distinguished geologist and alpine explorer, and a close friend of Senebier's.

Nicholas de Saussure was born in Geneva and attended the Geneva Academy (Hart, 1930). He developed an interest in natural philosophy and assisted his father on a number of alpine expeditions (Pilet, 1975b). Then he became intensely interested in chemistry and plant physiology.

There was a gap of well over a decade between Senebier's achievements in plant physiology and de Saussure's. Younger than his immediate predecessors in plant physiology, de Saussure did not have to labor under the weight of the phlogiston theory. He was an outstanding experimenter. (Fig. 30.10 shows examples of his experimental arrangements.)

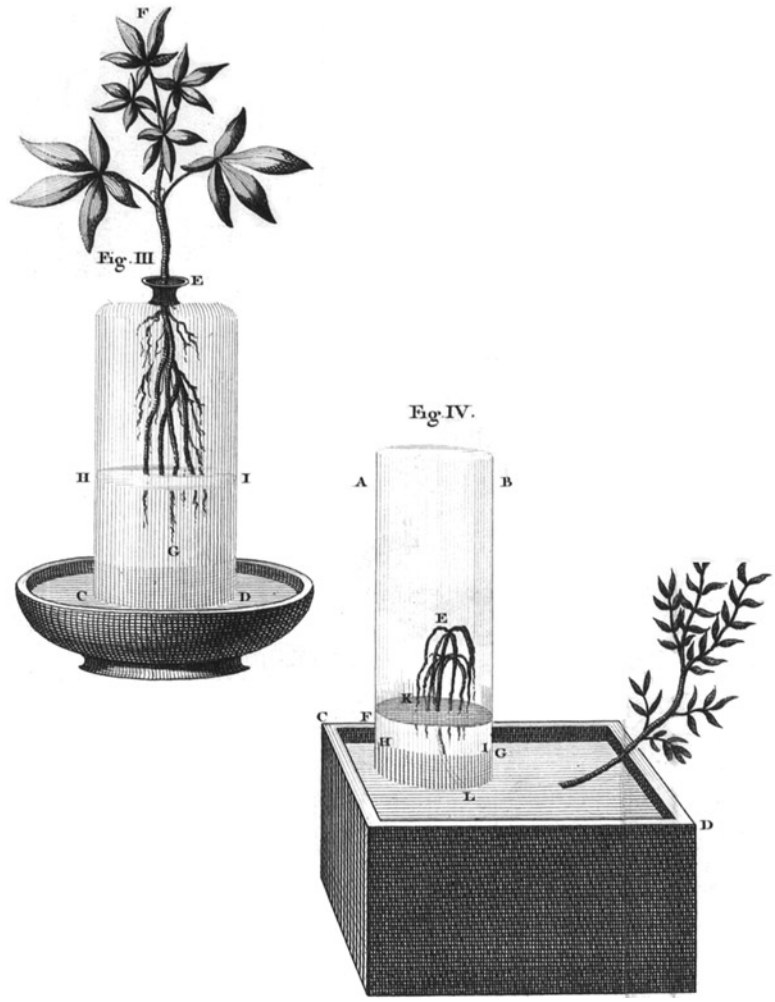

Fig. 30.10. Some experimental arrangements used by Nicholas de Saussure (1804, Plate). Fig. III: The roots of a horse chestnut (*FG*) were inserted into a recipient and the neck was sealed at E; the vessel was then filled with distilled water, and a gas (such as nitrogen, hydrogen, or carbon dioxide) was introduced through the opening CD; the upper part of the root was in contact with the gas, and the lower part with the water, HI. The vessel rested in a basin of mercury. The survival times of plants in the various gases, and the uptake of gases, were compared. Fig. IV: Mercury bath (*CD*) with recipient (*AB*) containing atmospheric air; the mercury surface in the recipient (*HI*) is covered with a layer of water (*FG*); a root (*EK*) of *Polygonum* was inserted from below. Water and oxygen, but not nitrogen, were taken up by the root.

Following the precedent of Lavoisier, de Saussure emphasized measurements of weight. Thus, instead of just observing whether his experimental plants did well, he measured the weight they gained. He also measured the weight of carbon and mineral elements that the plants contained. He carried out his research with relatively few experiments (in contrast to Senebier's extensive and repetitious work) and aimed his work at particular problems (Nash, 1952). From small, experimental plants grown with their shoots exposed to the open atmosphere and their roots in distilled water, de Saussure learned that, despite the unnatural conditions, the plants contained more total carbon than did plants that were at the same stage of development as his experimental plants had been when he had begun his experiments (Nash, 1952). He concluded that plants obtain all their carbon from the carbon dioxide of the atmosphere (Partington, 1962). His findings disproved Senebier's hypothesis that carbon dioxide dissolved in water is the source of the carbon in plants. (Ingen-Housz (1796) was in essential agreement with de Saussure on this point, but had not shown it quantitatively.)

Further, in contrast to his immediate predecessors in plant nutrition research, de Saussure did not focus solely on aerial nutrition, but also studied water and soil, both of which had long histories as suspected plant nutrients. Van Helmont (1648) had concluded from his willow-tree experiment that transmuted water forms the dry weight of plants, but his experiment did not really prove a role for water. Similarly, others had conjectured, without experimental evidence, that water is important for plants to gain in dry weight, because, knowing that plants contain abundant hydrogen, they could point to no other logical source.

De Saussure provided the first real evidence for a role for water. He showed that growing plants gain more in weight than can be accounted for by the assimilation of the carbon in the carbon dioxide that they take up. He reasoned that, since the amount of oxygen lost to the atmosphere is approximately equivalent to the amount of oxygen in the carbon dioxide absorbed, the extra weight gain must come from the water (Rabinowitch, 1945). De Saussure wrote (1804, pp. 269–270) that the results of his quantitative experiments "prove equally that the extract from the earth, the gas, and all the soluble principles in the water… do not make up the major part of the plant's dry weight if the water is excepted…. One will recognize that the water that the plant draws up and solidifies, be it from the soil or from the atmosphere, makes up, in weight, the greater part of the dry substance of the plant." De Saussure thus confirmed the guess that Van Helmont had made 200 years earlier, as Priestley and Ingen-Housz had confirmed the guesses of Stephen Hales (Rabinowitch and Govindjee, 1969).

De Saussure also demonstrated that plants take up various minerals from the soil. He contended that minerals found in the ash of plants had not been accidentally absorbed with the soil water, but were essential to the plant as nutrients, though they were often present in very small quantities (Hart, 1930).

With his highly targeted experiments and broad research program, de Saussure, according to Nash (1952, p. 117), "developed a conceptual scheme that allowed him to assign the source and route of supply of every major element that analysis showed to be present in mature plants." He presented his findings and interpretations in his single book, *Recherches Chimiques sur la Végétation* (1804), which laid the foundations of a new science, phytochemistry. The book was an immediate success. It was translated into German in 1805 and again in 1890. Despite an initial positive reception, however, there was a delay of 20–30 years before his ideas were fully understood and appreciated, because the old humus theory retained a strong hold on thinking about plant physiology (Hart, 1930).

In 1802, expecting to occupy a promised chair in plant physiology at the Geneva Academy, de Saussure was instead named honorary professor of mineralogy and geology. Disappointed at not being able to teach plant chemistry, he obtained a leave of absence. He never gave a course (Pilet, 1975b). Starting in 1808, he published a series of articles, mostly analyzing biochemical reactions in plant cells.

XI. Summary of the Early Contributions to the Modern Chemical Theory of Plant Nutrition

Sachs summed up the early work on photosynthesis (but said nothing about the role of light) (1890 translation of Sachs (1875), p. 491): "The discoveries of Priestley, Ingen-Housz and Senebier, and the quantitative determinations of de Saussure in

the years between 1774 and 1804, supplied the proof that the green parts of plants, and the leaves therefore especially, take up and decompose a constituent of the air, while they at the same time assimilate the constituents of water and increase in weight in a corresponding degree; but that this process only goes on copiously and in the normal way, when small quantities of mineral matter are introduced at the same time into the plant through the roots." Schofield (2004, p. 139) echoes Sachs: "It took the combined efforts of Jan Ingenhousz, Priestley, Jean Senebier, and Nicolas T. De Saussure to demonstrate, against much hostile criticism, that green vegetation, under the action of sunlight, absorbed carbon dioxide and released oxygen to the air." Hart (1930) concludes, "With Ingen-Housz and Senebier, de Saussure is responsible for founding the modern theory of plant nutrition."

The chemical equation of photosynthesis could now be written:

$$CO_2 + H_2O \xrightarrow[\text{green plant}]{\text{light}} O_2 + \text{organic matter}$$

Nash (1952, p. 106) considered de Saussure and Van Helmont transitional figures:

> Each represented the culmination of an extensive tradition, yet the work of each foreshadowed a new era. Van Helmont was still a partisan of alchemy, yet he dealt with subjects that became major fields of inquiry in the 'new experimental philosophy.' In similar fashion, de Saussure brought the studies of plant nutrition, begun by Priestley, Ingen-Housz, and Senebier, close to completion: he finished the fundamental experimental work and supplied a convincing theoretical interpretation of the whole. But de Saussure also opened up new vistas of experiment and thought in this field...

The new era that de Saussure inaugurated was long in coming, however. After de Saussure, there was "…a rapid decline, both in investigation and in the presentation of existing knowledge on the whole subject of plant metabolism…. The beautiful experiments [of Ingen-Housz, Senebier, and de Saussure]…were either forgotten or directly misinterpreted…"(Spoehr, 1919). No substantial advance was made in the overall inquiry on plant nutrition until roughly 60 years after the appearance of de Saussure's 1804 book (Rabinowitch, 1971).

It should be pointed out that, starting with the early pioneers, an erroneous view about photosynthesis was perpetuated. Ingen-Housz (1796), Senebier (1788), and de Saussure (1804) all expressed the belief that the oxygen emitted by plants originates in the carbon dioxide rather than in the water. For example, De Saussure (1804, p. 237) wrote, "plants, in no case, directly decompose water, assimilating its hydrogen and eliminating its oxygen in a gaseous state; they do not exhale oxygen gas except by immediate decomposition of carbonic acid gas."

Arnon (1991) comments, "...photodecomposition of CO_2, as proposed by Ingen-Housz, was the universally accepted concept; and with some elaboration it became a dogma that persisted well into the twentieth century." Then Dutch-born microbiologist Cornelis B. van Niel (1897–1985) proposed that the oxygen produced in green-plant photosynthesis comes from water, not carbon dioxide (Van Niel, 1931). He was subsequently proven correct.

XII. Julius Robert Mayer (1814–1878): The Final Component, Energy

The early workers in photosynthesis, from Van Helmont through de Saussure, were "chemical" pioneers. When they were doing their experiments, the concept of energy had not yet been formulated, although Ingen-Housz had discovered that light was essential to plants. Then, in the mid-nineteenth century, German physician Julius Robert Mayer (Fig. 30.11), the "physical" pioneer

Fig. 30.11. A portrait of Robert Mayer.

in our story, applied the newly emerging concept of energy to biological systems. For a detailed treatment of the development of Mayer's thought, see Caneva (1993). For a briefer account of his life and thought, see Turner (1975).

Mayer discounted the possibility that energy (a term that was not widely used at the time; his word was "kraft," which is translated as "force" or "power") was created in organic systems, since it was not created in inorganic ones (Nash, 1952). In his book *Die Organische Bewegung in ihrem Zusammenhang mit dem Stoffwechsel* (1845), Mayer contended that energy is conserved in biological as well as in physical systems, and that plants, in carrying out photosynthesis, store the energy of sunlight in the form of chemical energy. Animals acquire this chemical energy through food and convert it to body heat and the mechanical force of muscle movements (Turner, 1975). Before Mayer, plants could only be understood as chemical manufacturers of organic matter; after him, plants could also be seen as storers of energy.

Mayer (1845) wrote (translation taken from Rabinowitch and Govindjee, 1969, p. 9):

> Nature has put itself the problem how to catch in flight light streaming to the earth and to store the most elusive of all powers in rigid form. To achieve this aim, it has covered the crust of earth with organisms which in their life processes absorb the light of the sun and use this power to produce a continuously accumulating chemical difference.
>
> These organisms are the plants; the plant kingdom forms a reservoir in which the fleeting sun rays are fixed and skillfully stored for future use; an economic provision to which the physical existence of mankind is inexorably bound.
>
> The plants take in one form of power, light; and produce another power: chemical difference.

Fig. 30.12. A photograph of the author with Govindjee, Aug 2006.

Rabinowitch and Govindjee (1969) note that the term 'chemical difference' is what we would call chemical energy.

The equation of photosynthesis could now be written:

$$CO_2 + H_2O + \text{light} \xrightarrow{\text{green plant}} O_2 + \text{organic matter} + \text{chemical energy}$$

This equation sums up the overall scheme of photosynthesis, showing both the material and the energy balance (Rabinowitch and Govindjee, 1969). It completes the story of the early pioneers' achievements in elucidating the process by which plants nourish themselves and ultimately much of the rest of life on Earth. The carbon dioxide component was due to Senebier; the water to de Saussure; the light to Ingen-Housz; the oxygen to Priestley; and the chemical energy to Mayer. The other pioneers provided important stepping-stones to the unraveling of this scheme.

I end my chapter by showing my photograph with Govindjee (Fig. 30.12), who provided inspiration and impetus for this chapter.

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References

Arnon DI (1991) Photosynthetic electron transport: emergence of a concept, 1949–59. Photosynth Res 29: 117–131

Badash L (1964) Joseph Priestley's apparatus for pneumatic chemistry. J Hist Med Allied Sci 19: 139–155

Bay JC (1931) Jean Senebier. Plant Physiol 6: 188–193
Bay JC (1933) An essay on the food of plants and the renovation of soils. Reprint of Ingen-Housz 1796. Oquawka, Illinois
Beale N and Beale E (2001) Sunlight at Southall Green. Persp Biol Med 44: 333–344
Beale N and Beale E (2005) Evidence-based medicine in the eighteenth century: the Ingen Housz-Jenner correspondence revisited. Med Hist 49: 79–98
Bell MS (2005) Lavoisier in the Year One: The Birth of a New Science in an Age of Revolution. WW Norton, New York and London
Boklund U (1975) Carl Wilhelm Scheele. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol XII, pp 143–150. C Scribner's Sons, New York
Bonnet C (1754) Recherches sur l'usage des feuilles dans les plantes. Elie Luzac, fils, Göttingen/Leiden
Boyle R (1661) The Sceptical Chymist, or Chymico-Physical Doubts and Paradoxes. Cadwell, London.
Brock WH (1993) The Norton History of Chemistry. WW Norton, New York and London
Caneva KL (1993) Robert Mayer and the Conservation of Energy. Princeton University Press, Princeton, New Jersey
Cavendish H (1766) Three papers containing experiments on factitious air. Phil Trans Roy Soc London 56: 141–184
Conant JB (1950) The overthrow of the phlogiston theory: the chemical revolution of 1775–1789. Harvard Case Histories in Experimental Science, Case 2. Harvard University Press, Cambridge, Massachusetts
Conley TK and Brewer-Anderson M (1997) Franklin and Ingen-Housz: a correspondence of interests. Proc Amer Philosoph Soc 141: 276–296
de Waele H (1947) J-B van Helmont. Office de Publicité, Bruxelles
Diderot D, Alembert JLR d' and Mouchon P (1765) "Vegetation." In: Encyclopédie ou Dictionnaire raisonné des sciences, des arts et des métiers. Nouvelle impression en facsimile de la premiére edition de 1751–1780, 16: 953–962, 1966. Stuttgart-Bad Cannstatt
Djerassi C and Hoffman R (2001) Oxygen: A Play in Two Acts. Wiley-VCH, Weinheim, New York
Donovan A (1993) Antoine Lavoisier: Science, Administration and Revolution. Blackwell, Oxford, United Kingdom; and Cambridge, Massachusetts
Eklund J (1975) The incompleat chemist: being an essay on the eighteenth-century chemist in his laboratory, with a dictionary of obsolete chemical terms of the period. Smithsonian Stud Hist Tech, 33. Smithsonian Institution Press, Washington, DC
Freshfield DW (1920) The Life of Horace Benedict de Saussure. Edward Arnold, London
Galston AW (1994) Life Processes of Plants. Scientific American Library, New York
Gest H (1988) Sun-beams, cucumbers, and purple bacteria: historical milestones in early studies of photosynthesis revisited. Photosynth Res 19: 287–308
Gest H (1991) Sunbeams, cucumbers, and purple bacteria: the discovery of photosynthesis revisited. Persp Biol Med 34: 254–274
Gest H (1997) A 'misplaced chapter' in the history of photosynthesis research; the second publication (1796) on plant processes by Dr Jan Ingen-Housz, MD, discoverer of photosynthesis. Photosynth Res 53: 65–72
Gest H (2000) Bicentenary homage to Dr Jan Ingen-Housz, MD (1730–1799), pioneer of photosynthesis research. Photosynth Res 63: 183–190
Golinski J (1992) Science as Public Culture: Chemistry and Enlightenment in Britain 1760–1820. Cambridge University Press, Cambridge, New York and Melbourne
Govindjee and Gest H (eds) (2002) Celebrating the millennium – historical highlights of photosynthesis research, Part 1. Photosynth Res 73: 1–308
Govindjee and Krogmann D (2004) Discoveries in oxygenic photosynthesis (1727–2003): a perspective. Photosynth Res 80: 15–57
Govindjee, Beatty JT and Gest H (eds) (2003) Celebrating the millennium – historical highlights of photosynthesis research, Part 2. Photosynth Res 76: 1–462
Govindjee, Allen JF and Beatty JT (eds) (2004) Celebrating the millennium – historical highlights of photosynthesis research, Part 3. Photosynth Res 80: 1–13
Govindjee, Beatty J, Gest H and Allen J (eds) (2006) Discoveries in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 20. Springer, Dordrecht
Guerlac H (1972) Stephen Hales. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol VI, pp 35–48. C Scribner's Sons, New York
Hales S (1727) Vegetable Staticks, or, an Account of Some Statical Experiments on the Sap in Vegetation. W Innys, London (Reprinted, Macdonald, London, 1961)
Hales S (1733) Statical Essays: Containing Haemastaticks; Or, an Account of some Hydraulick and Hydrostatical Experiments on the Blood and Blood-Vessels of Animals. Printed for W Innys and R Manby, at the West End of St Paul's; And T Woodward, at the Half Moon between the Temple-Gates, Fleetstreet, London
Hart H (1930) Nicolas Théodore de Saussure. Plant Physiol 5: 424–429
Harvey RB (1929) Joannes Baptista van Helmont. Plant Physiol 4: 542–546
Harvey RB and Harvey HMW (1930) Jan Ingen-Housz. Plant Physiol 5: 283–287
Harvey W (1628) An anatomical disquisition on the motion of the heart and blood in animals. Willis's translation revised and edited by Alex Bowie. In: Camac CNB (1909) Epoch-making contributions to medicine, surgery and the allied sciences… , pp 27–111. Saunders, Philadelphia
Hill R (1970) The growth of our knowledge of photosynthesis. In: Needham J (ed) The Chemistry of Life: Eight Lectures on the History of Biochemistry, pp 1–14. Cambridge University Press, London

Hill R (1972) Joseph Priestley (1733–1804) and his discovery of photosynthesis in 1771. In: Forti G, Avron M and Melandri A (eds) Photosynthesis, Two Centuries after its Discovery by Joseph Priestley, pp 1–18. Dr W Junk Publisher, The Hague

Hoff HE (1964) Nicolaus of Cusa, Van Helmont, and Boyle: The first experiment of the Renaissance in quantitative biology and medicine. J Hist Med Allied Sci 19: 99–117

Hoffman R and Torrence V (1993) Chemistry Imagined: Reflections on Science. Smithsonian Institution Press, Washington and London

Hoskin MA (1961) Foreword to reprint edition of Hales (1727) Vegetable Staticks. Macdonald, London

Höxtermann E (1992) Fundamental discoveries in the history of photosynthesis research. Photosynthetica 26: 485–502

Höxtermann E (2007) Der Stoffwechsel: die Chemie des Lebens — Zur Geschichte der Biochemie. In: Höxtermann E and Hilger HH (eds) Lebenswissen. Eine Einführung in die Geschichte der Biologie, pp 142–177. Natur & Text, Rangsdorf

Howe HM (1965) A root of Van Helmont's tree. Isis 56: 408–419

Huzisige H and Ke B (1993) Dynamics of the history of photosynthesis research. Photosynth Res 38: 185–209

Ingen-Housz J (1779) Experiments upon Vegetables--Discovering Their Great Power of Purifying the Common Air in the Sunshine and of Injuring It in the Shade and at Night, to Which is Joined a New Method of Examining the Accurate Degree of Salubrity of the Atmosphere. Printed for P Elmsly and H Payne, London

Ingen-Housz J (1780) Expériences sur les végétaux: spécialement sur la propriété qu'ils possèdent à un haut degré, soit d'améliorer l'air quand ils sont au soleil, soit de la corrompre la nuit, ou lorsqu'ils sont a l'ombre; auxquelles on a joint une méthode nouvelle de juger de degré de salubrité de l'atmosphere. Translated from English by the author. Chez P Fr Didot le jeune, Libraire-Imprimeur de Monsieur, M, Paris.

Ingen-Housz J (1786) Versuche mit Pflanzen, hauptsächlich über die Eigenschaft, welche sie in einem hohen Grade besitzen, die Luft im Sonnenlichte zu reinigen und in der Nacht und im Schatten zu verderben … Wappler, Wien.

Ingen-Housz J (1787) Expériences sur les végétaux, spécialement sur la propriété qu'ils possèdent à un haut degré, soit d'améliorer l'air quand ils sont au soleil, soit de la corrompre la nuit, ou lorsqu'ils sont a l'ombre; auxquelles on a joint une méthode nouvelle de juger de degré de salubrité de l'atmosphere. A new revised and enlarged edition, Vol I. Translated from English by the author. Chez Théophile Barrois le jeune, Libraire, Paris

Ingen-Housz J (1789) Expériences sur les végétaux, spécialement sur la propriété qu'ils possèdent à un haut degré, soit d'améliorer l'air quand ils sont au soleil, soit de la corrompre la nuit, ou lorsqu'ils sont a l'ombre; auxquelles on a joint une méthode nouvelle de juger de degré de salubrité de l'atmosphere. A new revised and enlarged edition, Vol. II. Translated from English by the author. Chez Théophile Barrois le jeune, Libraire, Paris

Ingen-housz J (1796) An essay on the food of plants and the renovation of soils. Appendix to the Outlines of the Fifteenth Chapter of the Proposed General Report from the Board of Agriculture. Elmsley and Payne, London

Jackson J (2005) A world on fire: a heretic, an aristocrat, and the race to discover oxygen. Viking, New York

Johnson S (1756) A Dictionary of the English Language: in which the words are deduced from their originals, explained in their different meanings, and authorized by the names of the writers in whose works they are found. Abstracted from the folio edition, by the author Samuel Johnson, A. M. To which is prefixed, a grammar of the English language. 2 vols. J Knapton, London

Johnson S (1785) A Dictionary of the English Language: in which the words are deduced from their originals, and illustrated in their different significations by examples from the best writers. 2 vols. 6th edition. Printed for JF and C Rivington, L Davis, T Payne and Son, T Longman, B Law (and 21 others in London)

Kedrov BM (1973) Mikhail Lomonosov. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol VIII, pp 467–472. C Scribner's Sons, New York

King J (1997) Reaching for the Sun: How Plants Work. Cambridge University Press, Cambridge

Klein U and Lefèvre W (2007) Materials in Eighteenth-Century Science. A Historical Ontology. MIT Press, Cambridge, Massachusetts

Kottler D (1973) Jean Senebier and the emergence of plant physiology, 1775–1802: from natural history to chemical science. Dissertation, Johns Hopkins University

Krikorian AD and Steward FC (1968) Water and solutes in plant nutrition: with special reference to Van Helmont and Nicholas of Cusa. BioScience 18: 286–292

Kuhn T (1996) The Structure of Scientific Revolutions. 3rd edition. University of Chicago Press, Chicago and London

Lane N (2002) Oxygen: the Molecule that Made the World. Oxford University Press, Oxford and New York

Lavoisier A (1770) Sur la nature de l'eau. Oeuvres de Lavoisier 2: 1–28. Imprimerie impériale, 1862, Paris

Lavoisier A (1775) Mémoire sur la nature du principe qui se combine avec les métaux pendant leur calcination et qui en augmente le poids. Oeuvres de Lavoisier 2: 122–128. Imprimerie impériale, 1862, Paris

Lavoisier A (1777) Expériences sur la respiration des animaux et sur les changements qui arrivent à l'air par leur poumon. Oeuvres de Lavoisier 2: 174–183. Imprimerie impériale, 1862, Paris

Lavoisier A (1789) Traité élémentaire de chimie, présenté dans un ordre nouveau et d'après les découvertes modernes; avec figures. 2 vols. Cuchet, Paris

Magiels G (2007) Dr Jan IngenHousz, or why don't we know who discovered photosynthesis? Paper presented at 1st Conference of the European Philosophy of Science Association, Madrid, 15–17 November 2007

Magiels G (2010) From sunlight to insight. Jan IngenHousz, the discovery of photosynthesis & science in the light of ecology. Brussels University Press, Brussels

Mayer JR (1845) Die organische Bewegung in ihrem Zussamenhag mit dem Stoffwechsel: Ein Beitrag Zur Naturkunde. Verlag der C Drechsler'schen Buchhandlung, Heilbronn

McDonald M (2003) Photobiology of Higher Plants. Wiley, Chichester, England

Morton O (2008) Eating the Sun: How Plants Power the Planet. HarperCollins, New York

Morveau G de, Lavoisier A-L, Berthollet C-L, Fourcroy A-P, Hassenfratz J-H and Adet P-A (1787) Méthode de nomenclature chimique. Cuchet, Paris

Nash LK (1952) Plants and the atmosphere. Harvard Case Histories in Experimental Science, Case 5. Harvard University Press, Cambridge, Massachusetts

Newman WR and Principe LM (2002) Alchemy Tried in the Fire: Starkey, Boyle, and the Fate of Helmontian Chymistry. University of Chicago Press, Chicago and London

Pagel W (1972) Johannes (Joan) Baptista van Helmont. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol VI, pp 253–259. C Scribner's Sons, New York

Parascandola J and Ihde AJ (1969) History of the pneumatic trough. Isis 60: 351–361

Partington JR (1957) A Short History of Chemistry. 3rd edition, revised and enlarged. MacMillan, London; St. Martin's Press, New York. (Reprint, Dover Publications, New York, 1989)

Partington JR (1962) A History of Chemistry, Vol 3. Macmillan, London; St Martin's Press, New York

Pilet PE (1970) Charles Bonnet. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol II, pp 286–287. C Scribner's Sons, New York

Pilet PE (1975a) Jean Senebier. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol XII, pp 308–309. C Scribner's Sons, New York

Pilet PE (1975b) Nicholas-Théodore de Saussure. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol XII, pp 123–124. C Scribner's Sons, New York

Poirier JP (1996) Lavoisier: Chemist, Biologist, Economist. Translated by R Balinski. University of Pennsylvania Press, Philadelphia

Priestley JP (1765) A Description of a Chart of Biography. Printed for J Johnson, London

Priestley J (1767) The History and Present State of Electricity, with Original Experiments. Printed for J Dodsley, J Johnson and B Davenport, and T Cadell, London

Priestley J (1772a) The History and Present State of Discoveries Relating to Vision, Light, and Colours. Printed for J Johnson, London

Priestley J (1772b) Observations on different kinds of air. Phil Trans Roy Soc London 62: 147–264

Priestley J (1774) Experiments and Observations on Different Kinds of Air. Vol I. Printed for J Johnson, London

Priestley J (1775a) Experiments and Observations on Different Kinds of Air. Vol II. Printed for J Johnson, London

Priestley J (1775b) Account of further observations on air. Phil Trans Roy Soc London 65: 384–394

Priestley J (1775c) Experiments and Observations on Different Kinds of Air. Vol I, second edition, corrected. Printed for J Johnson, London

Priestley J (1776) Experiments and Observations on Different Kinds of Air. Vol II, second edition, corrected. Printed for J Johnson, London

Priestley J (1777) Experiments and Observations on Different Kinds of Air. Vol. III. Printed for J Johnson, London

Priestley J (1779) Experiments and Observations relating to various Branches of Natural philosophy, with a Continuation of the Observations on Air. Vol. I. Printed for J Johnson, London

Priestley J (1781) Experiments and Observations relating to various Branches of Natural Philosophy; with a Continuation of the Observations on Air. Vol. II. Printed for J Johnson, Birmingham, England

Priestley J (1786) Experiments and Observations relating to various Branches of Natural Philosophy; with a Continuation of the Observations on Air. Vol. III. Printed for J Johnson, Birmingham, England

Priestley J (1790) Experiments and Observations on Different Kinds of Air, and Other Branches of Natural Philosophy, connected with the subject, Being the former six volumes abridged and methodized, with many Additions. Birmingham. Printed by Thomas Pearson and sold by J Johnson, St Paul's Church-yard, London

Pringsheim EG (1932) Julius Sachs, der Begründer der neueren Pflanzenphysiologie. Gustav Fischer, Jena

Rabinowitch EI (1945) Photosynthesis and Related Processes. Vol I. Chemistry of Photosynthesis, Chemosynthesis, and Related Processes *in vitro* and *in vivo*, Chapter 2, pp 12–28. Interscience Publishers, New York

Rabinowitch EI (1971) An unfolding discovery. Proc Natl Acad Sci USA 68: 2875–2876

Rabinowitch EI and Govindjee (1969) Photosynthesis. John Wiley & Sons, New York

Redgrove HS and Redgrove IML (1922) Joannes Baptista van Helmont, Alchemist, Physician and Philosopher. W Rider & Son, Ltd, London. (Republished, Kessinger, Whitefish, Montana, 2003)

Reed HS (1949) Jan Ingenhousz, plant physiologist, with a history of the discovery of photosynthesis. Chronica Botanica Vol 11, No 5/6. Chronica Botanica Co, Waltham, Massachusetts

Sachs J von (1875) History of botany (1530–1860). 1890 English translation by HEF Garnsey, revised by IB Balfour, Oxford University Press, Oxford

Saussure NT de (1804) Recherches chimiques sur la Végétation. Didot jeune, Paris

Scheele CW and Bergman T (1777) Chemische Abhandlung von der Luft und dem Feuer. Uppsala u.a. English translation: Scheele CW (1780) Chemical observations and experiments on air and fire, with introduction by Torbern Bergman; translated from the German by JR Forster. Printed for J Johnson, London

Schofield RE (ed) (1966) A Scientific Autobiography of Joseph Priestley (1733–1804): Selected Scientific Correspondence Edited with Commentary by RE Schofield. MIT Press, Cambridge, Massachusetts, and London, England

Schofield RE (1975) Joseph Priestley. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol XI, pp 139–147. C Scribner's Sons, New York

Schofield RE (1997) The Enlightenment of Joseph Priestley: A Study of his Life and Work from 1733 to 1773. Pennsylvania State University Press, University Park, Pennsylvania

Schofield RE (2004) The Enlightened Joseph Priestley: A Study of his Life and Work from 1733 to 1804. Pennsylvania State University Press, University Park, Pennsylvania

Senebier J (1775) L'Art d'observer. 2 vols. Chez Cl Philibert & Bart Chirol, Geneva

Senebier J (1782) Mémoires physico-chymiques sur l'influence de la lumière solaire pour modifier les êtres des trois règnes de la Nature, & sur-tout ceux du règne vegetal. 3 vols. Barthelemi Chirol, Geneva

Senebier J (1783) Recherches sur l'influence de la lumière solaire pour metamorphoser l'air fixe en air pur par végétation. Barthelemi Chirol, Geneva

Senebier J (1788) Expériences sur l'action de la lumière solaire dans la végétation. Chez Barde, Manget et Co, Geneva

Senebier J (1791) Physiologie végétale. Encyclopédie méthodique 92: 1–296.

Senebier J (1792) Le gaz acide carbonique est décomposé par les plantes. Observations périodiques sur la physique, sur l'histoire naturelle et sur les arts 41: 205–213

Senebier J (1800) Traité de Physiologie Végétale. 5 vols. JJ Paschoud, Geneva

Senebier J (1802) Essai sur l'art d'observer et de faire des expériences. 3 volumes. 2nd edition. Chez JJ Paschoud, Geneva

Smit P (1980) Jan Ingen-Housz (1730–1799): Some new evidence about his life and work. Janus/Revue Internatl de l'Hist Sci, Méd, Pharm, et Tech 67: 125–139

Spoehr HH (1919) The development of conceptions of photosynthesis since Ingen-Housz. Science Monthly 9: 32–46

Turner RS (1975) Julius Robert Mayer. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol IX, pp 235–240. C Scribner's Sons, New York

Van der Pas PW (1973) Jan Ingen-Housz. In: Gillespie CC (ed) Dictionary of Scientific Biography, Vol VII, pp 11–16. C Scribner's Sons, New York

Van Helmont JB (1648) Ortus Medicinae. In: Van Helmont FW (ed) The Collected Works. Amsterdam

Van Helmont JB (1662) Oriatrike, or, Physick Refined. The common Errors therein Refuted, And the whole Art Reformed & Rectified: Being a New Rise and Progress of Phylosophy and Medicine for the Destruction of Diseases and Prolongation of Life. Translation of Ortus Medicinae by J Chandler. Printed for Lodowick Loyd, London

Van Niel CB (1931) On the morphology and physiology of the purple and green sulfur bacteria. Arch Mikrobiol 3: 1–112

White PR (1942) "Vegetable Dynamicks" and plant tissue cultures. Plant Physiol 17: 153–164

Wiesner J von (1905) Jan Ingen-Housz. Sein Leben und Sein Wirken als Naturforscher und Arzt. Verlagsbuchhandlung Carl Konegen, Vienna

Part VIII

The Career of Govindjee

Chapter 31

Contributions of Govindjee, 1955–1969

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Summary

I was the first, among Govindjee's graduate students, to finish a PhD under his supervision. Since the time I finished my PhD (in Biophysics in 1969), we have been friends, and research collaborators. Even now, after more than 40 years, we continue to exchange ideas and publish together. Two accounts have summarized Govindjee's career. The first [J.J. Eaton-Rye (2007) Photosynth Res 93: 1–5] celebrated Govindjee's 50 years in photosynthesis with a short article, and the second [J.J. Eaton-Rye (2007) Photosynth Res 94: 153–178] provided snapshots of the Govindjee's lab from the late 1960s to the late 1990s and beyond. Here, I present Govindjee's research contributions from 1955, when he published his first research paper in India, till 1969 when I graduated, and the famous Rabinowitch and Govindjee book on Photosynthesis was published by John Wiley & Sons, New York.

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DOI 10.1007/978-94-007-1579-0_31,

I. Early Training (1952–1954) and Research (1954–1956) in Plant Physiology, Allahabad, India

Govindjee obtained his B.Sc., in Chemistry, Zoology and Botany, in the first division, in 1952, from Allahabad University, Allahabad, India. He was awarded the Sri Vilas Scholarship since he had obtained the highest marks in Botany. This was followed by his M.Sc., in Botany, also in the first division, in 1954, from the same University. He was trained in Plant Physiology by Shri Ranjan, who had studied under Frederick Frost Blackman of England. Govindjee was the "topper" of his class (first on the Merit list), and this, I am told, led to his appointment as a Lecturer in Botany in 1954. He taught Plant Physiology to first-year M.Sc. students during 1954–1956. It was during this brief period that he did research on the effects of virus infection on amino acid metabolism in higher plants. At the suggestion, particularly of T. Rajarao, a 16-sector circular paper chromatogram was used in this research, and this allowed measurements on several samples all at the same time. Manmohan M. Laloraya (a classmate) and he discovered, in 1956, an increase in specific free amino acids, and amides, when tobacco leaves were infected with tobacco-leaf curl and tobacco mosaic virus; this work was published in *Nature*. More importantly, he met Rajni Verma during this period, and they were married in 1957 at Urbana, Illinois. For some photographs from this early period see Fig. 31.1.

A. Publications on the Effects of X-ray Treatment and Virus Infection on Amino Acid Metabolism

Govindjee conducted preliminary research, by himself, on the effects of X-rays on the metabolism of plants. In collaboration with Manmohan M. Laloraya, T. Rajarao, Rajni Verma, and Professor Shri Ranjan, Govindjee published one regular paper, and several short papers, during 1955–1957, in the area of virus infection on the amino acid and sugar metabolism of plants. These publications are listed below.

Laloraya MM, Govindjee and Rajarao T **(1955)** A chromatographic study of the amino acids (and sugars) of healthy and diseased leaves of *Acalypha indica*. Curr Sci 24: 203

Ranjan S, Govindjee and Laloraya MM **(1955)** Chromatographic studies on the amino acid metabolism of healthy and diseased leaves of *Croton sparsiflorus* morong. Proc Nat Inst Sci (India) 21: 42–47

Govindjee **(1956)** Effect of X-rays on the oxygen uptake of *Cicer arietinum* T87 seedlings. Naturwissenschaften 43: 524

Govindjee, Laloraya MM and Rajarao T **(1956)** Formation of asparagine and increase in the free amino acid content in virus infected leaves of *Abelmoschus esculentus*. Experientia 12: 180

Laloraya MM and Govindjee **(1956)** Effect of tobacco leaf-curl and tobacco mosaic virus on the amino acid content of *Nicotiana* sp. Nature 175: 907

Laloraya MM, Govindjee, Varma R and Rajarao T **(1956)** Increased formation of asparagine in carica-curl virus infected leaves. Experientia 12: 58

Rajarao T, Laloraya MM and Govindjee **(1956)** Absence of some free amino acids from the diseased leaves of *Trichosanthes angiuna*. Naturwissenschaften 43: 301

Govindjee **(1957)** Effect of X-rays on the content of free amino acids and amides of *Cicer arietinum* T87 Seedlings. Naturwissenschaften 44: 183

II. Graduate Training and Photosynthesis Research, Urbana, Illinois (1956–1960)

Govindjee's interest in photosynthesis research was sparked by his own studies and activities, during 1952–1954 at Allahabad. In particular, he had organized a 'Mock Symposium on Discoveries in Photosynthesis', where students played the role of the various discoverers (Otto Warburg and Robert Emerson were two of the several chosen). He acted as Emerson, not realizing then that he will work with him later. A second, but most important event was that he chose to write a term paper on "The Role of Chlorophyll in Photosynthesis" and read it in an Advanced Plant Physiology Seminar course, with Shri Ranjan as the professor-in-charge. In this paper, he had discussed the work of Richard Willstätter, Otto Warburg, and Robert Emerson, among others. According to notes provided by Govindjee to me, he was particularly fascinated

Abbreviations: Chl – Chlorophyll; PS – Photosystem; UIUC – University of Illinois at Urbana-Champaign

Fig. 31.1. **Top row:** (*Left*) Govindjee (left) and his contemporary Manmohan Laloraya, holding their M.Sc. (Botany) degrees in their hands, Allahabad University, 1954. (*Right*) Govindjee's photograph in his Indian passport, 1956. **Second row:** A 1956 group photograph at Urbana, Illinois. Left to right: Govindjee's first professor, Robert Emerson (holding one of Lavorels' children), Mary-Jo Lavorel, Ruth V. Chalmers (Emerson's assistant), Govindjee (holding another child of the Lavorels), Jean Lavorel and Paul Latimer. **Third row:** (*Left*) Govindjee on his new bicycle, 1965. (*Right*) Govindjee studying in his apartment, at 201 North Goodwin Avenue, Urbana, Illinois, 1959 (Emerson's photograph is in the background). **Bottom row:** A 1961 photograph taken in Stockholm, Sweden, at the International Biophysics Congress. Eugene I. Rabinowitch (second from left) reading a Swedish newspaper, Rajni Govindjee and Govindjee. Figure adapted from Eaton-Rye (2007).

by the anomalous "Red Drop" beyond 680 nm, in the action spectrum of the quantum yield of oxygen evolution, discovered by Emerson and Lewis (1943) in the green alga *Chlorella*.

Govindjee wrote to Emerson, in 1955, to be accepted as his PhD student in 1956. He received a Fulbright Travel Award, admission and a graduate fellowship in the *Physico-Chemical-Biology* Program at the University of Illinois at Urbana-Champaign (UIUC). He reached Urbana in September, 1956, to become Emerson's PhD student. He took extended course work for 2 years (1956–1958) in basic mathematics, physical chemistry, biochemistry, physics (especially optics), genetics, photobiology, and photosynthesis. After the tragic death of Emerson on February 4, 1959, Eugene Rabinowitch became his PhD advisor. Govindjee obtained his PhD in Biophysics in 1960, and worked for 1 year as a US Public Health Service Postdoctoral Trainee, under Rabinowitch. During his graduate research, he made three discoveries: (1) Two different spectral forms of chlorophyll (Chl) *a* function in two different photochemical systems in photosynthesis (Govindjee and Rabinowitch, in *Science*, in 1960); (2) An inhibitory effect of extreme-red light (720–760 nm light) on photosynthesis in red light (Rabinowitch, Govindjee and Jan B. Thomas, in *Science*, in 1960)—a phenomena that still needs research and evaluation (in all likelihood, it is due to effects on respiration), and (3) A decrease in the yield of Chl *a* fluorescence when far-red light was superimposed on the shorter wavelength light, a phenomenon that was inverse of the Emerson Enhancement Effect[1] in photosynthesis, discovered earlier by Emerson and his coworkers Carl N. Cederstrand and Ruth V. Chalmers, in 1957 (see Emerson et al., 1957). This fluorescence work was published as a preliminary note by Govindjee, S. Ichimura, C. Cederstrand and E. Rabinowitch, in *Archives of Biochemistry*, in 1960.

In 1961, Govindjee was appointed Assistant Professor of Botany to teach a beginning Plant Physiology course, and to do research in the area of photosynthesis.

A. Publications on the Emerson Enhancement Effect, the Two-Light Effect and the Two-Pigment System

In collaboration mainly with his PhD thesis advisor Professor Eugene Rabinowitch, Visiting Professor Jan B. Thomas (of the Netherlands; invited to Urbana to look after the late Emerson's duties), and Carl Cederstrand (who was earlier Emerson's research assistant), Govindjee published his graduate research work in three regular, and four short papers; in addition, some of the work was summarized in two papers in the Symposium on 'Light and Life' (these references are *italicized*).

Govindjee and Rabinowitch E **(1960a)** Two forms of chlorophyll *a* in vivo with distinct photochemical function. Science 132: 355–356

Govindjee and Rabinowitch E **(1960b)** Action spectrum of the second Emerson effect. Biophys J 1: 73–89

Govindjee, Ichimura S, Cederstrand C and Rabinowitch E **(1960a)** Effect of combining far-red light with shorter wave light on the excitation of fluorescence in *Chlorella*. Arch Biochem Biophys 89: 322–323

Govindjee, Rabinowitch E and Thomas JB **(1960b)** Inhibition of photosynthesis in certain algae by extreme red light. Biophys J 1: 91–97

Rabinowitch E, Govindjee and Thomas JB **(1960)** Inhibition of photosynthesis in some algae by extreme-red light. Science 132: 422

Thomas JB and Govindjee **(1960)** Changes in quantum yield of photosynthesis in the red alga *Porphyridium cruentum* caused by the stepwise reduction in the intensity of light preferentially absorbed by the phycobilins. Biophys J 1: 63–72

*Rabinowitch E and Govindjee **(1961)** Different Forms of chlorophyll* a *in vivo and their photochemical function. In: McElroy WD and Glass B (eds) Light and Life, pp 378–387. The Johns Hopkins Press, Baltimore*

*Thomas JB and Govindjee **(1961)** On the long-wave decline of the quantum yield of photosynthesis in the red alga Porphyridium cruentum. In: McElroy WD and Glass B (eds) Light and Life, pp 475–478. The Johns Hopkins Press, Baltimore*

[1]Emerson Enhancement Effect refers to the following: at low light intensities, when the rate of photosynthesis increases linearly with increasing light intensity, two-light beams, of different wavelengths of light (say e.g., 650 and 710 nm) given simultaneously give higher rates of photosynthesis than the sum of the rates of photosynthesis when these two-light beams are given separately to the photosynthetic organism.

III. Two-Light Reaction and Two-Pigment System of Photosynthesis, 1961–1965

Emerson's death (see Rabinowitch (1961), for Emerson's biography) was a very sad event for both Govindjee and Rajni Govindjee. Neither could publish their work with him since most of it took shape after his death (see Section II.A; and Govindjee et al., 1960, 1961). Emerson's work, however, appeared in Emerson and Rabinowitch (1960). Govindjee was appointed an Assistant Professor of Botany in 1961 to teach an undergraduate course in Plant Physiology at UIUC. By then, the crucial papers on the two-light reactions and the two-pigment systems were already published (see e.g., Hill and Bendall, 1960; Duysens et al., 1961; Kok and Hoch, 1961; see Papageorgiou and Govindjee, 2011). Govindjee and Rabinowitch (1960a; see Section II.A) showed that a short-wavelength form of Chl *a* (Chl *a* 670) was present in the same pigment system that had Chl *b* in the green alga *Chlorella* and that had Chl *c* in the diatom *Navicula*. On the other hand, Rajni Govindjee et al. (1960a,b) showed the same result in a benzoquinone-supported Hill reaction in intact cells of *Chlorella*, that had their respiration inhibited; this clearly implied that the Emerson Enhancement Effect was not related to respiration, in contrast to the earlier conclusion of Blinks (1957); further, it also indicated that carbon dioxide fixation (i.e., the Calvin-Benson cycle) may not be involved in this Emerson Enhancement Effect.

During 1961–1965, Govindjee explored several aspects of the two-light reaction and two-pigment system of photosynthesis: (1) With Carl Cederstrand (a PhD student of Eugene Rabinowitch), he discovered, in 1961, a new pigment in a cyanobacterium *Anacystis nidulans*, that had an absorption peak at 750 nm with no apparent function (published in *Science*); (2) With Rajni Govindjee, and George Hoch, he discovered, during 1962–1964, the existence of the Emerson Enhancement Effect in NADP reduction, showing clearly that this effect was in the NADP Hill reaction, and thus without involvement of respiration and of the Calvin-Benson cycle (published in *Biochemical Biophysical Research Communications*, and in *Plant Physiology*; (3) Using mass spectroscopy, and in collaboration with Olga Owens and George Hoch (Baltimore, MD), he showed, in 1963, that even after correcting for oxygen exchange that was due to respiration, the Emerson Enhancement Effect persists in *Chlorella*, and, thus, it is truly a photosynthetic effect (published in *Biochimica Biophysica Acta*); (4) In 1964, together with his then graduate student Anne Krey, he began to use fluorescence as powerful tool to explore the details of the two-pigment systems in the red alga *Porphyridium cruentum*; it lead to the discovery of a new emission band around 693 nm when photosynthesis is light-saturated, or inhibited (this was his first paper in the *Proceedings of the National Academy of Sciences, USA*). In today's terminology, the 693 nm emission band must originate in Photosystem II (PS II). (5) With Rajni Govindjee, he further explored the Emerson Enhancement Effect in the red alga *Porphyridium cruentum*, in 1965, using light pulses, instead of continuous light, and they showed that corrections due to non-linearity of light curves need to be taken into account, but even after such corrections, the Emerson Enhancement Effect was shown to be real and significant. Further, they noticed two different manifestations of this effect that still need to be explored and evaluated; it was published in *Photochemistry and Photobiology*); (6) Again, with Rajni Govindjee, he explored, also in 1965, the existence of the two-light effect and two-pigment systems by measuring absorption changes at 520 nm. Until that time, he had used either oxygen evolution, or Chl *a* fluorescence measurements. The 520 nm absorption changes, however, were not pursued further as by that time other research groups were already using much more direct measurements of the intermediates involved in the entire process.

A. Publications on the Two-Light Effect and the Two-Pigment System, using Absorption Spectroscopy, Mass Spectroscopy, and Fluorescence Spectroscopy

The publication list follows.

Govindjee, Cederstrand C and Rabinowitch E **(1961)** Existence of absorption bands at 730–740 and 750–760 mμ (millimicrons) in algae of different divisions. Science 134: 391–392

Govindjee R, Govindjee and Hoch G **(1962)** The Emerson enhancement effect in TPN-photoreduction by spinach chloroplasts. Biochem Biophys Res Com 9: 222–225

Govindjee, Owens OvH and Hoch G **(1963)** A mass spectroscopic study of the Emerson enhancement effect. Biochim Biophys Acta 75: 281–284

Govindjee, Govindjee R and Hoch G **(1964)** Emerson enhancement effect in chloroplast reactions. Plant Physiol 39: 10–14

Krey A and Govindjee **(1964)** Fluorescence changes in *Porphyridium* exposed to green light of different intensity: a new emission band at 693 nm and its significance to photosynthesis. Proc Nat Acad Sci USA 52: 1568–1572

Govindjee and Govindjee R **(1965a)** Two different manifestations of enhancement in the photosynthesis of *Porphyridium cruentum* in flashing monochromatic light. Photochem Photobiol 4: 401–415

Govindjee and Govindjee R **(1965b)** Action spectra for the appearance of difference absorption bands at 480 and 520 nm in illuminated *Chlorella* cells and their possible significance to a two-step mechanism of photosynthesis. Photochem Photobiol 4: 675–683

B. Invited Reviews and his first article in Scientific American

During 1963, Govindjee received his first invitation to present his unpublished work on the topic of the Emerson Enhancement Effect, the two light reactions and the two pigment systems, at a Conference 'Photosynthetic Mechanisms of Green Plants', held at Airlie House in Warrenton, Virginia (see Rurainski, 2005). It was followed by invitations, in 1965, for reviews from two Indian journals, and his first invitation from the most prestigious scientific magazine for the general readers, *Scientific American*; it was in this article that "P680" was proposed to identify the Chl *a* of the reaction center of PS II (see Fig. 33.1 in Chapter 33). Several years later the same term was adopted after its discovery in Berlin. The publication list follows.

Govindjee **(1963)** Emerson enhancement effect and two-light reactions in photosynthesis: dedicated to the memory of late professor Robert Emerson. In: Kok B, and Jagendorf AT (eds) Photosynthetic Mechanisms of Green Plants. Nat Acad Sci Nat Res Council Publication 1145, pp 318–334. Washington, DC

Govindjee **(1965)** Modern trends in photobiology: Energy conversion in photosynthesis. Sci Culture (India) 31: 468–476

Govindjee and Rabinowitch E **(1965a)** The photochemical stage of photosynthesis. J Sci Industrial Res (India) 24: 591–596

Rabinowitch E and Govindjee **(1965b)** The role of chlorophyll in photosynthesis. Sci Am 213: 74–83

IV. Exploiting Chlorophyll *a* Fluorescence as a Tool for Understanding Photosynthesis, and Putting the Controversy on the Maximum Quantum Yield of Photosynthesis to Rest, 1966–1969

In 1965, Govindjee became Associate Professor of Biophysics and Botany. His collaborative spirit and a desire to interact with others were quite visible during this period. This included working with the research groups of: Martin Kamen (Gif-sur-Yvette, France), Joseph Katz (Argonne National Lab), Jean Lavorel (Gif-sur-Yvette, France), as well as Henri Merkelo and Eugene Rabinowitch (both at Urbana, IL). However, the major work was done with his own highly dedicated graduate students (Maarib Bazzaz, Glenn Bedell, Carl Cederstrand (jointly under Rabinowitch), Fredrick (Fred) Cho, Anne Krey, Ted Mar, John C. Munday Jr., George Papageorgiou (the author) and Louisa Yang (Ni)), outstanding research scientists (Mrinmoyee Das; Ashish Ghosh; Rajni Govindjee) and a highly skilled assistant (Jobie D. Spencer).

A. Use of Chlorophyll Fluorescence in Understanding Photosystems

Govindjee was fascinated with fluorescence of photosynthetic pigments as a non-invasive, sensitive, and reproducible tool for the study of photosynthetic reactions, particularly of PS II: there was already the 1960 discovery of the two-light effect (Section II.A), and the 1964 discovery of a new emission band as photosynthesis became saturated (Section III.A) in the red alga *Porphyridium*. In collaboration with Henri Merkelo's group in the Electrical Engineering Department, his student Ted Mar, and Gauri Singhal (a research associate of Rabinowitch) he designed and constructed a one-of-a-kind lifetime of fluorescence instrument, using mode-locked lasers; they used it for photosynthetic systems; this paper was published in *Science,* in 1969. Further Jobie Spencer, Govindjee's technical assistant, in collaboration with his other graduate students, assembled a one-of-a-kind automatic fluorimeter that not only measured Excitation (Action) and Emission Spectra of fluorescence, as a function of temperature down

to liquid helium (4 K) temperature, but also measured Fluorescence Induction (Transient) in algal suspensions. Emphasis was either on (1) Spectroscopy, or on (2) Fluorescence Induction. Discussions with the late Gregorio Weber were an added impetus to his interest in using fluorescence. This was a period where:

(1) The spectrofluorometer, mentioned above, along with Carl Cederstrand's home-built unique regular dodecahedron (a platonic solid) integrating absorption spectrophotometer, was used to discover the absorption and fluorescence characteristics of the two-pigment systems in intact algae and in intact and physically-separated photosystems in spinach. At room temperature, Chl *a* 670 and Chl *a* 680 were directly seen in vivo (Cederstrand). At 77 K, there were three major emission bands (F685; F696 (discovered independently by three groups, including Govindjee's group in 1963); and F730, discovered by Brody (1958)). Through a combination of excitation (action) and emission spectra, pigments responsible for these bands were deciphered both in spinach chloroplasts (Louisa Yang; Carl Cederstrand; Mrinmoyee Das), and in cells of cyanobacteria (Ashish Ghosh; Carmela Shimony), green alga *Chlorella* (Fred Cho; Laszlo Szalay) and red alga *Porphyridium* (Anne Krey). For details, see the list of publications below (Section IV.B).

(2) This same spectrofluorometer, used in kinetic mode, where exciting light was turned on with a fast camera shutter, was exploited fully by two graduate students of Govindjee: George Papageorgiou (the author) and John C. Munday, Jr., the former focused on the slower changes that lasted minutes, and the latter focused on changes that lasted up to 1 s. Munday and Govindjee, who published two papers in 1969 in the *Biophysical Journal*, pioneered the beginnings of the understanding of the "P" level of fluorescence, which was suggested to be due to a traffic jam of electrons on the acceptor side of PS I. Further, they showed that PS I light, when superimposed on PS II light, did affect the shape and intensity of fluorescence induction: O (origin); I (inflection); D (dip); P (peak) and S (steady state). On the other hand, Papageorgiou and Govindjee (published in 1967 and 1968, also in the *Biophysical Journal*) showed that the emission spectra during the slow fluorescence induction changed with time, and the slow changes were sensitive to uncouplers of phosphorylation; fluorescence changes continued even when the electron transfer was blocked by the addition of diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea or DCMU) suggesting that structural changes played an important role in the S (Semi-steady state)—M (Maximum) and T (Terminal steady state) of fluorescence induction. There was already, here, a hint of the concept of what became known later as the "state" changes, but never clearly expressed (see Papageorgiou and Govindjee, 2011, for a discussion). For details, see the list of papers of both Munday and Papageorgiou (also see Papageorgiou and Govindjee, 2011). While in Gif-sur-Yvette (France), Govindjee discovered, in 1969, in collaboration with Martin Kamen and Jean Lavorel, age-related fluorescence induction differences in anoxygenic photosynthetic bacteria (published in the *Proceedings of the National Academy of Sciences USA*). Photographs of Govindjee's students during this period are shown in Fig. 31.2.

1. List of Publications on the Spectral and Kinetic Characteristics of the Two-Pigment Systems Through Fluorescence

Cederstrand C and Govindjee **(1966)** Some properties of spinach chloroplast fractions obtained by digitonin solubilization. Biochim Biophys Acta 120: 177–180

Cederstrand C, Rabinowitch E and Govindjee **(1966a)** Absorption and fluorescence spectra of spinach chloroplast fractions obtained by solvent extraction. Biochim Biophys Acta 120: 247–258

Cederstrand C, Rabinowitch E and Govindjee **(1966b)** Analysis of the red absorption band of chlorophyll a in vivo. Biochim Biophys Acta 126: 1–12

Cho F, Spencer J and Govindjee **(1966)** Emission spectra of *Chlorella* at very low temperatures (−269 C to −196 C). Biochim Biophys Acta 126: 174–176

Ghosh AK and Govindjee **(1966)** Transfer of the excitation energy in *Anacystis nidulans* grown to obtain different pigment ratios. Biophys J 6: 611–619 (R)

Ghosh A, Govindjee, Crespi HL and Katz JJ **(1966)** Fluorescence studies on deuterated *Chlorella vulgaris*. Biochim Biophys Acta 120: 19–22

Govindjee and Yang [Ni] L **(1966)** Structure of the red fluorescence band in chloroplasts. J General Physiol 49: 763–780

Krey A and Govindjee **(1966)** Fluorescence studies on a red alga *Porphyridium cruentum*. Biochim Biophys Acta 120: 1–18

Das M and Govindjee **(1967)** A long-wave absorbing form of chlorophyll a responsible for the red drop in fluorescence at 298 K and the F723 band at 77 K. Biochim Biophys Acta 143: 570–576

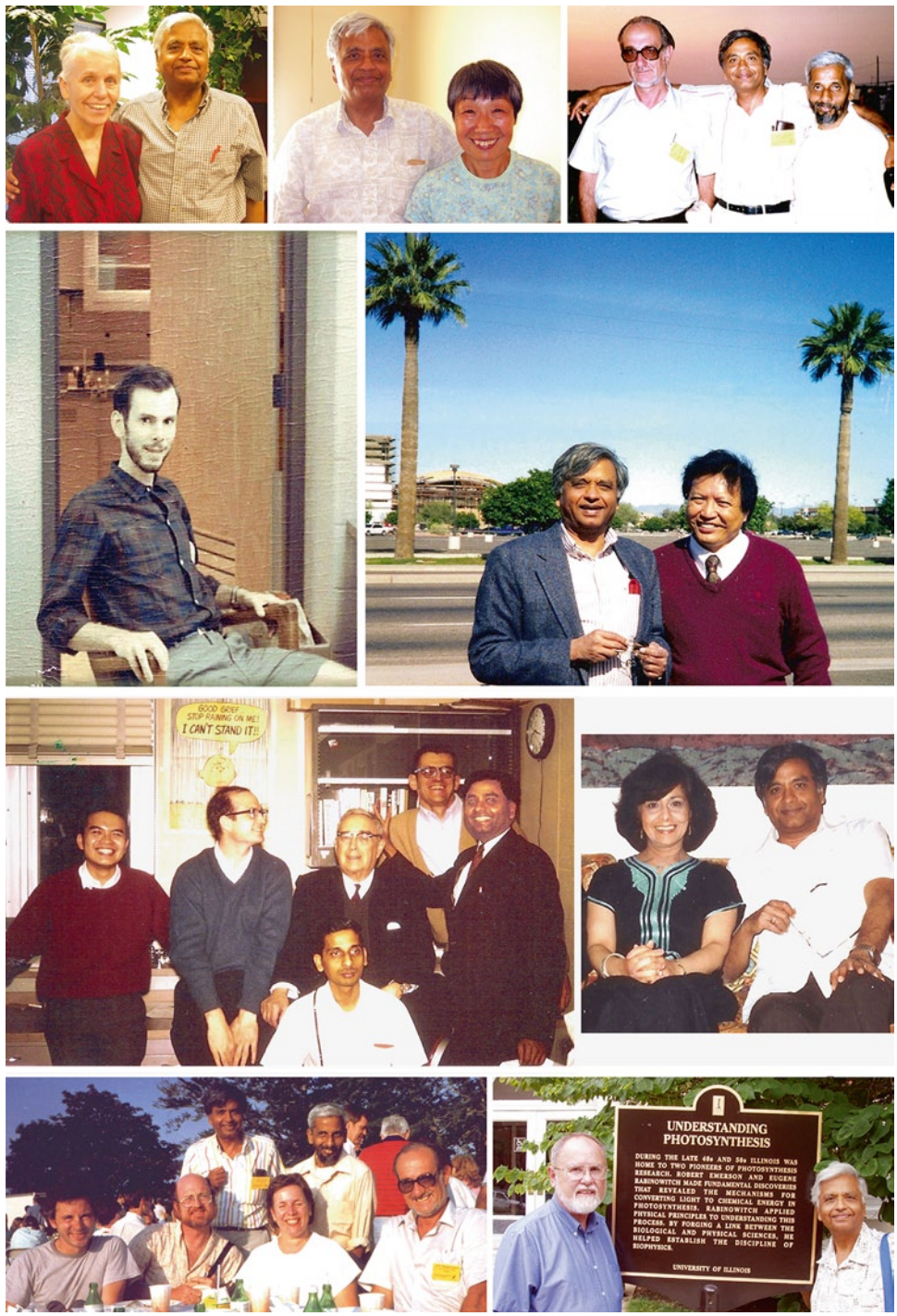

Fig. 31.2. Govindjee's graduate students from 1962 to 1972. **Top row:** (*Left*) Anne Krey (Anne (left) was in the lab in the early 1960s; photo taken in 2005) and Govindjee. (*Middle*) Govindjee, left, and Louisa Yang Ni (in the lab in the early 1960s; photo taken in 2005). (*Right*) Left to right: George C. Papageorgiou (PhD, 1968), Govindjee, and Prasanna Mohanty (PhD, 1972), photo taken at a conference, ~1987. **Second row:** (*Left*) John C. Munday, Jr. (PhD, 1968), photo taken in 283 Morrill Hall, in 1968. (*Right*) Left to right: Govindjee and Frederick Y.-T. Cho (PhD, 1969), photo taken in 1990 in Tempe, Arizona. **Third row:** (*Left*) Left to right: Ted Mar (PhD, 1971), Alan J. Stemler (PhD, 1974), Eugene Rabinowitch, Patrick Breen (then Govindjee's post-doctoral associate) and Govindjee; sitting in white shirt is Prasanna Mohanty (PhD, 1972), photo taken in 283 Morrill

Papageorgiou G and Govindjee **(1967a)** Oxygen evolution from lyophilized Anacystis with carbon dioxide as oxidant. Biochim Biophys Acta 131: 173–178
Papageorgiou G and Govindjee **(1967b)** Changes in intensity and spectral distribution of fluorescence. Effect of light pretreatment on Normal and DCMU-Poisoned *Anacystis nidulans*. Biophys J 7: 375–390
Shimony C, Spencer J and Govindjee **(1967)** Spectral characteristics of *Anacystis* particles. Photosynthetica 1: 113–125
Szalay L, Rabinowitch E, Murty N and Govindjee **(1967a)** Relationship between the absorption and emission spectra and the red drop in the action spectra of fluorescence in vivo. Biophys J 7: 137–149
Szalay L, Török M and Govindjee **(1967b)** Effect of secondary fluorescence on the emission spectrum and quantum yield of fluorescence in chlorophyll *a* solutions and algal suspensions. Acta Biochim Biophys Acad Sci Hungaria 2: 425–43
Papageorgiou G and Govindjee **(1968a)** Light induced changes in the fluorescence yield of chlorophyll *a* in vivo. I. *Anacystis nidulans*. Biophys J 8: 1299–1315
Papageorgiou G and Govindjee **(1968b)** Light induced changes in the fluorescence yield of chlorophyll *a* in vivo. II. *Chlorella pyrenoidosa*. Biophys J. 8: 1316–1328
de Klerk H, Govindjee, Kamen MD and Lavorel J **(1969)** Age and fluorescence characteristics in some species of Athiorhodaceae. Proc Nat Acad Sci USA 62: 972–978
Merkelo H, Hartman SR, Mar T, Singhal GS and Govindjee **(1969)** Mode locked lasers: measurements of very fast radiative decay in fluorescent systems. Science 164: 301–302
Munday JC Jr and Govindjee **(1969a)** Light-induced changes in the fluorescence yield of chlorophyll *a* in vivo. III. The dip and the peak in the fluorescence transient of *Chlorella pyrenoidosa*. Biophys J 9: 1–21
Munday JC Jr and Govindjee **(1969b)** Light-induced changes in the fluorescence yield of chlorophyll *a* in vivo. IV. The effect of preillumination on the fluorescence transient of *Chlorella pyrenoidosa*. Biophys J 9: 22–35

B. The Old Question: Minimum Quantum Requirement of Photosynthesis

The long-standing controversy between Govindjee's first professor Robert Emerson and Emerson's professor Otto Warburg was still in the air: a minimum of 8–12 photons (Emerson) versus 3–4 (Warburg) photons per oxygen molecule evolved. Although it was clear by 1961 that this number had to be at least eight because evidence for two-light reactions and two-pigment systems had poured in from all over the World from biochemical, biophysical, genetic, and structural studies, yet Warburg (1958, 1963) challenged all of Emerson's measurements by stating that Emerson had not used high enough CO_2, blue catalytic light and synchronous cultures of algae, necessary conditions for maximum quantum yield. With Rajni Govindjee, in 1968, these experiments, under Warburg's conditions, were performed and Emerson's values were confirmed (published in *Biochimica et Biophysica Acta*). In addition, with his student Glenn Bedell, Govindjee not only showed, in a paper in *Science*, that even in deuterated algae, that was expected to change the energetics of oxygen evolution, both the "Red Drop" (i.e., decline in oxygen evolution in the long-wavelength region of the spectrum), and the Emerson enhancement effect were present, but that the minimum quantum requirement per oxygen molecule was still 8–10 confirming Emerson's values. (For a story of this controversy on minimum quantum requirement, see Nickelsen and Govindjee, 2011.)

Bedell G and Govindjee **(1966)** Quantum yield of oxygen evolution and the Emerson enhancement effect in deuterated *Chlorella*. Science 152: 1383–1385
Govindjee and Bazzaz M **(1967)** On the Emerson enhancement effect in the ferricyanide Hill reaction in chloroplast fragments. Photochem Photobiol 6: 885–894
Govindjee R, Rabinowitch E and Govindjee **(1968)** Maximum quantum yield and action spectra of photosynthesis and fluorescence in *Chlorella*. Biochim Biophys Acta 162: 530–544

C. General Publications

In 1966, Govindjee was invited to present the research of his group at the Second Western European Conference, held in The Netherlands as well as at the Brookhaven Symposium on Energy

Fig. 31.2. (continued) Hall, in ~1969. (*Right*) Maarib D.L. Bakri (Bazzaz) (left, PhD, 1972) and Govindjee, photo taken at 1101 McHenry Avenue, Urbana, IL (where Govindjee lived), in ~1967. **Bottom row:** (*Left*) A group photograph at a conference on photosynthesis research ~1981**.** Left to right: Standing: Govindjee and Prasanna Mohanty (PhD, 1972); sitting: Paul A. Jursinic (PhD, 1977), Alan Stemler (PhD, 1974), Barbara A. Zilinskas (PhD, 1975) and George Papageorgiou (PhD, 1968). (*Right*) Glenn Bedell (PhD, 1972), with a plaque honoring Robert Emerson and Eugene Rabinowitch, and Govindjee (photo taken in 2006). Missing from these photographs are Carl N. Cederstrand (PhD, 1965; jointly under Rabinowitch) and Raymond Chollet (PhD, 1972) who worked jointly with William Ogren. Figure adapted from Eaton-Rye (2007).

Conversion by the Photosynthetic Apparatus. Further, in 1968, he received an invitation to present his work at the First International Congress on Photosynthesis Research, held in Freudenstadt, Germany. At these conferences he talked about the research done mainly by George Papageorgiou and John C. Munday, his first two PhD students, both in Biophysics. More importantly, I was fortunate to be a part of a major review that Govindjee was invited to write on Chl fluorescence for a book, edited by Guilbault (1967).

This period of Govindjee's career ended with his famous little book on Photosynthesis, with Rabinowitch, published by John Wiley in 1969. I am told that it sold almost 9,000 copies. It was used throughout the World. Fortunately, for all of us, its electronic copy is available free at his web site (http://www.life.illinois.edu/govindjee/photosynBook.html)

The list of these general publications follows.

Govindjee **(1966a)** Photosynthesis. In: Catholic Encyclopedia for Home and Schools, pp 425–429. McGraw-Hill Publishers, New York

Govindjee **(1966b)** Fluorescence studies on algae, chloroplasts and chloroplast fragments. In: Thomas JB and Goedheer JCH (eds) Currents in Photosynthesis, pp 93–103. Ad Donker Publisher, Rotterdam

Govindjee **(1967)** Transformation of light energy into chemical energy: photochemical aspects of photosynthesis. Crop Sci 7: 551–560

Govindjee, Munday JC Jr and Papageorgiou G **(1967a)** Fluorescence studies with algae: changes with time and preillumination. In: Olson JM (ed) Energy Conversion by the Photosynthetic Apparatus, Vol 19: 434–445. Brookhaven Symposia in Biology

Govindjee, Papageorgiou G and Rabinowitch E **(1967b)** Chlorophyll fluorescence and photosynthesis. In: Guilbault GG (ed) Fluorescence Theory, Instrumentation and Practice, pp 511–564. Marcel Dekker Inc, New York

Rabinowitch E, Szalay L, Das M, Murty N, Cederstrand C and Govindjee **(1967)** Spectral properties of cell suspensions. In: Olson JM (ed) Energy Conversion by the Photosynthetic Apparatus, Vol 19: 1–7. Brookhaven Symposia in Biology

Munday JC Jr and Govindjee **(1969)** Fluorescence transients in *Chlorella*: Effects of supplementary light, anaerobiosis and methyl viologen. Progress in Photosynthesis Res, Vol II: 913–922

Papageorgiou G and Govindjee **(1969)** The second wave of fluorescence induction in *Chlorella pyrenoidosa*. Progress in Photosynthesis Res, Vol II: 905–912

Rabinowitch E and Govindjee **(1969)** Photosynthesis (273 pages). John Wiley and Sons Inc, New York

Acknowledgements

I thank Julian Eaton-Rye for inviting me to participate in writing about Govindjee's contributions from his graduate days till he became, in 1969, a full Professor of Biophysics and Botany at the University of Illinois at Urbana-Champaign. I thank Govindjee for providing his autobiographical notes (also see: http://www.life.illinois.edu/govindjee/) that helped me in preparing this chapter.

References

Blinks LR (1957) Chromatic transients of photosynthesis in red algae. In: Gaffron H, Brown AH, French CS, Livingston R, Strehler BL, and Tolbert NE (eds) Research in Photosynthesis, pp 444–449. Interscience, New York

Brody SS (1958) New excited state of chlorophyll. Science 128: 838–839

Duysens LNM, Amesz J and Kamp BM (1961) Two photochemical systems in photosynthesis. Nature 190: 510–511

Eaton-Rye JJ (2007) Snapshots of the Govindjee lab from the late 1960s to the late 1990s, and beyond. Photosynth Res 94: 153–178

Emerson R and Lewis CM (1943) The dependence of quantum yield of *Chlorella* photosynthesis on wavelength of light. Am J Bot 30: 165–178

Emerson R and Rabinowitch E (1960) Red drop and role of auxiliary pigments in photosynthesis. Plant Physiol 35: 477–485

Emerson R, Chalmers and Cederstrand CN (1957) Some factors influencing the longwave limit of photosynthesis. Proc Natl Acad Sci USA 43: 133–143

Govindjee R, Thomas JB and Rabinowitch E (1960) Second Emerson effect in the Hill reaction of *Chlorella* cells. Science 132: 421

Govindjee R, Thomas JB and Rabinowitch E (1961) Studies on the Second Emerson effect in the Hill reaction in algal cells. Biophys J 1: 377–388

Hill R and Bendall F (1960) Function of the two cytochrome components in chloroplasts: A working hypothesis. Nature 186: 136–137

Kok B and Hoch G (1961) Spectral changes in photosynthesis. In: McElroy WD and Glass B (eds) Light and Life, pp 397–461. The Johns Hopkins Press, Baltimore

Nickelsen K and Govindjee (2011) The maximum quantum yield controversy: Otto Warburg and the 'midwest gang'. Bern Studies in the History and Philosophy of Science, University of Bern, Switzerland. ISBN: 978-3-9523421-9-0

Papageorgiou GC and Govindjee (2011) Photosystem II fluorescence: Slow changes–scaling from the past. J Photochem Photobiol B: Biol 104: 258–270

Rabinowitch E (1961) Robert Emerson, 1903–1959. Natl Acad Sci Biographical Memoirs 35: 112–131

Rurainski H J (2005) The conference at Airlie House in 1963. In: Govindjee; Beatty JT, Gest H and Allen JF (eds) Discoveries in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 20, pp 1241–1248. Springer, Dordrecht

Warburg O (1958) Photosynthesis experiments at the Max Planck Institute for Cell Physiology, Berlin-Dahlem. Science 128: 68–74

Warburg O (1963) The photolyte of photosynthesis. In: La Photosynthese (President du Colloque, Wurmser MR), pp 221–228. Editions du Centre National de la Recherche Scientifique, Paris

Chapter 32

Contributions of Govindjee, 1970–1999

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J.J. Eaton-Rye, B.C. Tripathy and T.D. Sharkey (eds.), Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation, Advances in Photosynthesis and Respiration 34, pp. 815–833,
DOI 10.1007/978-94-007-1579-0_32,

Summary

I received my PhD in Biology in 1987 under Govindjee's supervision. Since then Govindjee and I have kept close contact with each other, no matter where I went. Here, I present Govindjee's publications in photosynthesis, beginning in 1970, just after he became a full Professor of Biophysics and Botany, at the University of Illinois at Urbana-Champaign, and I end this description with his publications in 1999 when he retired as Professor Emeritus of Biochemistry, Biophysics and Plant Biology. The ~30-year period, I have covered here, has been broken in three sections: (I) 1970—1979; (II) 1980—1989, and (III) 1990—1999.

I. The 1970–1979 Period

This was one of the most intense and exciting periods in Govindjee's research career. The number and type of collaborators took a quantum jump. First, he had the collaboration of several of his graduate students. In alphabetical order, they were: Maarib Bazzaz; Glenn Bedell; Fred Cho; Julian Eaton-Rye; James Fenton. Paul Jursinic; Rita Khanna; Ted Mar; Prasanna Mohanty; Ralph Schooley; Alan Stemler; David VanderMeulen; Daniel Wong; Thomas Wydrzynski, and Barbara Zilinskas. For photographs of Govindjee's students and collaborators, see Figs. 32.1–32.3 (also see Fig. 31.2 in Chapter 31). This period also included several visiting scholars: John Anton (Illinois, USA); Jean-Marie Briantais (France); Ralph Gasanov (Azerbaijan); Elizabeth Gross (Ohio, USA); Ismael Moya (France); Barbara Prezlin (California, USA); Beazy Sweeney (California, USA); Karel Vacek (Czech Republic), and Jack van Rensen (The Netherlands). In addition, he collaborated with the following, on the campus of the University of Illinois at Urbana-Champaign (UIUC): Henri Merkelo (Electrical Engineering); Herb Gutowsky (Chemistry); Ken Kaufmann (Chemistry), and Charles Swenberg (Physics). Collaboration with Rajni Govindjee continued, and work during his sabbatical leaves was carried out in The Netherlands (with Lou Duysens; Hans van Gorkom; Tiny Pulles; Arnold Hoff); in India (with P.V. (Raj) Sane, T.S. Desai; V.G. Tatake); in France (with Jean Lavorel), and in Germany (with Gunter Döring). Further Stemler went to California and collaborated with Jerry Babcock.

Abbreviations: Chl – Chlorophyll; PS – Photosystem; UIUC – University of Illinois at Urbana-Champaign

This period was full of new initiatives and discoveries in several areas, all related to the primary events, rather than carbon assimilation. Exploitation of Chlorophyll *a* Fluorescence (**A**) continued to be the major theme; to it Govindjee added two other related ways of light emission: Delayed Light Emission (**B**) and Thermoluminescence (**C**). Studies on primary photochemical reactions in Photosystem II (PS II) (starting in Horst Witt's Lab) and Photosystem I (PS I) (in Urbana) were pioneered (**D**). However, the most exotic discovery was the unique role of bicarbonate in PS II (**E**). The Oxygen-Evolving Complex was studied through modeling of kinetics of oxygen evolution, preparation of antibodies against it, and by the first application of NMR (**F**). Govindjee played a while in the area of photophosphorylation, and ATP synthase (**G**) and even a bit on anoxygenic photosynthetic bacteria (**H**), but these areas were not pursued further. During this period, he wrote general articles for education purposes; and edited a book "Bioenergetics of Photosynthesis" (in 1975). A significant educational contribution was a 1974 Scientific American article on 'Primary Events in Photosynthesis', written in collaboration with Rajni Govindjee (**I**).

In each section of this chapter, publications are arranged chronologically.

A. Exploitation of Chlorophyll Fluorescence: Intensity and Lifetime of Fluorescence in Understanding the Two Pigment Systems, Excitation Energy Transfer, Its Regulation, as Well as Circadian Rhythm

Cho F and Govindjee (**1970a**) Low temperature (4–77 K) spectroscopy of *Anacystis*: Temperature dependence of energy transfer efficiency. Biochim Biophys Acta 216: 151–161

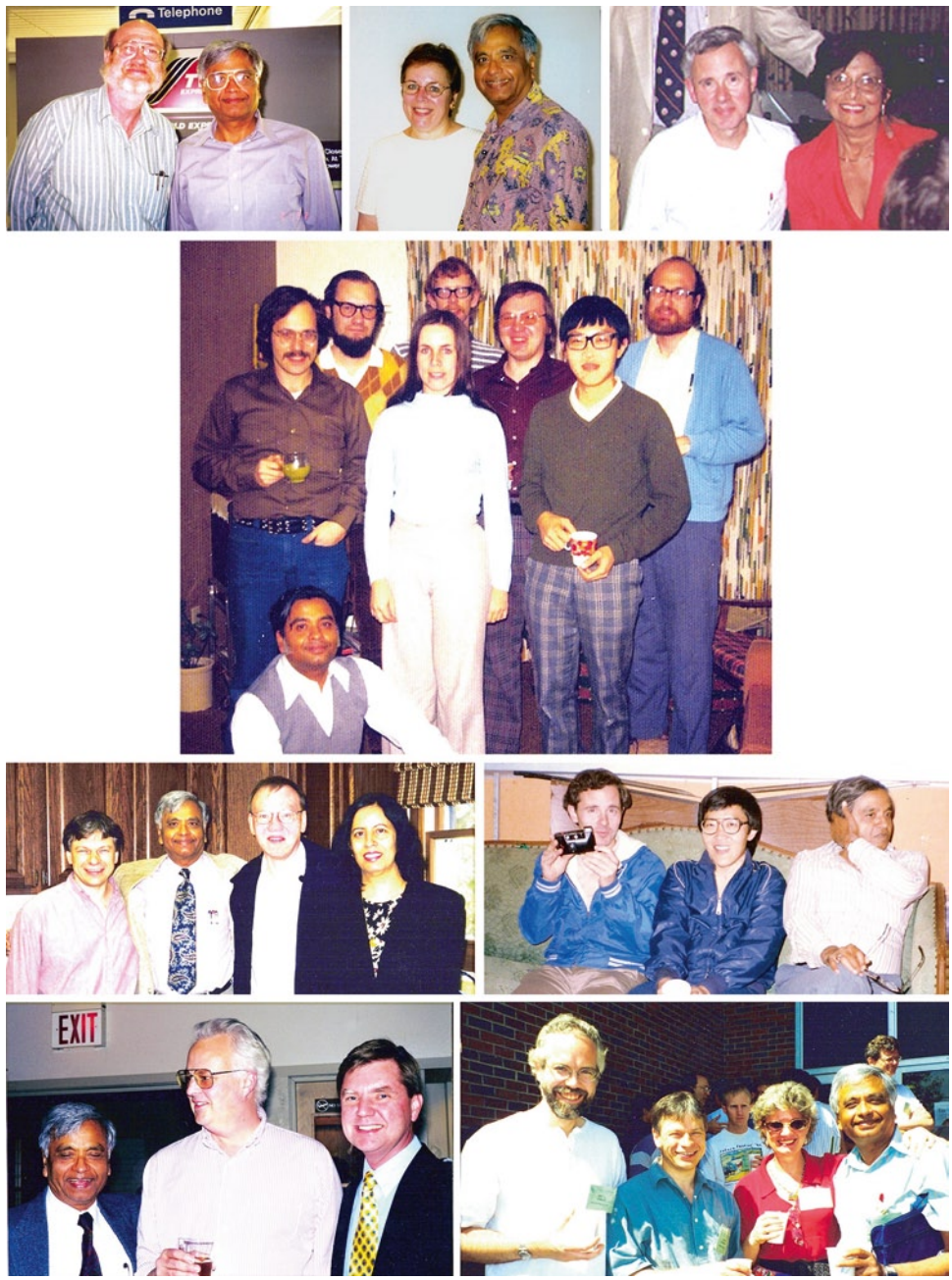

Fig. 32.1. Govindjee's graduate students in the mid 1970–early 1980s. **Top row:** (*Left*) Alan Stemler (PhD, 1974, *left*; photo taken ~2004) and Govindjee. (*Middle*) Barbara Zilinskas (PhD, 1975, *left*), and Govindjee (photo taken in 2004). (*Right*) Paul Jursinic (PhD, 1977) and Maarib Bazzaz (PhD, 1972; photo taken in 1999). **Second row:** A ~1976 group photograph. *Left* to *right*: R. Slovacek (with moustache), Ralph Schooley (with beard), Barbara Zilinskas; behind her is David VanderMeulen (with glasses; PhD, 1977), Thomas J. Wydrzynski (in red shirt; PhD, 1977), Daniel Wong (PhD, 1979), and Alan Stemler; sitting on the floor is Govindjee; photo taken at Govindjee's home, 1101 McHenry avenue, Urbana. **Third row:** (*Left*) *Left* to *right*: Julian J. Eaton-Rye (PhD, 1987), Govindjee, Thomas Wydrzynski and Rita Khanna (PhD, 1980). (*Right*) Paul Jursinic, Daniel Wong and Govindjee (photo taken ~1977). **Bottom row:** (*Left*) Govindjee, James M. Fenton, and Michael Wasielewski (from Northwestern University, Chicago; photo taken in 1999). (*Right*) Willem J. Vermaas, Julian Eaton-Rye, Christa Critchley and Govindjee (photo taken around 2000). Figure adapted from Eaton-Rye (2007b).

Cho F and Govindjee (**1970b**) Low-temperature (4–77 K) spectroscopy of *Chlorella*: Temperature dependence of energy transfer efficiency. Biochim Biophys Acta 216: 139–150

Cho F and Govindjee (**1970c**) Fluorescence spectra of *Chlorella* in the 295–77 K range. Biochim Biophys Acta 205: 371–378

Govindjee R, Govindjee, Lavorel J and Briantais JM (**1970**) Fluorescence characteristics of lyophilized maize chloroplasts suspended in buffer. Biochim Biophys Acta 205: 361–370

Mohanty P, Munday JC Jr and Govindjee (**1970**) Time-dependent quenching of chlorophyll *a* fluorescence from (pigment) system II by (pigment) system I of photosynthesis in *Chlorella*. Biochim Biophys Acta 223: 198–200

Mohanty P, Mar T and Govindjee (**1971a**) Action of hydroxylamine in the red alga *Porphyridium cruentum*. Biochim Biophys Acta 253: 213–221

Mohanty P, Papageorgiou G and Govindjee (**1971b**) Fluorescence induction in the red alga *Porphyridium cruentum*. Photochem Photobiol 14: 667–682

Papageorgiou G and Govindjee (**1971**) pH control of the chlorophyll *a* fluorescence in algae. Biochim Biophys Acta 234: 428–432

Briantais JM, Merkelo H and Govindjee (**1972**) Lifetime of the excited state τ in vivo. III. Chlorophyll during fluorescence induction in *Chlorella pyrenoidosa*. Photosynthetica 6: 133–141

Govindjee and Briantais JM (**1972**) Chlorophyll *b* fluorescence and an emission band at 700 nm at room temperature in green algae. FEBS Lett 19: 278–280

Mar T and Govindjee (**1972**) Decrease in the degree of polarization of chlorophyll fluorescence upon the addition of DCMU to algae. In: Forti G, Avron M and Melandri A (eds) Photosynthesis, Two Centuries After its Discovery by Joseph Priestley, pp 271–281. Dr W Junk NV Publishers, Den Haag

Mar T, Govindjee, Singhal GS and Merkelo H (**1972**) Lifetime of the excited state in vivo. I. Chlorophyll *a* at liquid nitrogen temperature; rate constant of radiationless deactivation and trapping. Biophys J 12: 797–808

Mohanty P, Braun (Zilinskas) BZ, Govindjee and Thornber JP (**1972**) Chlorophyll fluorescence characteristics of system I chlorophyll *a*-protein complex and system II particles at room and liquid nitrogen temperatures. Plant Cell Physiol 13: 81–91

Bazzaz MB and Govindjee (**1973a**) Photochemical properties of mesophyll and bundle sheath chloroplasts of maize. Plant Physiol 52: 257–262

Bazzaz MB and Govindjee (**1973b**) Absorption and chlorophyll *a* fluorescence characteristics of tris-treated and sonicated chloroplasts. Plant Sci Lett 1: 201–206

Mohanty P and Govindjee (**1973a**) Light-induced changes in the fluorescence yield of chlorophyll *a* in *Anacystis nidulans*. I. Relationships of slow fluorescence changes with structural changes. Biochim Biophys Acta 305 95–104

Mohanty P and Govindjee (**1973b**) Light-induced changes in the fluorescence yield of chlorophyll *a* in *Anacystis nidulans*. II. The fast changes and the effect of photosynthetic inhibitors on both the fast and slow fluorescence induction. Plant Cell Physiol 14: 611–629

Mohanty P and Govindjee (**1973c**) Effect of phenazine methosulfate and uncouplers on light-induced chlorophyll *a* fluorescence yield changes in intact algal cells. Photosynthetica 7: 146–160

Mohanty P, Braun (Zilinskas) BZ and Govindjee (**1973**) Light-induced slow changes in chlorophyll *a* fluorescence in isolated chloroplasts: Effects of magnesium and phenazine methosulfate. Biochim Biophys Acta 292: 459–476

Bazzaz MB and Govindjee (**1974a**) Effects of lead chloride on chloroplast reactions. Environ Lett 6: 175–191

Bazzaz MB and Govindjee (**1974b**) Effects of cadmium nitrate on spectral characteristics and light reactions of chloroplasts. Environ Lett 6: 1–12

Bazzaz MB, Govindjee and Paolillo DJ (**1974**) Biochemical, spectral, and structural study of olive necrotic 8147 mutant in *Zea mays* L. Z Pflanzenphysiol 72: 181–192

Gasanov R and Govindjee (**1974**) Chlorophyll fluorescence characteristics of photosystems I and II from grana and photosystem I from stroma lamellae. Z Pflanzenphysiol 72: 193–202

Mohanty P and Govindjee (**1974**) The slow decline and the subsequent rise of chlorophyll fluorescence transients in intact algal cells. Plant Biochem J 1: 78–106

Mohanty P, Govindjee and Wydrzynski T (**1974**) Salt-induced alterations of the fluorescence yield and of emission spectra in *Chlorella*. Plant Cell Physiol 15: 213–224

VanderMeulen DL and Govindjee (**1974a**) Relation of membrane structural changes to energy spillover in oats and spinach chloroplasts: use of fluorescence probes and light scattering. Biochim Biophys Acta 368: 61–70

VanderMeulen DL and Govindjee (**1974b**) 12-(9-anthroyl)-stearic acid and atebrin as fluorescence probes for energetic status of chloroplasts. FEBS Lett 45: 186–190

Das M and Govindjee (**1975**) Action spectra of chlorophyll fluorescence in spinach chloroplast fractions obtained by solvent extraction. Plant Biochem J 2: 51–60

Gross EL, Wydrzynski T, VanderMeulen D and Govindjee (**1975**) Monovalent and divalent cation-induced changes in chlorophyll *a* florescence and chloroplast structure. In: Avron M (ed) Proc 3rd Int Cong on Photosynthesis, Vol I, pp 345–361. Elsevier Publ Co, Amsterdam

VanderMeulen DL and Govindjee (**1975**) Anthroyl Stearate: a fluorescent probe for chloroplasts. In: Avron M (ed) Proc 3rd Int Cong on Photosynthesis, Vol I, pp 1095–1105. Elsevier Publ Co, Amsterdam

Wydrzynski T, Gross EL and Govindjee (**1975**) Effects of cations (sodium and magnesium) on the dark and light-induced chlorophyll *a* fluorescence yields in sucrose-washed spinach chloroplasts. Biochim Biophys Acta 376: 151–161

Schooley RE and Govindjee (**1976**) Cation-induced changes in the circular dichroism spectrum of chloroplasts. FEBS Lett 65: 123–125

VanderMeulen DL and Govindjee (**1976**) Anthroyl stearate as a fluorescent probe of chloroplast membranes. Biochim Biophys Acta 449: 340–356

Wong D and Govindjee (**1976**) Effects of lead ions on photosystem I in isolated chloroplasts: studies on the reaction center P700. Photosynthetica 10: 241–254

Hoff AJ, Govindjee and Romijn JC (**1977**) Electron spin resonance in zero magnetic field of triplet states of chloroplasts and subchloroplast particles. FEBS Lett 73: 191–196

Moya I, Govindjee, Vernotte C and Briantais JM (**1977**) Antogonistic effect of mono-and divalent cations on lifetime τ and quantum yield of fluorescence φ in isolated chloroplasts. FEBS Lett 75: 13–18

Vacek K, Wong D and Govindjee (**1977**) Absorption and fluorescence properties of highly enriched reaction center particles of photosystem I and of artificial systems. Photochem Photobiol 26: 269–276

Anton JA, Loach PA and Govindjee (**1978**) Transfer of excitation energy between porphyrin centers of a covalently-linked dimer. Photochem Photobiol 28: 235–242

Govindjee and Wong D (**1978**) Regulation of excitation energy transfer among the two pigment systems in photosynthesis. In: Fiala J (ed) Proc of 3rd Int Seminar on Excitation Energy Transfer in Condensed Matter, pp 19–28. Charles University, Prague, Czechoslovakia

Van Rensen JJS, Wong D and Govindjee (**1978**) Characterization of the inhibition of photosynthetic electron transport in pea chloroplasts by the herbicide 4,6 dinitro-o-cresol by comparative studies with 3-(3-4-dichlorophenyl)-1,1-dimethylurea. Z Naturforsch 33c: 413–420

Wong D, Vacek K, Merkelo H and Govindjee (**1978**) Excitation energy transfer among chlorophyll *a* molecules in polystyrene: Concentration dependence of quantum yield, polarization and lifetime of fluorescence. Z Naturforsch 33c: 863–869

Gasanov R, Abilov ZK, Gazanchyan RM, Kurbanova UM, Khanna R and Govindjee (**1979**) Excitation energy transfer in photosystems I and II from grana and in photosystem I from stroma lamellae, and identification of emission bands with pigment-protein complexes at 77 K. Z Pflanzenphysiol 95: 149–169

Govindjee, Wong D, Prezelin BB and Sweeney BM (**1979a**) Chlorophyll *a* fluorescence of *Gonyaulax polydera* grown on a light-dark cycle and after transfer to constant light. Photochem Photobiol 30: 405–411

Govindjee, Mathis P, Vernotte C, Wong D, Saphon S, Wydrzynski T and Briantais JM (**1979b**) Cation effects on system II reactions in thylakoids: Measurements on oxygen evolution, the electrochromic change at 515 nm, the primary acceptor and the primary donor. Z Naturforsch 34c: 826–830

Sweeney BM, Prezelin BB, Wong D and Govindjee (**1979**) In vivo chlorophyll *a* fluorescence transients and the circadian rhythm of photosynthesis in *Gonyaulax polydera*. Photochem Photobiol 30: 309–311

Wong D and Govindjee (**1979**) Antagonistic effects of mono- and divalent cations on polarization of chlorophyll fluorescence in thylakoids and changes in excitation energy transfer. FEBS Lett 97: 373–377

Wong D, Merkelo H and Govindjee (**1979**) Regulation of excitation transfer by cations: wavelength-resolved fluorescence lifetimes and intensities at 77 K in thylakoid membranes of pea chloroplasts. FEBS Lett 104: 223–226

B. Delayed Light Emission and Its Significance

Stacy WT, Mar T, Swenberg CE and Govindjee (**1971**) An analysis of a triplet exciton model for the delayed light in *Chlorella*. Photochem Photobiol 14: 197–219

Jursinic P and Govindjee (**1972**) Delayed light emission in DCMU-treated *Chlorella*: Temperature effects. In: Forti G, Avron M and Melandri A (eds) Photosynthesis, Two Centuries After its Discovery by Joseph Priestley, pp 223–232. Dr W Junk NV Publishers, Den Haag

Mohanty P, Braun (Zilinskas) BZ and Govindjee (**1972**) Fluorescence and delayed light emission in tris-washed chloroplasts. FEBS Lett 20: 273–276

Mar T, Roy G and Govindjee (**1974**) Effect of chloride and benzoate anions on the delayed light emission in DCMU-treated spinach chloroplasts. Photochem Photobiol 20: 501–504

Zilinskas BA and Govindjee (**1975**) Silicomolybdate and silicontungstate mediated dichlorophenyldimethylurea-insensitive photosystem II reaction: Electron flow, chlorophyll *a* fluorescence and delayed light emission changes. Biochim Biophys Acta 387: 306–319

Jursinic P and Govindjee (**1977a**) Temperature dependence of delayed light emission in the 6 to 340 microsecond range after a single flash in chloroplasts. Photochem Photobiol 26: 617–628

Jursinic P and Govindjee (**1977b**) The rise in chlorophyll *a* fluorescence yield and decay in delayed light emission in tris-washed chloroplasts in the 6–100 microsecond time range after an excitation flash. Biochim Biophys Acta 461: 253–267

Jursinic P, Govindjee and Wraight CA (**1978**) Membrane potential and microsecond to millisecond delayed light emission after a single excitation flash in isolated chloroplasts. Photochem Photobiol 27: 61–71

Wong D, Govindjee and Jursinic P (**1978**) Analysis of microsecond fluorescence yield and delayed light emission changes after a single flash in pea chloroplasts: Effects of mono- and divalent cations. Photochem Photobiol 28: 963–974

C. Thermoluminescence

Mar T and Govindjee (**1971**) Thermoluminescence in spinach chloroplasts and in *Chlorella*. Biochim Biophys Acta 226: 200–203

Fig. 32.2. Govindjee's graduate students, several from the late 1980–1990s. **Top row:** (*Left*) Julian Eaton-Rye (PhD, 1987), Govindjee and William J. Coleman (PhD, 1987, right), photo taken in ~1989. (*Right*) Julian Eaton-Rye (left), and Govindjee, photo taken in ~1998. **Second row:** (*Left*) Hyunsuk Shim (PhD, 1992, jointly under Professor Peter Debrunner), photo taken in 1992. (*Right*) A ~1995 group photograph. Left to Right: Julian Eaton-Rye, Govindjee, Danny J. Blubaugh (PhD, 1987; behind Govindjee), Thomas Wydrzynski (PhD, 1977), Prasanna Mohanty (PhD, 1972; holding a Conference bag) and George Papageorgiou (PhD, 1968; wearing glasses and with beard). **Third row:** (*Left*) Left to right: Govindjee and Chunhe Xu (PhD, 1992), photo taken in ~2000. (*Right*) Govindjee and Jin Xiong (PhD, 1998), photo taken ~2002. **Bottom row:** A 1999 group photograph, taken at Govindjee's retirement party. Left to right: Paul Spilotro (now MD), Thomas Wydrzynski (in yellow tie), Maarib Bazzaz (PhD, 1972; in white dress), Barbara Zilinskas (PhD, 1975; standing high), Alan Stemler (PhD, 1974), Rita Khanna (PhD, 1980), James Fenton and Jack J.S. van Rensen. Govindjee is sitting, defending himself! Missing in the photographs are Jiancheng Cao (PhD, 1992), and Fatma El-Shintinawy. Figure adapted from Eaton-Rye (2007b).

Jursinic P and Govindjee (**1972**) Thermoluminescence and temperature effects on delayed light emission (Corrected for changes in quantum yield of fluorescence) in DCMU-treated algae. Photochem Photobiol 15: 331–348

Sane PV, Desai TS, Tatake VG and Govindjee (**1977**) On the origin of glow peaks in *Euglena* cells, spinach chloroplasts and subchloroplast fragments enriched in system I or II. Photochem Photobiol 26: 33–39

D. Primary Photochemical Reactions, the First Measurement on Photosystem I

Govindjee, Döring G and Govindjee R (**1970**) The active chlorophyll a_{II} in suspensions of lyophilized and tris-washed chloroplasts. Biochim Biophys Acta 205: 303–306

Fenton JM, Pellin MJ, Govindjee and Kaufmann K (**1979**) Primary photochemistry of the reaction center of photosystem I. FEBS Lett 100: 1–4

E. Discovery of the Unique Role of Bicarbonate in Photosystem II

Stemler A and Govindjee (**1973**) Biocarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol 52: 119–123

Stemler A and Govindjee (**1974a**) Bicarbonate stimulation of oxygen evolution, ferricyanide reduction and photoinactivation using isolated chloroplasts. Plant Cell Physiol 15: 533–544

Stemler A and Govindjee (**1974b**) Effects of bicarbonate ion on chlorophyll *a* fluorescence transients and delayed light emission from maize chloroplasts. Photochem Photobiol 19: 227–232

Stemler A and Govindjee (**1974c**) Bicarbonate stimulation of oxygen evolution in chloroplast membranes. In: Packer L (ed) International Symposium in Biomembranes, pp 319–330. Academic Press, New York

Stemler A, Babcock GT and Govindjee (**1974**) The effect of bicarbonate on photosynthetic oxygen evolution in flashing light in chloroplast fragments. Proc Nat Acad Sci USA 71: 4679–4683

Govindjee, Stemler AJ and Babcock GT (**1975**) A critical role of bicarbonate in the reaction center II complex during oxygen evolution in isolated broken chloroplasts. In: Avron M (ed) Proc 3rd Int Cong on Photosynthesis, Vol I, pp 363–371. Elsevier Publ Co, Amsterdam

Wydrzynski T and Govindjee (**1975**) A new site of bicarbonate effect in photosystem II of photosynthesis: Evidence from chlorophyll fluorescence transients in spinach chloroplasts. Biochim Biophys Acta 387: 403–408

Govindjee, Pulles MPJ, Govindjee R, Van Gorkom HJ and Duysens LNM (**1976**) Inhibition of the reoxidation of the secondary electron acceptor of photosystem II by bicarbonate depletion. Biochim Biophys Acta 449: 602–605

Jursinic P, Warden J and Govindjee (**1976**) A major site of bicarbonate effect in system II reaction: Evidence from ESR signal II vf, fast fluorescence yield changes and delayed light emission. Biochim Biophys Acta 440: 323–330

Khanna R, Govindjee and Wydrzynski T (**1977**) Site of bicarbonate effect in Hill reaction: evidence from the use of artificial electron acceptors and donors. Biochim Biophys Acta 462: 208–214

Siggel U, Khanna R, Renger G and Govindjee (**1977**) Investigation of the absorption changes of the plastoquinone system in broken chloroplasts: the effect of bicarbonate depletion. Biochim Biophys Acta 462: 196–207

Govindjee and Khanna R (**1978**) Bicarbonate: its role in photosystem II. In: Metzner H (ed) Photosynthetic Oxygen Evolution, pp 269–282. Academic Press, London

Govindjee and Van Rensen JJS (**1978**) Bicarbonate effects on the electron flow in isolated broken chloroplasts. Biochim Biophys Acta 505: 183–213

F. The Oxygen-Evolving Complex: Mechanism, Biochemistry and First Application of NMR

Braun (Zilinskas) BZ and Govindjee (**1972**) Antibodies against an intermediate on the water side of photosystem II of photosynthesis. FEBS Lett 25: 143–146

Mar T and Govindjee (**1972**) Kinetic models of oxygen evolution in photosynthesis. J Theor Biol 36: 427–446

Braun (Zilinskas) BZ and Govindjee (**1974**) Antisera against a component of the oxygen-evolving side of system II reaction: antisera prepared against an extract from frozen and thawed chloroplasts. Plant Sci Lett 3: 219–227

Wydrzynski T, Zumbulyadis N, Schmidt PG and Govindjee (**1975**) Water proton relaxation as a monitor of membrane-bound manganese in spinach chloroplasts. Biochim Biophys Acta 408: 349–354

Wydrzynski T, Zumbulyadis N, Schmidt PG, Gutowsky HS and Govindjee (**1976a**) Proton relaxation and charge accumulation during oxygen evolution in photosynthesis. Proc Nat Acad Sci USA 73: 1196–1198

Wydrzynski T, Govindjee, Zumbulyadis N, Schmidt PG and Gutowsky HS (**1976b**) NMR studies on chloroplast membranes. In: Resing HA and Wade GG (eds) Magnetic Resonance in Colloid and Interface Science. American Chemical Society (ACS) Symposium Series 34, pp 471–487

Zilinskas B and Govindjee (**1976**) Stabilization by glutaraldehyde fixation of chloroplast membranes against inhibitors of oxygen evolution. Z Pflanzenphysiol 77: 302–314

Govindjee, Wydrzynski T and Marks SB (**1977**) The role of manganese in the oxygen evolving mechanism of photosynthesis. In: Packer L, Papageorgiou G and Trebst A (eds) Bioenergetics of Membranes, pp 305–316. Elsevier/North Holland Biomedical Press

Marks SB, Wydrzynski T, Govindjee, Schmidt PG and Gutowsky HS (**1978**) An NMR study of manganese in chloroplast membranes. In: Agris PF (ed) Biomolecular

Structure and Function, pp 95–100. Academic Press, New York

Wydrzynski TJ, Marks SB, Schmidt PG, Govindjee and Gutowsky HS (**1978**) Nuclear magnetic relaxation by the manganese in aqueous suspensions of chloroplasts. Biochemistry 17: 2155–2162

G. Playing with Photophosphorylation and ATP Synthase

Bedell GW and Govindjee (**1973**) Photophosphorylation in intact algae: effects of inhibitors, intensity of light, electron acceptors and donors. Plant Cell Physiol 14: 1081–1097

VanderMeulen D and Govindjee (**1975**) Interactions of fluorescent analogs of adenine nucleotides with coupling factor protein isolated from spinach chloroplasts. FEBS Lett 57: 272–275

VanderMeulen DL and Govindjee (**1977**) Binding of modified adenine nucleotides to isolated coupling factor from chloroplasts as measured by polarization of fluorescence. Eur J Biochem 78: 585–598

Younis HM, Boyer JS and Govindjee (**1979**) Conformation and activity of chloroplast coupling factor exposed to low chemical potential of water in cells. Biochim Biophys Acta 548: 228–240

H. Playing with Anoxygenic Photosynthetic Bacteria

Govindjee, Hammond JH and Merkelo JH (**1972**) Lifetime of the excited state in vivo. II. Bacteriochlorophyll in photosynthetic bacteria at room temperature. Biophys J 12: 809–814

Govindjee R, Smith WR Jr and Govindjee (**1974**) Interaction of viologen dyes with chromatophores and reaction-center preparations from *Rhodospirillum rubrum*. Photochem Photobiol 20: 191–199

Govindjee, Hammond JH, Smith WR, Govindjee R and Merkelo H (**1975**) Lifetime of the excited states in vivo. IV. Bacteriochlorophyll and bacteriopheophytin in *Rhodospirillum rubrum*. Photosynthetica 9: 216–219

Govindjee, Desai TS, Tatake VG and Sane PV (**1977**) A new glow peak in *Rhodopseudomonas sphaeroides*. Photochem Photobiol 25: 119–122

I. General Publications: Reviews, Edited Books, Edited Journals

Govindjee (**1971**) Bacterial photosynthesis; photosynthesis. In: McGraw-Hill Encyclopedia of Science and Technology, pp 62–66; 201–210. McGraw-Hill Book Co Inc, NY

Govindjee and Mohanty P (**1971**) Chlorophyll *a* fluorescence in the study of photosynthesis. In: Passwater RA and Welker P (eds) Fluorescence News, Vol 6 (2), pp 1–4. Publ by Biochemical Instrumentation Div American Instrument Co Silver Spring, MD

Govindjee and Papageorgiou G (**1971a**) Chlorophyll fluorescence; fluorescence compounds, plants. In: McGraw-Hill Encyclopedia of Science and Technology, pp 86–87; 382–386. McGraw-Hill Book Co Inc, New York

Govindjee and Papageorgiou G (**1971b**) Chlorophyll fluorescence and photosynthesis: fluorescence transients. In: Giese AC (ed) Photophysiology Vol 6, pp 1–46. Academic Press, New York

Govindjee (Guest Editor) (**1972**) Photosynthesis, July issue of Biophysical Journal, Dedicated to Eugene I. Rabinowitch.

Govindjee and Mohanty PK (**1972**) Photochemical aspects of photosynthesis in blue-green algae. In: Desikachary T (ed) Biology and Taxonony of Blue-Green Algae, pp 171–196. U Madras, Madras, India

Govindjee, Papageorgiou G and Rabinowitch E (**1973**) Chlorophyll fluorescence and photosynthesis (Revision of 1967 article). In: Guilbault GG (ed) Practical Fluorescence Theory, Methods, and Techniques, pp 543–575. Marcel Dekker Inc, NY

VanderMeulen D and Govindjee (**1973**) Is there a role of triplet state of chlorophyll in photosynthesis? J Sci Indust Res 32: 62–69

Govindjee and Govindjee R (**1974**) Primary events in photosynthesis. Sci Am 231: 68–82

Govindjee and Braun BZ (Zilinskas) (**1974**) Light absorption, emission and photosynthesis. In: Stewart WDP (ed) Algal Physiology and Biochemistry, pp 346–390. Blackwell Scientific Publication Ltd, Oxford

Govindjee (ed) (**1975**) Bioenergetics of Photosynthesis. Academic Press, New York

Govindjee and Govindjee R (**1975**) Introduction to photosynthesis, In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 1–50. Academic Press, New York

Govindjee (**1977**) Chlorophyll *a* fluorescence as a probe for locating the site of bicarbonate action in photosystem II of photosynthesis. Acta Phys Chem Nova Series 23: 49–60

Govindjee and Govindjee R (**1977a**) Light energy conversion by photosynthesis. J Sci Indust Res 36: 662–671

Govindjee and Govindjee R (**1977b**) Photosynthesis (Revision of 1971 article) In: McGraw-Hill Encyclopedia of Science and Technology, Vol 10, pp 200–210

Govindjee and Warden J (**1977**) Green plant photosynthesis: upconversion or not? J Am Chem Soc 99: 8088–8090

Govindjee (**1978a**) (Guest Editor) Photochem Photobiol 28: 935–1039

Govindjee (**1978b**) Ultrafast reactions in photosynthesis. Photochem Photobiol 28: 935–938

Govindjee (**1978c**) Pulsed nuclear magnetic resonance and thylakoid membranes. Nat Acad Sci Lett (India) 1: 3–6

Govindjee, Wydrzynski T and Marks SB (**1978**) Manganese and chloride: their roles in photosynthesis. In: Metzner H

Fig. 32.3. Some of Govindjee's research collaborators. **Top row:** (*Left*) Rajni Govindjee and Govindjee in California in 1959. (*Middle*) Ashish Ghosh and Govindjee, photo taken in 2005. (*Right*) Mrinmoyee Das and Govindjee, photo taken in 2005. **Second row:** (*Left*) Gauri S. Singhal, photo taken in 1990. (*Middle*) Govindjee and Jean-Marie Briantais, photo taken in 1995. (*Right*) A 1999 group photograph of Teruo Ogawa, Jin Xiong, Govindjee and André Jagendorf. **Third row:** A 1999 group photograph of Jack J.S. van Rensen, Thomas Wydrzynski, Julian Eaton Rye, Alan Stemler, Govindjee, Rita Khanna and Govindjee's grand daughter Sunita. **Fourth row:** (*Left*) Vladimir Shinkarev and Govindjee. (*Middle*) Govindjee, Christa Critchley and Wim Vermaas, photograph taken in ~1990. (*Right*) Subhash Padhye and Govindjee, photograph taken in 2005. **Bottom row:** (*Left*) Adam Gilmore, photo taken in ~1997. (*Right*) Rhanor Gillette (Govindjee taught Biology 121 jointly with Rhanor, at Urbana), Govindjee, Manfredo Seufferheld (Govindjee's last postdoc), and his wife Alejandra Maria Seufferheld, photo taken in 2007. A very large number of Govindjee's collaborators, including, e.g., Laszlo Szalay, Ralph Gasanov, S. Rajan, G. Sarojini, Don DeVault, A. William Rutherford, T. Kambara, Michael Wasielewski and Michel Seibert are missing in this Figure. Figure adapted from Eaton-Rye (2007b).

(ed) Symposium on Photosynthetic Oxygen Evolution, pp 321–344. Academic Press, London

Govindjee and Jursinic PA (**1979**) Photosynthesis and fast changes in light emission by green plants. In: Smith KC (ed) Photochem Photobiol Reviews, Vol 4, pp 125–205. The Plenum Press, New York

II. The 1980–1989 Period

This was an equally intense and exciting period in Govindjee's research career. He had the collaboration of four of his graduate students. In alphabetical order, they were: Danny Blubaugh, William Coleman, Julian Eaton-Rye and Rita Khanna (Daniel Wong had continued from the last period). This period included a larger number of visiting scholars: Ian Baianu (Romania); Christa Critchley (Australia); Takeshi Kambara (Japan); Shmuel Malkin (Israel); Teruo Ogawa (Japan); Subhash Padhye (India); S. Rajan (India); G. Sarojini (India); Jack van Rensen (The Netherlands), and Willem (Wim) F.J. Vermaas (The Netherlands). In addition, he continued his collaboration with Henri Merkelo (Electrical Engineering) and Herbert (Herb) S. Gutowsky (Chemistry) at the UIUC. Further, he initiated new collaborations with Antony (Tony) R. Crofts (Biophysics); Don C. DeVault (Biophysics); William (Bill) Ogren (Agronomy); Jack Widholm (Agronomy), and Colin Wraight (Biophysics). Research collaboration with Rajni Govindjee had essentially ended as she had moved to work with the *Bacteriorhodopsin System*, in the group of Thomas (Tom) G. Ebrey (in Biophysics), where she made her own elegant discoveries. During his sabbatical years, Govindjee carried out exciting research in India on thermoluminescence (with T.S. Desai, P.V. (Raj) Sane and V.G. Tatake): in Germany (with Uli Siggel and Wolfgang Junge on the bicarbonate effect in PS II), and in Japan (with Yorinao Inoue, William Rutherford and H. Koike on thermoluminescence). In addition, Govindjee began interactions with C. John Whitmarsh and Donald (Don) R. Ort (both at the UIUC).

This period of the 1980s was also full of new initiatives and discoveries in several areas including: (**A**) Exploitation of Chl fluorescence intensity and lifetime of fluorescence in understanding the two pigment systems, excitation energy transfer, and its regulation that was continued, although at a slightly reduced rate; (**B**) Delayed light emission, but interest in it diminished drastically as other interests took over; (**C**) Primary photochemical reactions: they were given much importance with Michael (Mike) W. Wasielewki's group (at Argonne National Lab), where the very first picosecond measurements on the primary charge separation in PS II were made (see Govindjee and Seibert, 2010); (**D**) Thermoluminescence: the first detailed, and correct, understanding of its mechanism was presented (together with Don DeVault and William Arnold); (**E**) Unique role of bicarbonate in PS II: it became the major area of focus of Govindjee's group, and it was during this period that the role of bicarbonate was established on the electron acceptor side of PS II, both biochemically and biophysically; (**F**) Oxygen-evolving system work continued, and chloride-NMR was introduced to understand the role of chloride on the donor side of PS II. The publications of this period are presented under the above listed headings and under 'General Publications' (**G**). A major educational contribution, during this period, was a two-volume thorough treatise on all major aspects of photosynthesis, edited by Govindjee (Academic Press, 1982); and another one on *Light Emission by Plants and Bacteria*, co-edited with Jan Amesz and David Fork (Academic Press, 1986).

Govindjee's papers are listed below in a manner similar to that done in Section I.

A. Exploitation of Chlorophyll Fluorescence: Intensity and Lifetime of Fluorescence in Understanding Two Pigment Systems, Excitation Energy Transfer, and Its Regulation

Fork DC and Govindjee (**1980**) Chlorophyll *a* fluorescence transients of leaves from sun and shade plants. Naturwishenschaften 67: 510–511

Freyssinet G, Rebeiz CA, Fenton JM, Khanna R and Govindjee (**1980**) Unequal distribution of novel chlorophyll *a* and *b* chromophores in subchloroplast particles of higher plants. Photobiochem Photobiophys 1: 203–212

Malkin S, Wong D, Govindjee and Merkelo H (**1980**) Parallel measurements on fluorescence lifetime and intensity changes from leaves during the fluorescence induction. Photobiochem Photobiophys 1: 83–89

Wong D, Govindjee and Merkelo H (**1980**) Effects of bulk pH and of monovalent and divalent cations on chlorophyll

a fluorescence and electron transport in pea thylakoids. Biochim Biophys Acta 592: 546–558

Govindjee, Downton WJS, Fork DC and Armond PA (**1981**) Chlorophyll *a* fluorescence transients as an indicator of water potential of leaves. Plant Sci Lett 20: 191–194

Singhal GS, Mohanty P and Govindjee (**1981**) Effects of preheating intact algal cells on pigments revealed by absorption and fluorescence spectra. Z Naturforsch 103c: 217–228

Wong D and Govindjee (**1981**) Action spectra of cation effects on the fluorescence polarization and intensity in thylakoids at room temperature. Photochem Photobiol 33: 103–108

Wong D, Merkelo H and Govindjee (**1981**) Estimation of energy distribution and redistribution among two photosystems using parallel measurements of fluorescence lifetimes and transients at 77 K. Photochem Photobiol 33: 97–101

Ogawa T, Grantz D, Boyer J and Govindjee (**1982**) Effects of abscisic acid on chlorophyll *a* fluorescence in guard cells of *Vicia faba*. Plant Physiol 69: 1140–1144

Sane PV, Desai TS, Tatake VG and Govindjee (**1984**) Heat-induced reversible increase in photosystem I emission in algae, leaves and chloroplasts: spectra, activities, and relation to state changes. Photosynthetica 18: 439–444

Spalding MH, Critchley C, Govindjee and Ogren WL (**1984**) Influence of carbon-di-oxide concentration during growth on fluorescence induction characteristics of the green alga *Chlamydomonas reinhardtii*. Photosynth Res 5: 169–176

Govindjee and Satoh K (**1986**) Fluorescence properties of chlorophyll *b*- and chlorophyll *c*-containing algae. In: Govindjee, Amesz J and Fork DC (eds) Light Emission by Plants and Bacteria, pp 497–537. Academic Press, Orlando

Xu C, Blair LC, Rogers SMD, Govindjee and Widholm JM (**1988**) Characterestics of five new photoautotropic suspension cultures including two *Amaranthus* species and a cotton strain growing on ambient CO_2 levels. Plant Physiol 88: 1297–1302

Xu C, Rogers SMD, Goldstein C, Widholm JM and Govindjee (**1989**) Fluorescence characterestics of photoautrophic soybean cells. Photosynth Res 21: 93–106

B. Delayed Light Emission

Jursinic P and Govindjee (**1982**) Effects of hydroxylamine and silicomolybdate on the decay in delayed light emission in the 6–100 microsecond range after a single 10 ns flash in pea thylakoids. Photosynth Res 3: 161–177

C. Thermoluminescence, Theory and Its Mechanism

Tatake VG, Desai TS, Govindjee and Sane PV (**1981**) Energy storage states of photosynthetic membranes: activation energies and lifetimes of electrons in the trap states by thermoluminescence method. Photochem Photobiol 33: 243–250

DeVault D, Govindjee and Arnold W (**1983**) Energetics of photosynthetic glow peaks. Proc Nat Acad Sci USA 80: 983–987

Rutherford W, Govindjee, Inoue Y (**1984a**) Charge accumulation and photochemistry in leaves studied by thermoluminescence and delayed light emission. Proc Nat Acad Sci USA 81: 1107–1111

Rutherford AW, Govindjee and Inoue Y (**1984b**) Thermoluminescence as a probe of photosystem II in leaves. In: Sybesma C (ed) Advances in Photosynthesis Research, pp 261–264. Martinus Nijhoff/Dr W Junk Publishers, Den Haag

Govindjee, Koike H and Inoue Y (**1985**) Thermoluminiscence and oxygen evolution from a thermophilic blue-green alga obtained after single-turnover light flashes. Photochem Photobiol 42: 579–585

Demeter S and Govindjee (**1989**) Thermoluminescence from plants. Physiol Plant 75: 121–130

D. Primary Photochemistry, First Measurements in Photosystem II

Wasielewski MR, Fenton JM and Govindjee (**1987**) The rate of formation of $P700^+$ -Ao^- in photosystem I particles from spinach as measured by picosecond transient absorption spectroscopy. Photosynth Res 12: 181–190

Govindjee and Wasielewski MR (**1989**) Photosystem II: from a femtosecond to a millisecond. In: Briggs WE (ed) Photosynthesis, pp 71–103. Alan Liss Publishers, New York

Wasielewski MR, Johnson DG, Govindjee, Preston C and Seibert M (**1989a**) Determination of the primary charge separation rate in photosystem II reaction centers at 15 K. Photosynth Res 22: 89–100

Wasielewski MR, Johnson DG, Seibert M and Govindjee (**1989b**) Determination of the primary charge separation rate in isolated photosystem II reaction centers with 500 femtosecond time resolution. Proc Nat Acad Sci USA 86: 524–548

E. Unique Role (Effect) of Bicarbonate on the Acceptor Side of Photosystem II

Jordan D and Govindjee (**1980**) Bicarbonate stimulation of electron flow in thylakoids. Golden Jubilee Commemoration Volume of the Nat Acad Sci (India), pp 369–378

Khanna R, Wagner R, Junge W and Govindjee (**1980**) Effects of CO_2-depletion on proton uptake and release in thylakoid membranes. FEBS Lett 121: 222–224

Khanna, R, Pfister K, Keresztes A, Van Rensen JJS and Govindjee (**1981**) Evidence for a close spatial location of the binding sites of and for photosystem II inhibitors. Biochim Biophys Acta 634: 105–116

Sarojini G and Govindjee (**1981a**) On the active species in bicarbonate stimulation of Hill reaction in thylakoid membranes. Biochim Biophys Acta 634: 340–343

Sarojini G and Govindjee (**1981b**) Is CO_2 an active species in stimulating the Hill reaction in thylakoid membranes? In: Akoyunoglou G (ed) Photosynthesis, Vol II: Photosynthetic Electron Transport and Photophosphorylation, pp 143–150. Balaban International Science Services, Philadelphia

Vermaas WFJ and Govindjee (**1981a**) Unique role(s) of carbon dioxide and bicarbonate in the photosynthetic electron transport system. Proc Indian Nat Sci Acad B47: 581–605

Vermaas WFJ and Govindjee (**1981b**) The acceptor side of photosystem II in photosynthesis. Photochem Photobiol 34: 775–793

Vermaas WFJ and Govindjee (**1982a**) Bicarbonate effects on chlorophyll *a* fluorescence transients in the presence and the absence of diuron. Biochim Biophys Acta 680: 202–209

Vermaas WFJ and Govindjee (**1982b**) Chapter 16: Bicarbonate or CO_2 as a requirement for efficient electron transport on the acceptor side of photosystem II. In: Govindjee (ed) Photosynthesis, Vol II: Development, Carbon Metabolism, and Plant Productivity, pp 541–558. Academic Press, New York

Vermaas WFJ, Van Rensen JJS and Govindjee (**1982**) The interaction between bicarbonate and the herbicide ioxynil in the thylakoid membrane and the effects of amino acid modification Biochim Biophys Acta 681: 242–247

Blubaugh DJ and Govindjee (**1984**) Comparison of bicarbonate effects on the variable chlorophyll *a* fluorescence of CO_2-depleted and non CO_2-depleted thylakoids in the presence of diuron. Z Naturforsch 39c: 378–381

Eaton-Rye JJ and Govindjee (**1984**) A study of the specific effect of bicarbonate on photosynthetic electron transport in the presence of methyl viologen. Photobiochem Photobiophys 8: 279–288

Govindjee, Nakatani HY, Rutherford AW and Inoue Y (**1984**) Evidence from thermoluminescence for bicarbonate action on the recombination reactions involving the secondary quinone electron acceptor of photosystem II. Biochim Biophys Acta 766: 416–423

Robinson HH, Eaton-Rye JJ, Van Rensen JJS and Govindjee (**1984**) The effects of bicarbonate depletion and formate incubation on the kinetics of oxidation-reduction reactions of the photosystem II quinone acceptor complex. Z Naturforsch 39c: 382–385

Sane PV, Govindjee, Desai TS and Tatake VG (**1984**) Characterization of glow peaks of chloroplast membranes: III. Effects of bicarbonate depletion on peaks I and II associated with photosystem II. Indian J Exp Biol 22: 267–269

Govindjee, Eaton-Rye JJ, Blubaugh DJ and Coleman W (**1985**) Action of bicarbonate and chloride anions on electron transport in thylakoid membranes. In: Proceedings of Ion Interactions in Energy Transport Systems, pp 75–80. Nuclear Research Center Demokritos, Athens, Greece

Blubaugh DJ and Govindjee (**1986**) Bicarbonate, not CO_2, is the species required for the stimulation of photosystem II electron transport. Biochim Biophys Acta 848: 147–151

Eaton-Rye JJ, Blubaugh DJ and Govindjee (**1986**) Action of bicarbonate on photosynthetic electron transport in the presence or absence of inhibitory anions. In: Papageorgiou G, Barber J and Papa S (eds) Ion Interactions in Energy Transfer Biomembranes, pp 263–278. Plenum Press, New York

Govindjee and Eaton-Rye JJ (**1986**) Electron transfer through photosystem II acceptors: interactions with anions. Photosynth Res 10: 365–379

Eaton-Rye JJ and Govindjee (**1987**) The effect of pH and flash frequency on electron transfer through the quinone acceptor complex of PSII in bicarbonate-depleted or anion-inhibited thylakoid membranes. Progress in Photosynth Res 2: 433–436

Blubaugh DJ and Govindjee (**1988a**) The molecular mechanism of the bicarbonate effect at the plastoquinone reductase site of photosynthesis. Photosynth Res 19: 85–128

Blubaugh DJ and Govindjee (**1988b**) Kinetics of the bicarbonate effect and the number of bicarbonate binding sites in thylakoid membranes. Biochim Biophys Acta 936: 208–214

Blubaugh DJ and Govindjee (**1988c**) Sites of inhibition by disulfiram in thylakoid membranes. Plant Physiol 88: 1021–1025

Cao J and Govindjee (**1988**) Bicarbonate effect on electron flow in cyanobacterium *Synechocystis PCC 6803*. Photosynth Res 19: 277–285

Eaton-Rye JJ and Govindjee (**1988a**) Electron transfer through the quinone acceptor complex of photosystem II in bicarbonate-depleted spinach thylakoid membranes as a function of actinic flash number and frequency. Biochim Biophys Acta 935: 237–247

Eaton-Rye JJ and Govindjee (**1988b**) Electron transfer through the quinone acceptor complex of photosystem II after one or two actinic flashes in bicarbonate-depleted spinach thylakoid membranes. Biochim Biophys Acta 935: 248–257

Garab Gy, Rozsa Zs and Govindjee (**1988**) Carbon-dioxide affects charge accummulation in leaves: measurements by thermoluminescence. Naturwissenschaften 75: 517–519

Govindjee, Robinson H, Crofts AR and Van Rensen JJS (**1989**) Bicarbonate does not influence electron transfer to the reaction center chlorophyll *a* of photosystem II: measurements by chlorophyll *a* fluorescence rise in microseconds. Naturwissenschaften 76: 119–121

Shopes RJ, Blubaugh D, Wraight CA and Govindjee (**1989**) Absence of a bicarbonate-depletion effect in electron transfer between quinones in chromatophores and reaction centers of *Rhodobacter sphaeroides*. Biochim Biophys Acta 974: 114–118

F. The Oxygen-Evolving System of Photosynthesis

Govindjee (**1980**) The oxygen evolving system of photosynthesis. Plant Biochem J Sicar Memorial Vol: 7–30

Govindjee and Wydrzynski T (**1981**) Oxygen evolution, manganese, ESR and NMR. In: Akoyunoglou G (ed) Photosynthesis, Vol II: Photosynthetic Electron Transport and Photophosphorylation, pp 293–306. Balaban International Science Services, Philadelphia

Khanna R, Rajan S, Steinback KE, Bose S, Govindjee and Gutowsky HS (**1981a**) ESR and NMR studies on the effects of magnesium ion on chloroplast manganese. Israel J Chem 21: 291–296

Khanna R, Rajan S, Govindjee and Gutowsky HS (**1981b**) NMR and ESR studies of thylakoid membranes. In: Akoyunoglou G (ed) Photosynthesis, Vol II: Photosynthetic Electron Transport and Photophosphorylation, pp 307–316. Balaban International Science Services, Philadelphia

Critchley C, Baianu IC, Govindjee and Gutowsky HS (**1982**) The role of chloride in O_2 evolution by thylakoids from salt-tolerant higher plants. Biochim Biophys Acta 682: 436–445

Govindjee, Baianu IC, Critchley C and Gutowsky HS (**1983**) Comments on the possible roles of bicarbonate and chloride ions in photosystem II. In: Inoue Y, Crofts AR, Govindjee, Murata N, Renger G and Satoh K (eds) The Oxygen Evolving System of Photosynthesis, pp 303–315. Academic Press, Tokyo and San Diego

Khanna R, Rajan S, Govindjee and Gutowsky HS (**1983**) Effects of physical and chemical treatments on chloroplast manganese: NMR and ESR studies. Biochim Biophys Acta 725: 10–18

Baianu IC, Critchley C, Govindjee and Gutowsky HS (**1984**) NMR study of chloride-ion interactions with thylakoid membranes. Proc Nat Acad Sci USA 81: 3713–3717

Coleman WJ, Baianu IC, Gutowsky HS and Govindjee (**1984**) The effect of chloride and other anions on the thermal inactivation of oxygen evolution in spinach chloroplasts. In: C. Sybesma (ed) Advances in Photosynthesis Research, Vol 1, pp 283–286. Martinus Nijhoff/Dr W Junk Publishers, Den Haag

Govindjee (**1984**) Photosystem II: the oxygen evolving system of photosynthesis. In: Sybesma C (ed) Advances in Photosynthesis Research, Vol 1, pp 227–238. Martinus Nijhoff/Dr W Junk Publishers, Den Haag

McCain DC, Selig TC, Govindjee and Markley JL (**1984**) Some plant leaves have orientation-dependent EPR and NMR spectra. Proc Nat Acad Sci USA 81: 748–752

Coleman W and Govindjee (**1985**) The role of chloride in oxygen evolution. In: Ovchinnikov YA (ed) Proceedings of the 16th FEBS Congress, Part B, pp 21–28. VNU Science Press, Utrecht

Govindjee, Kambara T and Coleman W (**1985**) The electron donor side of photosystem II: the oxygen evolving complex. Photochem Photobiol 42: 187–210

Kambara T and Govindjee (**1985**) Molecular mechanism of water oxidation in photosynthesis based on the functioning of manganese in two different environments. Proc Nat Acad Sci USA 82: 6119–6123

Renger G and Govindjee (**1985**) The mechanism of photosynthetic water oxidation. Photosynth Res 6: 33–55

Govindjee (**1986**) Mechanism of oxygen evolution in photosynthesis (translated into Russian). Soviet - J DI Mendeleeva Chem Soc 31: 514–524

Padhye S, Kambara T, Hendrickson DN and Govindjee (**1986**) Manganese-histidine cluster as the functional center of the water oxidation complex in photosynthesis. Photosynth Res 9: 103–112

Coleman WJ and Govindjee (**1987a**) A model for the mechanism of chloride activation of oxygen evolution in photosystem II. Photosynth Res 13: 199–223

Coleman WJ and Govindjee (**1987b**) Applications of [35] Cl[−]NMR to the study of chloride-binding in the oxygen-evolving complex of photosystem II. In: Rajamanickam C (ed) Current Trends in Life Sciences, XIII Biomembranes: Structure, Biogenesis and Transport, pp 215–220. Today and Tomorrow's Printers and Publishers, New Delhi

Coleman WJ, Govindjee and Gutowsky HS (**1987a**) [35] Cl^- NMR measurement of chloride binding to the oxygen-evolving complex of spinach photosystem II. Biochim Biophys Acta 894: 443–452

Coleman WJ, Govindjee and Gutowsky HS (**1987b**) The location of the chloride binding sites in the oxygen evolving complex of spinach photosytem II. Biochim Biophys Acta 894: 453–459

Coleman WJ, Govindjee and Gutowsky HS (**1987c**) Involvement of $Ca2^+$ in Cl^- binding to the oxygen evolving complex of photosystem II. Progress in Photosynth Res 1: 629–632

Coleman WJ, Govindjee and Gutowsky HS (**1988**) The effect of chloride on the thermal inactivation of oxygen evolution. Photosynth Res 16: 261–276

Govindjee (**1989**) The role of chloride in oxygen evolution. In: Singhal GS, Barber J, Dilley R, Govindjee, Haselkorn R and Mohanty P (eds) Photosynthesis: Molecular Biology and Bioenergetics, pp 147–162. Narosa Publishers, New Delhi

Govindjee and Homann PH (**1989**) Function of chloride in water oxidation in photosynthesis. In: Kotyk A, Skoda J, Paces V and Kostka V (eds) Highlights of Modern Biochemistry, pp 933–960. VSP International Science Publishers Ziest

G. General Publications: Some Reviews, Some of Historical Interest (H) and Edited Books

Govindjee and Barber J (**1980**) Photosynthesis session of the British Photobiology Society Meeting. Photobiochem Photobiophys 1: 183–187 (H)

Govindjee (ed) (**1982**) Photosynthesis in 2 Volumes, **Vol I**: Energy Conversion by Plants and Bacteria (799 pp)

and **Vol II**: Development, Carbon Metabolism and Plant Productivity (580 pp). Academic Press, New York

Govindjee and Whitmarsh J (**1982**) Chapter 1: Introduction to photosynthesis. In: Govindjee (ed) Photosynthesis, Vol I: Energy Conversion by Plants and Bacteria, pp 1–16. Academic Press, New York

Govindjee (**1983**) Bacterial photosynthesis; chlorophyll; fluorescence compounds, plant (revisions of earlier versions). In: McGraw Hill Encyclopedia of Science and Technology, pp 71–75; 127–129; 510–514. New York

Inoue Y, Crofts AR, Govindjee, Murata N, Renger G and Satoh K (eds) (**1983**) The Oxygen Evolving System of Photosynthesis (459 pages). Academic Press, Tokyo and San Diego

Govindjee (**1986**) Publications of Warren L Butler on photosynthesis. Photosynth Res 10: 151–159 (H)

Govindjee, Amesz J and Fork DC (eds) (**1986**) Light Emission by Plants and Bacteria. Academic Press, Orlando

Govindjee, Barber J, Cramer WA, Goedheer JHC, Lavorel J, Marcelle R and Zilinskas B (eds) (**1987a**) Excitation Energy and Electron Transfer in Photosynthesis [**Dedicated to Warren Butler**]. Martinus Nijhoff/Dr W Junk Publishers, The Netherlands

Govindjee, Govindjee R and Shopes R (**1987b**) Photosynthesis: Plant Photosynthesis and Bacterial Photosynthesis. McGraw Hill Encyclopedia of Science and Technology, 6th ed, Vol 13: 430–444

Ort DR and Govindjee (**1987**) Introduction to oxygenic photosynthesis. In: Krasnovsky AA and Litvin FF (eds) Photosynthesis, edited by Govindjee, Vol I, pp 8–89 (translated into Russian by Ganago AO, Ganago EV and Melkosernova AA). Mir Publishers, Moscow

Govindjee (**1988**) The discovery of chlorophyll-protein complex by Emil L Smith during 1937–1941. Photosynth Res16: 285–289 (H)

Govindjee (**1989**) My association with Stacey French. In: Briggs WE (ed) Photosynthesis, pp 1–3. Alan Liss Publishers, New York (H)

Govindjee, Bohnert HJ, Bottomley W, Bryant DA, Mullet JE, Ogren WL, Pakrasi H and Somerville CR (eds) (**1989**) Molecular Biology of Photosynthesis, pp 815. Kluwer Academic Publishers, Dordrecht

III. The 1990–1999 Period

I had left Govindjee's lab during the third decade of Govindjee's full professorship. He continued to work with graduate students. In alphabetical order, they were: Jiancheng Cao; Robin Roffey (visiting from Columbus, Ohio); Fatima El-Shintinawy (visiting from Egypt); Hyunsuk Shim (jointly with Peter Debrunner); Paul Spilotro (who later moved to become an M.D.); Jin Xiong, and Chunhe Xu. During this period, his most versatile and productive research associate was Adam M. Gilmore, with whom Govindjee pioneered the use of lifetime of Chl fluorescence measurements to study how plants protect themselves against excess light. During this period Govindjee collaborated with several other research groups. At Urbana, he had the benefit of working with: Tony Crofts (as well as David Kramer, Jun Minagawa and Shinichi Taoka), Don DeVault, Peter Debrunner, Enrico Gratton (as well as Julie Auger, Theodor (Chip) Hazlett, Cathie Royer and Martin Van de Ven), Vladimir Shinkarev, Shankar Subramaniam and Colin Wraight (as well as Peter Maroti and Xutong Wong).

Throughout this time, Govindjee also worked with a large number of international collaborators on his many research projects. Knowing Govindjee, he would have liked me to recognize them all. Thus, I have attempted to list as many as possible. These include the following in: (1) *Canada* (Dave Turpin and Harold Weger); (2) *China* (Chunhe Xu and his many coworkers); (3) *Finland* (Eva-Mari Aro, Pirko Mäenpää, P. Mulo, M. Keranen, Esa Tyystjärvi and Tina Tyystjärvi); (4) *France* (Chantal Astier, Jean-Marie Briantais (his first-doc in the 1970s, i.e., during the period covered in this chapter), Jean-Marc Ducruet, Ann-Lise Etienne, Diana Kirilovsky and Claudie Vernotte); (5) *Germany* (Dieter Osterhelt, W. Zinth and their coworkers); (6) *Greece* (George Papageorgiou and Kostas Stamatakis); (7) *India* (Sudhakar Bharti and Anjana Jajoo); (8) *Israel* (Yossi Hirschberg and N. Ohad); (9) *Japan* (Mamoru Mimuro and Norio Murata); (10) *The Netherlands* (D. Naber, Oscar de Voss, Gert Schanskar, Jan Snel and Jack van Rensen); (11) *Switzerland* (Peter Eggenberg, Klaus Pfister, Jean-David Rochaix, Beatrix Schwarz, Alexandra Stirbet, Bruno Strasser, Reto Strasser and Alaka Srivastava), and (12) *USA*: (a) *Arizona* (Wim Vermaas, Govindjee's own former student); (b) *Colorado* (Micheal Seibert and his group); (c) *Illinois* (Mike Wasielewski and his group, particularly Scott Greenfield and Doug Johnson), and (d) *Ohio* (Ron Hutchison, Robin Roffey and Richard Sayre).

This period was equally full of new initiatives and discoveries under the following categories: (**A**) *Chlorophyll a Fluorescence and Photosynthesis*. Research on the use of Chl *a* fluorescence peaked during this period. Here, parallel measurements on intensity and lifetime of fluorescence were used in understanding PS II reactions when the reaction centers are open or closed; the Chl *a* fluorescence transient was used as a tool to monitor inactive and active PS IIs; A nomenclature of OJIP was coined (with Reto Strasser) for the fluorescence transient (see Stirbet and Govindjee, 2011); new insight was provided for fluorescence quenching by oxidized reaction center II Chls ($P680^+$); two-light effects were measured by comparing effects on both PS II (fluorescence) and PS I (through P700); fluorescence was used to understand effects of different D1 mutants, particularly herbicide-resistant mutants; differential effects of different quinones on Chl fluorescence were examined; models and numerical solutions of fast Chl fluorescence transient (OJIP) were presented (with Reto Strasser's group); anion-induced state changes were discovered; fluorescence was used to understand ATP synthase mutants, lipid unsaturation effects and greening of plants, but most importantly pioneering experiments were made on lifetime of fluorescence measurements to understand the photoprotective mechanism of the xanthophyll-cycle pigments. In 1999, George Papageorgiou (his first PhD student) and Govindjee edited an advanced treatise 'Chlorophyll *a* Fluorescence: A Signature of Photosynthesis', summarizing this entire area and the conflicting views on it. (**B**) *Primary Photochemical Reactions.* It was during this period that the *time* of the charge separation in PS II was clearly established to be in the low picosecond range (with Mike Wasielewski's group; see Govindjee and Seibert, 2010). (**C**) *Thermoluminescence*. The theory of the mechanism of thermoluminescence was finalized and reviewed for the future students of this area. (**D**) *Unique Role of Bicarbonate in Photosystem II*. This effect was discovered in cyanobacteria; both the donor and acceptor side effects of bicarbonate on PS II were observed in the same leaves; using various mutants with site-directed mutations in the D1 and D2 proteins, a general idea of the regions on the acceptor side where bicarbonate acted was obtained; several arginine mutants were considered key to identifying the binding vicinity of bicarbonate; the absence of the bicarbonate effect in bacterial reaction centers began to provide further clues to its binding in PS II, and the field was opened for future researchers to find the final answers. (**E**) *Oxygen-Evolving System.* Research activities in this area were slowly phased out seeing the enormous progress being made in this area around the World using the most modern state-of-the art instrumentation and exciting creative ideas in other top laboratories. (**F**) *General Publications.* In addition to several highly readable reviews, Govindjee edited special issues of *Photosynthesis Research* honoring Bessel Kok (discoverer of P700 and the S-state cycle for oxygen evolution); and William Arnold (co-discoverer of the Photosynthetic Unit, Thermoluminescence and Delayed Light Emission). It was during this period that Govindjee founded the 'Advances in Photosynthesis and Respiration' series in 1994, currently in its 34th volume, now published by Springer. Other important educational contributions were: (1) A 1990 Scientific American article on 'How Plants Make Oxygen' (with William Coleman), and (2) A Web article on "Photosynthesis and the World Wide Web" (with Larry Orr).

Finally, his long-standing interest in the 'History of Photosynthesis Research' became a more serious educational interest during this period, with a 1999 article on the minimum requirement of four versus eight photons for the evolution of one molecule of oxygen: an article detailing the controversy between the Nobel-laureate Otto Warburg (exponent of four photons) and his student Robert Emerson (exponent of 8–12 photons), who was Govindjee's first professor. (For a story of this controversy on minimum quantum requirement, see Nickelsen and Govindjee, 2011.)

A. Chlorophyll a Fluorescence and Photosynthesis

Cao J and Govindjee (**1990**) Chlorophyll *a* fluorescence transient as an indicator of active and inactive photosystem II in thylakoid membranes. Biochim Biophys Acta 1015: 180–188

Govindjee, Van de Ven M, Preston C, Seibert M and Gratton E (**1990a**) Chlorophyll *a* fluorescence lifetime distributions in open and closed photosystem II reaction center

preparations: Analysis by multifrequency phase fluorometry. Biochim Biophys Acta 1015: 173–179

Govindjee, Van de Ven M, Preston C, Seibert M and Gratton E (**1990b**) Recombinational light emission from photosystem II reaction centers. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol I.2: 459–462. Kluwer Academic Publishers, Dordrecht

Xu C, Auger J and Govindjee (**1990**) Chlorophyll *a* fluorescence measurements of isolated spinach thylakoids using single-laser-based flow cytometry. Cytometry 11: 349–358

Strasser RJ and Govindjee (**1992**) On the O-J-I-P fluorescence transient in leaves and D1 mutants of *Chlamydomonas reinhardtii*. In: Murata N (ed) Research in Photosynthesis, Vol II: 29–32. Kluwer Academic Publishers, Dordrecht

Strasser R, Eggenberg P, Pfister K and Govindjee (**1992**) An equilibrium model for electron transfer in photosystem II acceptor complex: an application to *Chlamydomonas reinhardtii* cells of D1 mutants and those treated with formate. Arch Science Genève 45: 207–224

Govindjee, Van de Ven M, Cao J, Royer C and Gratton E (**1993a**) Multifrequency cross-correlation phase fluorometry of open and closed reaction centers in thylakoid membranes and PSII-enriched membranes. Photochem Photobiol 58: 437–444

Govindjee, Snel JFH, deVos OJ and Van Rensen JJS (**1993b**) Antagonistic effects of light I and II on chlorophyll *a* fluorescence yield and P700 turnover as monitors of carbon dioxide depletion in intact algal and cyanobacterial cells. Physiol Plant 89: 143–148

Shinkarev VP and Govindjee (**1993**) Insight into the relationship of chlorophyll *a* fluorescence yield to the concentration of its natural quenchers in oxygenic photosynthesis. Proc Nat Acad Sci USA 90: 7466–7469

Gilmore AM, Hazlett TL and Govindjee (**1995a**) Xanthophyll cycle-dependent quenching of photosystem II chlorophyll *a* fluorescence: formation of a quenching complex with a short fluorescence lifetime. Proc Natl Acad Sci USA 92: 2273–2277

Gilmore AM, Hazlett TL and Govindjee (**1995b**) Xanthophyll cycle dependent non-photochemical quenching of chlorophyll *a* fluorescence at low physiological temperatures. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol IV, pp 825–828. Kluwer Academic Publishers, Dordrecht

Govindjee (**1995**) Sixty-three years since Kautsky: chlorophyll *a* fluorescence. Aust J Plant Physiol 22: 131–160

Srivastava A, Strasser RJ and Govindjee (**1995a**) Differential effects of dimethylbenzoquinone and dichlorobenzoquinone on chlorophyll fluorescence transient in spinach thylakoids. J Photochem Photobiol B: Biology 31: 163–169

Srivastava AK, Strasser RJ and Govindjee (**1995b**) Polyphasic rise of chlorophyll *a* fluorescence in herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii*. Photosynth Res 43: 131–141

Stirbet AD, Govindjee, Strasser BJ and Strasser RJ (**1995**) Numerical simulation of chlorophyll *a* fluorescence induction in plants. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol II, pp 919–922. Kluwer Academic Publishers, Dordrecht

Strasser RJ, Srivastava A and Govindjee (**1995**) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochem Photobiol 61: 32–42

Gilmore AM, Hazlett TL, Debrunner PG and Govindjee (**1996**) Comparative time-resolved photosystem II chlorophyll *a* fluorescence analyses reveal distinctive differences between photoinhibitory reaction center damage and xanthophyll cycle dependent energy dissipation. Photochem Photobiol 64: 552–563

Gilmore AM, Hazlett TL, Debrunner PG and Govindjee (**1996**) Photosystem II chlorophyll *a* fluorescence lifetimes and intensity are independent of the antenna size differences between barley wild-type and chlorina mutants: Photochemical quenching and xanthophyll cycle dependent non-photochemical quenching of fluorescence. Photosynth Res 48: 171–187

Gilmore A, Shinkarev VP, Hazlett TL and Govindjee (**1998a**) Quantitative analysis of the effects of intrathylakoid pH and the xanthophyll cycle pigments on chlorophyll *a* fluorescence lifetime distributions and intensity in thylakoids. Biochemistry 37: 13582–13593

Gilmore AM, Shinkarev VP, Hazlett TL and Govindjee (**1998b**) Quantitative analysis of intrathylakoid pH and xanthophyll cycle effects on PSII fluorescence lifetime distributions and intensity. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 2297–2300. Kluwer Academic Publishers, Dordrecht

Jajoo A, Bharti S and Govindjee (**1998a**) Anion-induced state changes in spinach thylakoid membranes. FEBS Lett 434: 193–196

Jajoo A, Bharti S and Govindjee (**1998b**) Inorganic anions induce state changes in spinach thylakoid membranes. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 1227–1230. Kluwer Academic Publishers, Dordrecht

Papageorgiou GC, Govindjee, Govindjee R, Mimuro M, Stamatakis K, Alygizaki-Zorba A and Murata N (**1998**) Temperature and lipid unsaturation effects on plasma and thylakoid membranes of *Synechocystis* sp. PCC6803. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 2485–2488. Kluwer Academic Publishers, Dordrecht

Spilotro PJ, Patil SC and Govindjee (**1998**) Chlorophyll *a* fluorescence measurements of an *Arabidopsis* mutant, altered in the gamma-subunit of the ATP synthase, display changes in non-photochemical quenching. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 2253–2256. Kluwer Academic Publishers, Dordrecht

Stirbet A, Govindjee, Strasser B and Strasser R (**1998**) Chlorophyll *a* fluorescence induction in higher plants: modeling and numerical simulation. J Theor Biol 193: 131–151

Papageorgiou GC, Govindjee, Govindjee R, Mimuro M, Stamatakis K, Alygizaki-Zorba A and Murata N (**1999**) Light-induced and osmotically-induced changes in chlorophyll *a* fluorescence in two *Synechocystis* sp. PCC 6803 strains that differ in membrane lipid unsaturation. Photosynth Res 59: 125–136

Srivastava, Strasser RJ and Govindjee (**1999**) Greening of peas: parallel measurements of 77 K emission spectra, OJIP chlorophyll *a* fluorescence transient, period four oscillation of the initial fluorescence level, delayed light emission, and P700. Photosynthetica 37: 365–392

B. Primary Photochemical Reactions

Wasielewski MR, Johnson DG, Govindjee, Preston C, Seibert M (**1990**) The primary charge-separation rate in isolated photosystem II reaction center complex. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol I.2: 451–454. Kluwer Academic Publishers, Dordrecht

Seibert M, Toon S, Govindjee, O'Neil MP and Wasielewski MR (**1992**) Primary charge separation in isolated photosystem II reaction centers. In: Murata N (ed) Research in Photosynthesis, Vol II: 41–44. Kluwer Academic Publishers, Dordrecht

Wiederrecht GP, Seibert M, Govindjee and Wasielewski MR (**1994**) Femtosecond dichroism studies of isolated photosystem II reaction centers. Proc Nat Acad Sci USA 91: 8999–9003

Greenfield SR, Wasielewski M, Govindjee and Seibert M (**1995**) Femtosecond spectroscopy of PSII reaction centers: new results. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol I, pp 663–666. Kluwer Academic Publishers, Dordrecht

Greenfield SR, Seibert M, Govindjee and Wasielewski MR (**1996**) Wavelength and intensity dependent primary photochemistry of isolated photosystem II reaction centers at 5°C. Chem Phys 210: 279–295

Greenfield SR, Seibert M, Govindjee and Wasielewski MR (**1997**) Direct measurement of the effective rate constant for primary charge separation in isolated photosystem II reaction centers. J Phys Chem 101: 2251–2255

C. Thermoluminescence

DeVault D and Govindjee (**1990**) Photosynthetic glow peaks and their relationship with the free energy changes. Photosynth Res 24: 175–181

Kramer DM, Roffey RA, Govindjee and Sayre RT (**1994**) The A(t) thermoluminescence band from *Chlamydomonas reinhardtii* and the effects of mutagenesis of histidine residues on the donor side of the photosystem II D1 polypeptide. Biochim Biophys Acta 1185: 228–237

Maenpaa P, Miranda T, Tyystjarvi E, Tyystjarvi T, Govindjee, Ducruet JM, Etienne AL and Kirilovsky D (**1995**) A mutation in the D-de loop of D1 modifies the stability of the $S_2Q_A^-$ and $S_2Q_B^-$ states in photosystem II. Plant Physiol 107: 187–197

Vass I and Govindjee (**1996**) Thermoluminescence from the photosynthetic apparatus. Photosynth Res 48: 117–126

Keranen M, Mulo P, Aro EM, Govindjee and Tyystjarvi E (**1998**) Thermoluminescence B and Q bands are at the same temperature in an autotrophic and a heterotrophic D1 protein mutant of *Synechocystis* sp. PCC 6803. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 1145–1148. Kluwer Academic Publishers, Dordrecht

D. Unique Role of Bicarbonate in Photosystem II

Cao J and Govindjee (**1990**) Anion effects on the electron acceptor side of photosystem II in a transformable cyanobacterium *Synechocystis* 6803. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol I.2: 515–518. Kluwer Academic Publishers, Dordrecht

El-Shintinawy F and Govindjee (**1990a**) Bicarbonate effect in leaf discs from spinach. Photosynth Res 24: 189–200

El-Shintinawy F and Govindjee (**1990b**) Reversible anion interactions between Q_A and Q_B and between Z (or D) and Q_A in leaves and green algae. In: Baltscheffsky (ed) Current Research in Photosynthesis, Vol I.2: 511–518. Kluwer Academic publishers, Dordrecht

El-Shintinawy F, Xu C and Govindjee (**1990**) A dual bicarbonate-reversible formate effect in *Chlamydomonas* cells. J Plant Physiol 136: 421–428

Govindjee, Vernotte C, Peteri B, Astier C and Etienne AL (**1990**) Differential sensitivity of bicarbonate-reversible formate effects on herbicide-resistant mutants of *Synechocystis* 6714. FEBS Lett 267: 273–276

Cao J, Vermaas WFJ and Govindjee (**1991**) Arginine residues in the D2 polypeptide may stabilize bicarbonate binding in photosystem II of *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 1059: 171–180

Govindjee (**1991**) A unique role of CO_2 in photosystem II. In: Abrol Y, Wattal PN, Gnanam A, Govindjee, Ort DR and Teramura (eds) Impact of Global Climatic Changes on Photosynthesis and Plant Productivity, pp 349–369. Oxford/IBH Private Ltd, New Delhi

Govindjee, Weger HG, Turpin DH, Van Rensen JJS, de Vos OJ and Snel JFH (**1991a**) Formate releases carbon dioxide/bicarbonate from thylakoid membranes: measurements by mass spectroscopy and infrared gas analyzer. Naturwissenschaften 78: 168–170

Govindjee, Schwarz B, Rochaix JD and Strasser RJ (**1991b**) The herbicide-resistant D1 mutant L275F of *Chlamydomonas reinhardtii* fails to show the bicarbonate-reversible formate effect on chlorophyll *a* fluorescence transients. Photosynth Res 27: 199–208

Xu C, Taoka S, Crofts AR and Govindjee (**1991**) Kinetic characteristics of formate/formic acid binding at the plastoquinone reductase site in spinach thylakoids. Biochim Biophys Acta 1098: 32–40

Cao J, Ohad N, Hirschberg J, Xiong J and Govindjee (**1992**) Binding affinity of bicarbonate and formate in herbicide-resistant D1 mutants of *Synechococcus* sp. PCC 7942. Photosynth Res 34: 397–408

Govindjee (**1992**) What about the bicarbonate effect? In: Murata N (ed) Research in Photosynthesis, Vol II: 143–146. Kluwer Academic Publishers, Dordrecht

Govindjee, Eggenberg P, Pfister K and Strasser RJ (**1992**) Chlorophyll *a* fluorescence yield decay in herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii* and the formate effect. Biochim Biophys Acta 1101: 353–358

Wang X, Cao J, Maroti P, Stilz HU, Finkele U, Lauterwasse C, Zinth W, Oesterhelt D, Govindjee and Wraight CA (**1992**) Is bicarbonate in photosystem II the equivalent of the glutamate ligand to the iron atom in bacterial reaction centers? Biochim Biophys Acta 1100: 1–8

Xu C, Zhu Y and Govindjee (**1992**) Differential inhibition and rephasing of photosystem II electron acceptor side by monohalogenated acetates of different hydrophobicity. Z Naturforsch 47c: 121–126

Govindjee (**1993**) Bicarbonate-reversible inhibition of plastoquinone reductase in photosystem II. Z Naturforsch 48c: 251–258

Govindjee and Van Rensen JJS (**1993**) Photosystem II reaction centers and bicarbonate. In: Deisenhofer J and Norris JR (eds) Photosynthetic Reaction Centers, Vol I, pp 357–389. Academic Press, Orlando

Oscar J. deVos, Van Rensen JJS and Govindjee (**1995**) Photosystem II electron flow requires bound bicarbonate. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol I, pp 567–570. Kluwer Academic Publishers, Dordrecht

Vernotte CJ, Briantais M, Astier C and Govindjee (**1995**) Differential effects of formate in single and double mutants of D1 in *Synechocystis* species PCC 6714. Biochim Biophys Acta 1229: 296–301

Xiong J, Hutchison R, Sayre R and Govindjee (**1995**) Characterization of a site-directed mutant (D1-arginine 269-glycine) of *Chlamydomonas reinhardtii*. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol I, pp 575–578. Kluwer Academic Publishers, Dordrecht

Xiong J, Subramaniam S and Govindjee (**1996**) Modeling of the D1/D2 proteins and cofactors of the photosystem II reaction center: implications for herbicide and bicarbonate binding. Protein Sci 5: 2054–2073

Govindjee, Xu C and Van Rensen JJS (**1997**) On the requirement of bound bicarbonate for photosystem II. Z Naturforsch 52c: 24–32

Govindjee, Xu C, Schansker G and Van Rensen JJS (**1997**) Chloroacetates as inhibitors of photosystem II: effects on electron acceptor side. J Photochem Photobiol B: Biology 37: 107–117

Mulo P, Tyystjarvi T, Tyystjarvi E, Govindjee, Maenpaa P and Aro EM (**1997**) Mutagenesis of the D-E loop of photosystem II reaction centre protein D1. Function and assembly of photosystem II. Plant Mol Biol 33: 1059–1071

Xiong J, Hutchison RS, Sayre RT and Govindjee (**1997**) Modification of the photosystem II acceptor side function in a D1 mutant (Arginine-269-Glycine) of *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1322: 60–76

Yu H, Zheng XH, Li KB, Song HY, Xu CH and Govindjee (**1997**) Comparison of different effects of chloroacetates on electron transport in PS II and in the reaction center of *Rb. sphaeroides* 601. Acta Biochim Biophys Sinica 29: 36–43

Xiong J, Minagawa J, Crofts AR and Govindjee (**1998**) Loss of inhibition by formate in newly constructed photosystem II D1 mutants, D1-R257E and D1-R257M, of *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1365: 473–491

Van Rensen JJS, Xu C and Govindjee (**1999**) Role of bicarbonate in the photosystem II, the water-plastoquinone oxido-reductase of plant photosynthesis. Physiol Plant 105: 585–592

E. Oxygen-Evolving System

Shim H, Cao J, Govindjee and Debrunner PG (**1990**) Purification of highly active oxygen-evolving photosystem II from *Chlamydomonas reinhardtii*. Photosynth Res 26: 223–228

Govindjee and Coleman W (**1993**) Oxidation of water to molecular oxygen. In: Abrol Y, Mohanty P and Govindjee (eds) Photosynthesis: Photoreactions to Productivity, pp 83–108. Oxford/IBH Private Ltd. New Delhi

Naber D, Van Rensen JJS and Govindjee (**1993**) High misses after odd flashes in thoroughly dark-adapted thylakoids from pea and *Chenopodium album*. Photosynth Res 38: 309–314

Roffey RA, Kramer DM, Govindjee and Sayre RT (**1994**) Lumenal side histidine mutations in the D1 protein of photosystem II affect donor aide electron transfer in *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1185: 257–270

Xu C, Li R, Shen Y and Govindjee (**1995**) The sequential release of three extrinsic polypeptides in the PS II particles by high concentrations of trichloroacetates. Naturwissenschaften 82: 477–478

Li R, Lin N, Xu C, Shen Y and Govindjee (**1997**) Trichloroacetate affects redox active tyrosine 160 of the D2 polypeptide of the photosystem II core. Z Naturforsch 52c: 782–788

Shinkarev VP, Xu C, Govindjee and Wraight CA (**1997**) Kinetics of the oxygen evolution step in plants determined from flash-induced chlorophyll *a* fluorescence. Photosynth Res 51: 43–49

Govindjee, Srivastava A and Strasser RJ (**1998**) The "Oxygen Clock" in greening pea leaves as probed by the period four oscillations in the fluorescence intensity at 50 micro-seconds and 2 milli-seconds after pre-flashing during the OJIP transient. In: Garab G (ed) Photosynthesis: Mechanisms

and Effects, pp 1467–1450. Kluwer Academic Publishers, Dordrecht

Xu C, Li R, Hou C, Yu X, Yu Y, Zeng X, Shen Y and Govindjee (**1998**) On differences in accessibility and redox activity of D1-Y161 and D2-Y160 of PSII as probed by halogenated acetates. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 1181–1184. Kluwer Academic Publishers, Dordrecht

F. General Publications

(H is for articles of historical importance)

Govindjee (**1990**) Photosystem II heterogeneity: electron acceptor side. Photosynth Res 25: 151–160

Govindjee and Coleman W (**1990**) How does photosynthesis make oxygen? Sci Am 262: 50–58

Govindjee and Renger G (**1993**) In appreciation of Bessel Kok. Photosynth Res 38: 211–213 (H)

Renger G and Govindjee (eds) (**1993**) How plants and cyanobacteria make oxygen: 25 years of period four oscillations. Photosynth Res 38: 211–468 (H)

Halls S, Downie SR and Govindjee (**1995**) Phylogenetic analysis of photosystems I and II. In: Mathis P (ed) Photosynthesis: from Light to Biosphere, Vol I, pp 955–958. Kluwer Academic Publishers, Dordrecht

Whitmarsh J and Govindjee (**1995**) Photosynthesis. Encycl Applied Phys 13: 513–532

Govindjee, Knox RS and Amesz J (eds) (**1996**) "Photosynthetic unit: antenna and reaction centers", a special issue of "Photosynthesis Research" dedicated to William A. Arnold. Photosynth Res 48: 1–319 (H)

Orr L and Govindjee (**1998**) Photosynthesis and the world wide web. In: Garab G (ed) Photosynthesis: Mechanisms and Effects, pp 4387–4400. Kluwer Academic Publishers, Dordrecht

Xiong J, Subramaniam S and Govindjee (**1998**) A knowledge-based three dimensional model of the photosystem II reaction center of *Chlamydomonas reinhardtii*. Photosynth Res 56: 229–254

Gilmore A and Govindjee (**1999**) How higher plants respond to excess light: energy dissipation in photosystem II. In: Singhal GS, Renger G, Irrgang K-D, Sopory S and Govindjee (eds) Concepts in Photobiology: Photosynthesis and Photomorphogenesis, pp 513–548. Narosa Publishers/Kluwer Academic Publishers, New Delhi/Dordrecht

Govindjee (**1999a**) On the requirement of minimum number of four versus eight quanta of light for the evolution of one molecule of oxygen in photosynthesis: a historical note. Photosynth Res 59: 249–254 (H)

Govindjee (**1999b**) Carotenoids in photosynthesis: an historical perspective. In: Frank HA, Young AJ, Britton G and Cogdell RJ (eds) The Photochemistry of Carotenoids, Advances in Photosynthesis and Respiration, Vol 8, pp 1–19. Kluwer Academic Publishers, Dordrecht

Renger G, Sopory SK, Singhal GS, Irrgang K-D and Govindjee (**1999**) Introduction to Photobiology, Photosynthesis and Photomorphogenesis.In: Concepts in Photobiology and Photomorphogenesis, pp 1–7. Narosa Publishers/Kluwer Academic Publishers, New Delhi/Dordrecht

Whitmarsh J and Govindjee (**1999**) The photosynthetic process. In: Singhal GS, Renger G, Irrgang K-D, Sopory S and Govindjee (eds) Concepts in Photobiology: Photosynthesis and Photomorphogenesis, pp 11–51. Narosa Publishers/Kluwer Academic Publishers, New Delhi/Dordrecht

IV. Concluding Remarks

In August, 1999, Govindjee formally retired, but he continues to do research, write and edit at the same rate as before. This is borne out by the content of Chapter 33 contributed by Professor Robert Clegg of UIUC, which covers the period 2000 to the present. In addition, Govindjee's contributions to the photosynthesis community were recognized by two special issues in *Photosynthesis Research* that contain 47 papers by more than 170 authors from all over the World (Eaton-Rye, 2007a, b).

References

Eaton-Rye JJ (ed) (2007a) Govindjee Special Issue: Part A—Celebrating Govindjee's 50 years in photosynthesis research and his 75th birthday. Photosynth Res 93: 1–244

Eaton-Rye JJ (ed) (2007b) Govindjee Special Issue: Part B—Celebrating Govindjee's 50 years in photosynthesis research and his 75th birthday. Photosynth Res 93: 153–466

Govindjee and Seibert M (2010) Picosecond spectroscopy of the isolated reaction centers from the photosystems of oxygenic photosynthesis-ten years (1987–1997) fun. A tribute to Michael R. Waisielewski on his 60th birthday. Photosynth Res 103:1–6

Nickelsen K and Govindjee (2011) The maximum quantum yield controversy: Otto Warburg and the 'midwest gang'. Bern Studies in the History and Philosophy of Science, University of Bern, Switzerland. ISBN: 978-3-9523421-9-0

Stirbet A and Govindjee (2011) On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and application of the OJIP fluorescence transient. J Photochem Photobiol B: Biol 104: 236–257

Chapter 33

Contributions of Govindjee, 2000–2011

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Summary

In this chapter, I have provided a complete list of contributions of Govindjee that covers the period 2000–2011, his retirement life thus far. I have provided information on: Research publications in photosynthesis; contributions of historical interest; contributions of educational interest; and recent recognition in the form of three prestigious Awards. He has been as active as when he was in service. He has served the International Society of Photosynthesis, and the wider scientific community, well, and is an example of intense dedication to the field of photosynthesis and its practitioners.

I. Introduction

I arrived in 1998 in the Department of Physics at the University of Illinois, Urbana, from the Max Planck Institute of Biophysical Chemistry in Göttingen, Germany. Soon thereafter I became acquainted with Govindjee, who worked in the area of Biophysics of Photosynthesis, and had a life-long interest and extensive contributions in using chlorophyll *a* fluorescence for understanding photosynthesis. Although Govindjee retired in 1999, our collaboration using FLIM (Fluorescence Lifetime Imaging Microscopy), which I had in my laboratory, on photosynthetic systems began and has continued over the years. In fact, we are now planning new experiments. After his retirement, Govindjee developed a new passion for the 'History of Photosynthesis Research' (see e.g., Fig. 33.1 that shows his 1965 scheme of photosynthesis), although he continues at full speed his earlier passion for 'Education' through his outstanding Series of books 'Advances in Photosynthesis and Respiration', now in its 34th volume.

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J.J. Eaton-Rye, B.C. Tripathy and T.D. Sharkey (eds.), Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation, Advances in Photosynthesis and Respiration 34, pp. 835–844,
DOI 10.1007/978-94-007-1579-0_33,

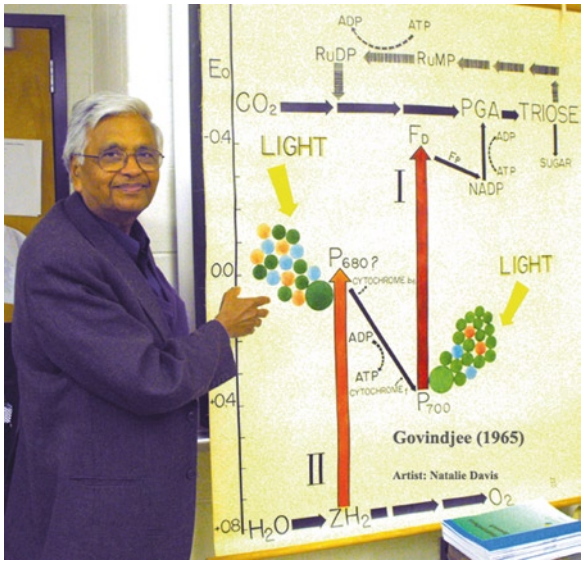

Fig. 33.1. Govindjee standing next to the Z-Scheme he made in 1965 to teach students at the University of Illinois at Urbana–Champaign. In this scheme, reaction center chlorophyll of Photosystem II was shown for the first time as "P_{680}", 4 years before its discovery in Berlin in the laboratory of Horst Witt; the reaction center of Photosystem I is shown as "P_{700}" (discovered by Bessel Kok in the 1950s). The diagram shows two light reactions (***I** and **II**, red arrows*) and two pigment systems (represented by colored balls), as well as the Calvin-Benson-Bassham cycle at the top. ZH_2 represents the electron donor to oxidized P_{680}; F_D represents ferredoxin; NADP is nicotinamide adenine di nucleotide phosphate; PGA is phosphoglyceric acid; RuMP and RuDP are ribulose monophosphate and ribulose diphosphate, respectively. Later, RuDP was named RuBP, ribulose bisphosphate; and P_{680} was shown to be at the bottom of the scheme, and "Q_A", the primary electron acceptor, to be at the top of arrow for Photosystem II. Rabinowitch and Govindjee (1965) used this scheme, in their Scientific American article. Artist: Natalie Davis. The photo is by Melih Sener; taken in the Fall of 2008.

During his retirement period, Govindjee had only one graduate student (Paul Spilotro, who later left to become an MD; he practices in Michigan) and one post-doctoral associate (Manfredo Seufferheld, who is now on the faculty in a different College at our University). However, six graduate students from other laboratories sought his collaboration: Oliver Holub and Yi-Chun Chen (in my research group); Xinguang Zhu (in Steve Long's group), Stuart Rose and Sean Padden (in Tony Crofts's group) and Yan Zhou (in Lance Schideman's group). In these collaborations, Govindjee successfully used chlorophyll fluorescence to characterize and understand: (1) an *Arabidopsis thaliana* mutant altered in the γ subunit of ATP synthase (Spilotro); (2) Xanthophyll cycle non-photochemical quenching (npq) mutants of *Chlamydomonas reinhardtii* (Holub; Seufferheld), related to photoprotection against excess light; (3) a model for Photosystem II reactions (Zhu), from microseconds up to 1 s after illumination; (4) D1-Arginine-257 mutants of *Chlamydomonas reinhardtii*, related to a unique role of bicarbonate/carbonate on the acceptor side of Photosystem II (Rose; Padden); (5) Lutein cycle involved in photoprotection of Avocado leaves (Chen in cooperation with Shizue Matsubara, from Germany), and (6) a special mutant of *Chlamydomonas reinhardtii*, made in the laboratory of Manfredo Seufferheld, which has unique potentials for its exploitation in the field of bioenergy (Zhou).

During this period of more than 10 years, Govindjee has collaborated with many other researchers in: (1) Australia (Fred Chow and A. B. Hope); (2) China (Chunhe Xu and coworkers); (3) Japan (Shigeru Itoh); (4) Czech Republic (Lada Nedbal and his coworkers); (5) Switzerland (Reto Strasser; Gert Schansker); (6) India (Neera Bhalla Sarin and coworkers); and (7) USA (R. Decampo and coworkers).

Currently, Govindjee's collaborators are in: (1) The Czech Republic (Ondrej Prášil and Radek Kaňa); (2) Greece (George Papageorgiou); (3) Germany (Shizue Matsubaara); (4) India (Baishnab C. Tripathy); (5) Iran (M. Mahdi Najafpour); (6) Mexico (Ernesto Garcia Mendoza); (7) Norway (Dmitryi Shevela); (8) Poland (Hazem M. Kalaji), and (9) USA (Robert M. Clegg, Manfredo Seufferheld and Lance Schideman and their coworkers; and Alexendrina Stirbet).

The 'historian in him' has had its collaborators also: John Allen (UK); J. Thomas Beatty (Canada); Clanton Black (USA); Howard Gest (USA); David Krogmann (USA), and Zdněk Šesták (Czech Republic), among others. The major current 'historical' collaborator is Kärin Nickelsen (of Switzerland and Germany). Further, the 'educator in him' had the following collaborators: G.A. Berkowitz (USA); Lars Björn (Sweden); Robert Blankenship (USA); Jan Kern (Germany); Johannes Messinger (Sweden); Larry Orr (USA); George Papageorgiou (Greece), and John Whitmarsh (USA).

Govindjee has also developed an interest in asking questions like "Is there photosynthesis,

Fig. 33.2. Govindjee making chlorophyll *a* fluorescence measurements on a leaf in 2002. Photo taken by Reto Strasser.

and thus life, in outer space?" (with Nancy Kiang, at NASA, and her coworkers). This interest now continues with Lars Olof Björn (of Sweden). His inquisitiveness has led him to ask "Why chlorophyll *a*?" (together with Björn, Blankenship and Papageorgiou).

I provide below his list of publications during 2000–2011, arranged in the following categories: (A) Research (he used FLIM with us; see Holub et al., 2000, 2007, Matsubara et al., 2011; see Section II); (B) History (book; conferences; edited journal; papers); (C) Education (edited books; reviews; web articles; his book Series on Advances in Photosynthesis and Respiration, Springer). Although still involved in experimental research (see Fig. 33.2), it is clear from the following list of publications that Govindjee's interest has significantly shifted to historical and educational aspects of photosynthesis, something that is unique to him and is much appreciated by the scientific community.

II. Research Publications

Chow WS, Funk C, Hope AB and Govindjee (**2000**) Greening of intermittent light-grown bean plants in continuous light: thylakoid components in relation to photosynthetic performance and capacity for photoprotection. Indian J Biochem Biophys 37: 395–404

Gilmore A, Itoh S and Govindjee (**2000**) Global spectral-kinetic analysis of room temperature chlorophyll *a* fluorescence from light harvesting antenna mutants of barley. Phil Trans R Soc London B 335: 1–14

Holub O, Seufferheld MJ, Gohlke C, Govindjee and Clegg RM (**2000**) Fluorescence life-time imaging (FLI)- a new technique in photosynthesis research. Photosynthetica 38: 583–601

Ruiz FA, Marchesini N, Seufferheld M, Govindjee and Decampo R (**2001**) The polyphosphate bodies of *Chlamydomonas reinhardtii* possess a proton pumping pyrophosphatase and are similar to acidocalcisomes. J Biol Chem 276: 46196–46203

Strasser RJ, Schansker G and Govindjee (**2001**) Simultaneous measurement of photosystem I and photosystem II probed by modulated transmission at 820 nm and by chlorophyll *a* fluorescence in the sub ms to second time range. PS2001 Proceedings, 12th International congress on Photosynthesis, Brisbane, CSIRO Publishing, ISBN 0643 06711 6, www.publish.csiro/au/ps2001; S14–003

Yu Y, Li R, Xu C, Ruan K, Shen Y and Govindjee (**2001**) N-bromosccinimide modification of tryptophan 241 at the C-terminus of the manganese stabilizing protein of plant photosystem II influences its structure and function. Physiol Plant 111: 108–115

Govindjee and Seufferheld MJ (**2002**) Non-photochemical quenching of chlorophyll *a* fluorescence: early history and characterization of two xanthophyll cycle mutants of *Chlamydomonas reinhardtii*. Funct Plant Biol 29: 1141–1155

Govindjee and Spilotro P (**2002**) An *Arabidopsis thaliana* mutant, altered in the γ subunit of the ATP synthase, has a different pattern of intensity dependent changes in non-photochemical quenching and kinetics of the P- to- S fluorescence decay. Funct Plant Biol 29: 425–434

Nedbal L, Brezina V, Adamec F, Stys D, Oja V, Laisk A and Govindjee (**2003**) Negative feedback regulation is responsible for the non-linear modulation of photosynthetic activity in plants and cyanobacteria exposed to a dynamic light environment. Biochim Biophys Acta 1607: 5–7

Schansker G, Srivastava A, Govindjee and Strasser RJ (**2003**) Characterization of the 820-nm transmission signal paralleling the chlorophyll *a* fluorescence rise (OJIP) in pea leaves. Funct Plant Biol 30: 1–10

Govindjee (**2004**) Chlorophyll *a* fluorescence: a bit of basics and history. In: Papageorgiou G and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration, Vol 19, pp 2–42. Springer, Dordrecht

Zhu XG, Govindjee, Baker NR, deSturler E, Ort DR and Long SP (**2005**) Chlorophyll *a* fluorescence induction kinetics in leaves predicted from a model describing each discrete step of excitation energy and electron transfer associated with photosystem II. Planta 223: 114–133

Holub O, Seufferheld MJ, Gohlke C, Govindjee, Heiss GJ and Clegg RM (**2007**) Fluorescence lifetime imaging microscopy of *Chlamydomonas reinhardtii*: non-photochemical quenching mutants and the effect of photosynthetic inhibitors on the slow chlorophyll fluorescence transient. J Microscopy 226: 90–120

Rose S, Minagawa J, Seufferheld M, Padden S, Svensson B, Kolling DRJ, Crofts AR and Govindjee (**2008**) D1-arginine mutants (R257E, K and Q) of *Chlamydomonas reinhardtii* have a lowered Q_B redox potential: analysis of thermoluminescence and fluorescence measurements. Photosynth Res 98: 449–468

Kaňa R, Prášil O, Komárek O, Papageorgiou GC and Govindjee (**2009**) Spectral characteristic of fluorescence induction in a model cyanobacterium, *Synechococcus* sp. (PCC 7942) Biochim Biophys Acta 1787: 1170–1178

Yusuf MA, Kumar D, Rajwanshi R, Strasser RJ, Tsimilli-Michael M, Govindjee and Sarin NM (**2010**) Overexpression of γ-tocopherol methyl transferase gene in transgenic *Brassica juncea* plants alleviates abiotic stress: Physiological and chlorophyll *a* fluorescence measurements. Biochim Biophys Acta 1797: 1428–1438

Kalaji HM, Govindjee, Bosa K, Kościelniak J, and Żuk-Gołaszewska K (**2010**) Effects of salt stress on photosystem II efficiency and CO_2 assimilation of two Syrian barley landraces. Special issue "Chlorophyll fluorescence: from theory to the (good) practice". Environ Exp Bot, *in the press*

Stirbet A and Govindjee (**2011**) On the relation between the Kautsky effect (chlorophyll *a* fluorescence induction) and Photosystem II: Basics and application of the OJIP fluorescence transient. J Photochem Photobiol B: Biol 104: 236–257

Matsubara S, Chen YC, Caliandro R, Govindjee and Clegg RM (**2011**) Photosystem II fluorescence lifetime imaging in avocado leaves: Contributions of the lutein-epoxide and violaxanthin cycles to fluorescence quenching. J Photochem Photobiol B: Biol 104: 271–284

Garcia-Mendoza E, Ocampo-Alvarez H and Govindjee (**2011**) Photoprotection in the brown alga *Macrocystis pyrifera*: Evolutionary implications. J Photochem Photobiol B: Biol 104: 377–385

III. Historical

Govindjee (**2001a**) Lighting the path: a tribute to Robert Emerson (1903–1959). PS2001 Proceedings, 12th International congress on Photosynthesis, Brisbane, CSIRO Publishing, ISBN 0643 06711 6, www.publish.csiro/au/ps2001; S43–001

Govindjee (**2001b**) Our greetings to Olle Bjorkman, Christopher Field, and Alexander Glazer. Photosynth Res 70: 241–243

Govindjee (**2001c**) Calvin and Hill prizes: 2001. Photosynth Res 70: 325–328

Govindjee and Gest H (**2002**) Editorial: Celebrating the historical highlights in photosynthesis research. Photosynth Res 73: 1–6; reproduced in Govindjee et al. (2005), see below, pp 5–10

Govindjee and Krogmann D (**2002**) A list of personal perspectives with selected quotations, list of tributes, historical notes, Nobel and Kettering wards, related to photosynthesis. Photosynth Res 73: 11–20; reproduced in Govindjee et al. (2005), see below, pp 1219–1228

Govindjee, Sestak Z and Peters WR (**2002**) The early history of "Photosynthetica," "Photosynthesis Research," and their publishers. Photosynthetica 40: 1–11

Govindjee, Beatty JT and Gest H (**2003**) Editorial: Celebrating the millennium-historical highlights of photosynthesis research, part 2. Photosynth Res 76: 1–11; reproduced in Govindjee et al. (2005), see below, pp 11–21

Govindjee (**2004a**) Robert Emerson and Eugene Rabinowitch: understanding photosynthesis. In: Hoddeson L (ed) No Boundaries: University of Illinois Vignettes, Ch 12, pp 181–194. University of Illinois Press, Urbana and Chicago

Govindjee (**2004b**) A list of photosynthesis conferences and of edited books in photosynthesis. Photosynth Res 80: 447–460; reproduced in Govindjee et al. (2005), see below, pp 1249–1262

Govindjee and Krogmann DW (**2004**) Discoveries in oxygenic photosynthesis (1727–2003): A perspective. Photosynth Res 80: 15–57; reproduced in Govindjee et al. (2005), see below, pp 63–105

Govindjee, Allen JF and Beatty JT (**2004**) Celebrating the millenium: Historical highlights of photosynthesis research, Part 3. Photosynth Res 80: 1–13; reproduced in Govindjee et al. (2005), see below, pp 23–35

Govindjee (**2005**) In Memoriam (List of 189 person's birth and death years), pp 1263–1265, In Govindjee et al. (2005), see right below

Govindjee, Beatty JT, Gest H and Allen JF (eds) (**2005**) Discoveries in Photosynthesis, Advances in Photosynthesis and Respiration, Vol 20, xxxviii + 1304 pp, Springer, Dordrecht

Govindjee (**2006a**) Editorial: Celebrating 20 years of historical papers in photosynthesis research. Photosynth Res 87: 151–158

Govindjee (**2006b**) Editorials: The two letters to the editors by Steve Vik and Wolfgang Junge. Photosynth Res 87: 229–229

Govindjee and Fork DC (**2006**) Charles Stacy French (1907–1995). Biographical Memoirs (National Academy of Sciences, Washington, DC) 88: 2–29

Govindjee and Knaff D (**2006**) International photosynthesis congresses (1968–2007). Photosynth Res 89: 1–2

Govindjee and Krogmann D (**2006**) Discoveries in oxygenic photosynthesis (1726–2003): A perspective. In: Chemistry and Biology: The Transition between the Two Centuries. Convegno Internazionale. Academia Nazionale dei Lincei; Fondazione <<Guido Donegani>>, Vol 36, pp 203–285. Bardi Editore, Roma

Govindjee (**2007**) Editorial: A tale of naming a photosynthetic bacterium. Photosynth Res 92: 1–2

Govindjee and Telfer A (**2007**) Six young research investigators were honored at an international conference in Russia. Photosynth Res 92: 139–141

Govindjee and Yoo H (**2007**) The international society of photosynthesis research (ISPR) and its associated international congress on photosynthesis (ICP): A pictorial report. Photosynth Res 91: 95–106

Govindjee, Rutherford AW and Britt RD (**2007**) Four young research investigators were honored at the 2006 Gordon research conference on photosynthesis. Photosynth Res 92: 137–138

Nozzolillo CG, Gorham HH and Govindjee (**2007**) Obituary: Paul R. Gorham (April 16, 1918–November 9, 2006). Photosynth Res 92: 3–5

Black CC and Govindjee (2008) Martin Gibbs and the peaceful uses of nuclear radiation, ^{14}C. Photosynth Res 99: 63–80

Govindjee (**2008**) Recollections of Thomas John Wydrzynski. Photosynth Res 98:13–31

Prasil O, Suggett J, Cullen JJ, Babin M and Govindjee (**2008**) Aquafluo 2007: chlorophyll fluorescence in aquatic sciences, an international conference held in Nové Hrady. Photosynth Res 95: 111–115

Govindjee (**2009a**) List of biography and history published mostly in Photosynthesis Research, 1988–2008. Photosynth Res 99: 139–153

Govindjee (**2009b**) A tribute to Achim Trebst, a friend. Photosynth Res 100:113–115

Govindjee (**2009c**) Young research investigators honored at the 2008 and 2009 Gordon research conferences on photosynthesis: ambiance and a personal perspective. Photosynth Res 102: 1–6

Govindjee (**2010a**) Obituary of Sam Aronoff. ASPB (American Society of Plant Biologists) News Letter 37: 21–22, See http://www.plantbio.org/newsletter/marapr10/09aronoff.cfm

Govindjee (**2010b**) Celebrating Andrew Alm Benson's 93rd birthday. Photosynth Res 105: 201–208

Govindjee and Seibert M (**2010**) Picosecond spectroscopy of the isolated reaction centers from the photosystems of oxygenic photosynthesis-ten years (1987–1997)' fun. A tribute to Michael R. Waisielewski on his 60th birthday. Photosynth Res 103:1–6

Govindjee and Srivastava SL (eds) (**2010**) A Tribute: Krishnaji (January 13, 1922–August 14, 1997), xii + 266 pages, Apex Graphics, Allahabad, India [*This is a Tribute to his own brother, a physicist at Allahabad University; it has family photographs*]

Hirsch RE, Rich M and Govindjee (**2010**) A tribute to Seymour Steven Brody: in memoriam (November 29, 1927 to May 25, 2010). Photosynth Res 106: 191–199

Raghavendra AS and Govindjee (**2011**) Sir Jagadish Chandra Bose (1858–1937): A pioneer in photosynthesis research and discoverer of unique carbon assimilation in *Hydrilla*. In: Raghavendra, AS and Sage RF (eds) C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms, Advances in Photosynthesis and Respiration, Vol 32, pp 3–11. Springer, Dordrecht

Nickelsen K and Govindjee (**2011**) The maximum quantum yield controversy: Otto Warburg and the 'midwest gang'. Bern Studies in the History and Philosophy of Science, University of Bern, Switzerland. ISBN: 978-3-9523421-9-0

IV. Educational

A major educational service that Govindjee has done to the international student and teaching community is through design, production and free distribution (in collaboration with his dentist friend Wilbert Veit) of Z-Scheme posters for electron transport from water to $NADP^+$ (nicotinamide adenine dinucleotide phosphate). In 2010, 1,000 copies of this valuable and up-to-date poster had already been distributed around the World. This poster includes his major discovery of bicarbonate on the electron acceptor side of photosystem II, one shared by Julian Eaton-Rye, chief editor of the current volume. For these and other services (see below), some have called him "Mr. Photosynthesis" and others state "The World is his laboratory."

A. Reviews, Encyclopedia Articles, Web Articles, and Announcements

Govindjee (**2000**) Milestones in photosynthesis research. In: Younis M, Pathre U and Mohanty P (eds) Probing Photosynthesis, pp 9–39. Taylor & Francis, UK; for a web version, see http://www.life.uiuc.edu/govindjee/papers/milestones.html

Govindjee and Nedbal L (**2000**) Seeing is believing. Photosynthetica 38: 481–482

Orr L and Govindjee (**2001**) Photosynthesis and the Web: 2001. Photosynth Res 68: 1–28

Whitmarsh J and Govindjee (**2001a**) Photosynthesis: Light Reactions. In: Robinson R (ed) Plant Sciences, pp 33–140. Macmillan Reference, Detroit, MI, USA (See http://www.mlr.com or call 1-800-877-GALE); for a web article by these authors "The Photosynthetic Process", see http://www.life.uiuc.edu/govindjee/paper/gov.html

Whitmarsh J and Govindjee (**2001b**) Photosystem II. Encyclopedia of Life Sciences. McMillan Reference Ltd, London, UK Web article (revised in 2009)

Govindjee (**2002**) A role for a light-harvesting antenna complex of Photosystem II in photoprotection (Editorial). Plant Cell 14: 1663–1668

Papageorgiou G and Govindjee (eds) (**2004**) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration, Vol 19. Springer, Dordrecht (reprinted in paperback in 2010)

Govindjee (**2005a**) Announcement: Advances in Photosynthesis and Respiration, Vols 17 and 18: Focus on plant respiration. Photosynth Res 85: 255–259

Govindjee (**2005b**) Announcement: Advances in Photosynthesis and Respiration, Vol 19: Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, edited by George C. Papageorgiou and Govindjee. Photosynth Res 83: 101–105

Govindjee (**2006a**) Announcement: Photoprotection, Photoinhibition, Gene Regulation, and Environment, Vol 21, Advances in Photosynthesis and Respiration. Photosynth Res 89: 53–57

Govindjee (**2006b**) Announcement: Discoveries in Photosynthesis, Vol 20, Advances in Photosynthesis and Respiration. Photosynth Res 87: 235–239

Govindjee (**2006c**) Announcement: Photosystem II: The Light-driven Water: Plastoquinone Oxidoreductase, Vol 22, Advances in Photosynthesis and Respiration. Photosynth Res 87: 331–335

Govindjee (**2006d**) Announcement: Advances in Photosynthesis and Respiration, Vol 23, Structure and Function of Plastids. Photosynth Res 89: 173–177

Govindjee (**2006e**) Announcement: Advances in Photosynthesis and Respiration, Vol 24 (Photosystem I) and Vol 25 (Chlorophylls and Bacteriochlorophylls). Photosynth Res 90: 91–96; for comments on the Series, by Robert Blankenship, see http://www.life.uiuc.edu/govindjee/newbook/Quotation.html

Berkowitz GA, Portis AR Jr and Govindjee (**2007**) Carbon dioxide fixation. The Encyclopedia of Science and Technology, 10th Edition, Vol 13, pp 475–481. McGraw Hill Publishers, New York

Björn LO and Govindjee (**2007**) The evolution of photosynthesis and its environmental impact. In: Björn LO (ed) Photobiology: The Science of Light and Life, pp 243–274. Springer, New York

Blankenship RE and Govindjee (**2007**) Photosynthesis. The Encyclopedia of Science and Technology, 10th Edition, Vol 13, pp 468–475. McGraw Hill Publishers, New York

Govindjee, Blankenship RE and Shopes R (**2007**) Bacterial Photosynthesis. The Encyclopedia of Science and Technology, 10th Edition, Vol 13, pp 481–486. McGraw Hill Publishers, New York

Kiang NY, Siefert J, Govindjee and Blankenship RE (**2007a**) Spectral signatures of photosynthesis I. Review of earth organisms. Astrobiology 7: 222–251

Kiang NY, Segura A, Tinetti G, Govindjee, Blankenship RE, Cohen M, Siefert J, Crisp D and Meadows VS (**2007b**) Spectral signatures of photosynthesis II. Coevolution with other stars and the atmosphere on extra-solar worlds. Astrobiology 7: 252–274

Orr L and Govindjee (**2007**) Photosynthesis and the web: 2008. Photosynth Res 91: 107–131; for 2008 version, see http://www.life.uiuc.edu/govindjee/photoweb/

Govindjee (**2008**) Teaching photosynthesis: Some thoughts. In: Allen JF, Gantt E, Golbeck JH and Osmond B (eds) Photosynthesis. Energy from the Sun, pp 1619–1624. Springer, Dordrecht

Björn LO and Govindjee (**2009**) The evolution of photosynthesis and chloroplasts. Curr Sci 96: 1466–1474

Björn LO, Papageorgiou GC, Blankenship R and Govindjee (**2009a**) A viewpoint: why chlorophyll *a*? Photosynth Res 99: 85–98

Björn LO, Papageorgiou GC, Dravins D and Govindjee (**2009b**) Detectability of life on exoplanets. Cur Sci 96: 1171–1175

Messinger J, Alia A and Govindjee (Editorial) (**2009a**) Special educational issue on 'Basics and application of biophysical techniques in photosynthesis and related processes' – [Part A]. Photsynth Res 101: 89–92

Messinger J, Alia A and Govindjee (Editorial) (**2009b**) Special educational issue on 'Basics and application of biophysical techniques in photosynthesis and related processes' – [Part B]. Photosynth Res 102: 103–106

Clegg RM, Sener M and Govindjee (**2010**) From Förster Resonance Energy Transfer (FRET) to Coherent Resonance Energy Transfer (CRET) and Back — A wheen o' mickles mak's a muckle. SPIE (Society for Promotion of Instrumentation and Engineering) Proceedings. In: Optical Biopsy VII, edited by Robert R. Alfano, Proceedings of SPIE, Vol. 7561 (SPIE, Bellingham, WA, 2010), paper number: 7561–12; article CID number: 75610C, 21 pages

Govindjee (**2010**) Book Review: Gernot Renger (ed): Primary processes of photosynthesis: principles and apparatus, parts 1 and 2. The Royal Society of Chemistry (RSC), 2008. Photosynth Res 103: 61–63

Govindjee, Kern JF, Messinger J and Whitmarsh J (**2010**) Photosystem II. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0000669.pub2

Orr L and Govindjee (**2010**) Photosynthesis online. Photosynth Res 105: 167–200

Govindjee and Björn LO (**2011**) Dissecting oxygenic photosynthesis: The evolution of the "Z"-scheme for thylakoid reactions, In**:** Itoh, S, Mohanty P and Guruprasad, KN (eds) Photosynthesis: Overviews on Recent Progress & Future Perspective, pp 1–27. Information and Knowledge (IK) International Publishers, New Delhi

Govindjee and Shevela D (**2011**) Adventures with cyanobacteria: A personal perspective. Front Plant Sci 2: 28. DOI: 10.3389/fpls.2011.0028 17 pages www.frontiersin.org

Najafpour MM and Govindjee (**2011**) Oxygen evolving complex in Photosystem II: Better than excellent. Dalton Trans 40: 1–9. DOI: 10.1039/c1dt10746a

Papageorgiou GC and Govindjee (**2011**) Photosystem II fluorescence: Slow changes-scaling from the past. J Photochem Photobiol B: Biol 104: 258–270

B. Advances in Photosynthesis and Respiration Series, Springer

Already in 1994, Govindjee had become the founding Editor of the Series "Advances in Photosynthesis" (AIPH), with the publication of its first volume (*Molecular Biology of Cyanobacteria*), edited by Donald Bryant. By the time he retired, a total of 8 volumes were published: volume 2 (*Anoxygenic Photosynthetic Bacteria*, 1995), edited by Robert Blankenship, Michael T. Madigan and Carl E. Bauer; volume 3 (*Biophysical Techniques in Photosynthesis*, 1996), edited by Jan Amesz and Arnold J. Hoff; volume 4 (*Oxygenic Photosynthesis: The Light Reactions*, 1996), edited by Donald R. Ort and Charles F. Yocum; volume 5 (*Photosynthesis and the Environment*, 1996), edited by Neil R. Baker; volume 6 (*Lipids in Photosynthesis: Structure, Function and Genetics*, 1998), edited by Paul-Andre Siegenthaler and Norio Murata; volume 7 (*The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*, 1998), edited by Jean David Rochaix, Michael Goldschmidt-Clermont and Sabeeha Merchant; and Volume 8 (*The Photochemistry of Carotenoids*, 1999), edited by Harry A. Frank, Andrew J. Young, George Britton and Richard J. Cogdell.

During 2000–2010, under Govindjee's leadership, 24 more volumes were published on almost all aspects of photosynthesis, from the time photons are absorbed till food and biomass are made. In addition, realizing that we must learn from the reverse process of Photosynthesis, that is Respiration, Govindjee changed the title of the Series to include Respiration. Beginning with volume 11, the title of the Series became 'Advances in Photosynthesis and Respiration'. Starting with volume 31, Govindjee invited Thomas D. Sharkey to join him as his co-Series Editor. In 2011, we will see in print the current volume "*Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation"*, edited by Julian J. Eaton-Rye, Baishnab C. Tripathy and Thomas D. Sharkey, as well as "*Functional Genomics and Evolution of Photosynthetic Systems*", edited by Robert L. Burnap and Willem F.J. Vermaas.

In addition to being a Series Editor, Govindjee has co-edited volumes 19, 20 and 29. I was invited to be one of the authors in volume 19 (Clegg, 2004). Volumes 9–32 are listed below.

2000

- Volume 9 (*Photosynthesis, Physiology and Metabolism*), edited by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer

2001

- Volume 10 (*Photosynthesis: Photobiochemistry and Photobiophysics*), authored by Bacon Ke
- Volume 11 (*Regulation of Photosynthesis*), edited by Eva-Mari Aro and Bertil Anderson

2002

- Volume 12 (*Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism*), edited by Christine Foyer and Graham Noctor

2003

- Volume 13 (*Light Harvesting Antennas*), edited by Beverley Green and William Parson
- Volume 14 (*Photosynthesis in Algae*), edited by Anthony Larkum, Susan Douglas and John Raven

2004

- Volume 15 (*Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers*), edited by Davide Zannoni
- Volume 16 (*Respiration in Archaea and Bacteria: Diversity of Prokaryotic Respiratory System*), edited by Davide Zannoni
- Volume 17 (*Plant Mitochondria: From Genome to Function*), edited by David A. Day, Harvey Millar and James Whelan
- Volume 19 (*Chlorophyll a Fluorescence : A Signature of Photosynthesis*), edited by George C. Papageorgiou and Govindjee

2005

- Volume 18 (*Plant Respiration: From Cell to Ecosystem*), edited by Hans Lambers and Miquel Ribas-Carbo
- Volume 20 (*Discoveries in Photosynthesis*), edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen
- Volume 21 (*Photoprotection, Photoinhibition, Gene Regulation and Environment*), edited by

Barbara-Demmig-Adams, William W. III Adams and Autar K. Mattoo
- Volume 22 (*Photosystem II: The Light-Driven Water-Plastoquinone Oxidoreductase*), edited by Thomas J. Wydrzynski and Kimiyuki Satoh

2006

- Volume 23 (*The Structure and Function of Plastids*), edited by Robert R. Wise and J. Kenneth Hoober
- Volume 24 (*Photosystem I: The Light-Driven Plastocyaninr-Ferredoxin Oxidoreductase*), edited by John H. Golbeck
- Volume 25 (*Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*), edited by Bernard Grimm, Robert Porra, Wolfhart Rüdiger and Hugo Scheer

2008

- Volume 26 (Biophysical Techniques in Photosynthesis), edited by Thijs J. Aartsma and Jörg Matysik
- Volume 27 (*Sulfur Metabolism in Phototrophic Organisms*), edited by Rudiger Hell, Christian Dahl, David B. Knaff and Thomas Leustek
- Volume 28 (*The Purple Phototrophic Bacteria*), edited by C. Neil Hunter, Fevzi Daldal, Marion Thurnauer and J. Thomas Beatty

2009

- Volume 29 (*Photosynthesis in silico: Understanding Complexity from Molecules to Ecosystem*), edited by Agu Laisk, Ladislav Nedbal and Govindjee
- Volume 30 (*Lipids in Photosynthesis: Essential and Regulatory Functions.*), edited by Hajime Wada and Norio Murata

2010

- Volume 31 (*The Chloroplast: Basics and Applications*), edited by Constantin Rebeiz, Hans Bohnert, Christoph Benning, Henry Daniell, J. Kenneth Hoober, Hartmut Lichtenthaler, Archie R. Portis and Baishnab C. Tripathy
- Volume 32 (*C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms*), edited by Agepati S. Raghavendra and Rowan Sage

In addition to the above books in his Series (that now has Thomas D. Sharkey as his co-Series Editor), Govindjee has co-edited a book, in **2010**, with Ashwani Pareek, Sudhir K. Sopory, and Hans J. Bohnert on another topic: *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation*, Springer, Dordrecht, XLI, 526 p. 25 illus. in color., Hardcover: ISBN: 978-90-481-3111-2

Govindjee has done a great service to the scientific community by bringing these books out on *Photosynthesis and Respiration* (often known by the older acronym AIPH) as they provide the backbone for obtaining the basic knowledge needed for applications of plant, algal and bacterial systems for our future needs: Biomass; Biofuel (Bioalcohol; Biodiesel); Bioelectricity, and Hydrogen.

Robert E. Blankenship, of Washington University at Saint Louis, Missouri, has given one of the testimonials of Govindjee's Series. He wrote: "Govindjee's mentor Eugene Rabinowitch wrote the story of photosynthesis in the 1940s and 1950s. No one could ever hope to do that again; the amount of information is just too vast for any one person to ever hope to do a proper job of giving the real state of knowledge. However, Govindjee has really duplicated Rabinowitch's accomplishment in the only way it could be done nowadays, by enlisting editors who are experts in areas of the field and having them in turn enlist expert authors. When I look at the AIPH books on my shelf I am struck with how effectively they collectively summarize the field. *I am continually impressed with how Govindjee has added new books to the series that make sense and really provide the level of detail that is needed.*' See http://www.life.uiuc.edu/govindjee/newbook/Quotation.html

V. Awards and Honors

I have already expressed my appreciation of Govindjee in Eaton-Rye (2007b) (see p. 168). I end this list of his contributions by mentioning that for his outstanding photosynthesis research, communication and outreach to others, he has been recognized, during his retirement years, by the following prestigious Awards.

- The first (**2006)** Lifetime Achievement Award for Basic (Biological) Research from the Rebeiz Foundation (Rebeiz et al., 2007).
- The **2007** Communication Award of the International Society of Photosynthesis Research Conference, held in Glasgow (see Blankenship,

2007; and http://www.sebiology.org/publications/Bulletin/October07/PS07.html).

- A special issue of Photosynthesis Research, in two parts, in **2007**, was published to honor his 50 years in Photosynthesis Research and his 75th birthday (Eaton-Rye, 2007a,b)
- The **2008** Liberal Arts & Sciences (LAS) Alumni Achievement Award of the University of Illinois at Urbana-Champaign. http://www.las.uiuc.edu/alumni/magazine/articles/2009/govindjee/

Further, his country of birth, India, held a special symposium on Photosynthesis in his honor, during November 27–29, 2008, in Indore, India (Jajoo et al., 2009). Currently a book " Photosynthesis: Overviews on Recent Progress & Future Perspective", honoring him, is in press; it is being edited by S. Itoh , P. Mohanty and K.N. Guruprasad , and will also be published in 2011, by Information and Knowledge (IK) International Publishers, New Delhi, India (see Fig. 33.3 for a photograph of P. Mohanty, with George Papageorgiou (author of Chapter 31) and Julian Eaton-Rye (author of Chapter 32)).

Lastly, he has received a new honor, from India, in being listed among the two Indian American Botanists in a **2010** book "Eminent Botanists: Past and Present (Biographies and Contributions)", edited by P. Suresh Narayana and T. Pullaiah, Regency Publications, Delhi (ISBN: 978-81-89233-63-1)

VI. Concluding Remarks

It is fitting to close this chapter by including the two following quotes:

Doug Peterson wrote, in connection with Govindjee's 2008 LAS Alumni Achievement Award: "Just as a plant absorbs light and creates energy, Govindjee's career has been one of absorbing everything he could learn about photosynthesis and then transforming it into energy—or, as one former student calls it, 'an infectious enthusiasm.' "

Donald R. Ort, a longtime colleague of Govindjee in the area of photosynthesis research, wrote, while nominating Govindjee for the LAS Alumni Achievement Award:

"Govindjee stands out as "perhaps the world's most recognized photosynthesis researcher; he added "Driven by a single-minded fascination with the process of photosynthesis, Govindjee's research contributions have been paradoxically far-reaching and diverse." See an excellent interview of Govindjee by Don Ort at http://www.annualreviews.org/page/audio#govindjee. Here, we learn about the story of his one name as well as how he entered photosynthesis research.

This chapter will not be complete without mentioning Govindjee's enthusiasm in recognizing excellence in biological research among graduate students at his alma mater, the University of Illinois at Urbana. He along with his wife Rajni Govindjee

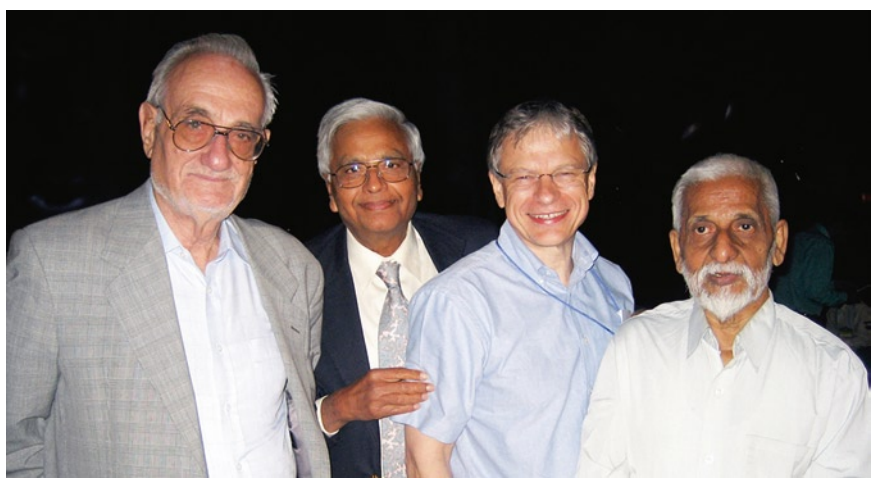

Fig. 33.3. Photograph of three past PhD students of Govindjee. *Left* to *right*: George Papageorgiou (PhD, 1968; author of Chapter 31), Govindjee, Julian Eaton-Rye (PhD, 1987, author of Chapter 32) and Prasanna Mohanty (PhD, 1972; co-editor of 'Photosynthesis: Overviews on Recent Progress & Future Perspective', being released in 2011, honoring Govindjee). This photo was taken during the special symposium held in Govindjee's honor in Indore, India, in November, 2008.

Fig. 33.4. Photograph of the 2010 winners of the Robert Emerson Award, as well as of the Govindjee (G) and Rajni Govindjee (RG) Award for Excellence in Biological Sciences. From *left* to *right*: Paul Nabity (Emerson Award), Ryan Kelly (G and RG Award), Kelly Gillespie (G and RG Award), Govindjee, and Rajni. Brian Steidinger (G and RG Award) was absent. Place: in front of a plaque honoring Govindjee's (and Rajni's) professors, Robert Emerson and Eugene Rabinowich on the University of Illinois Campus in Urbana-Champaign, Illinois. To honor Emerson, Govindjee is wearing his lab apron; and the ties were to symbolize respect for both Emerson as well as Eugene Rabinowitch. Photo by Martha Plummer; June 10, 2010.

(a well-known biophysicist in the area of energy transduction in bacteriorhodopsin) have established an annual Govindjee and Rajni Govindjee Award for Excellence in Biological Sciences (see http://sib.illinois.edu/grants_Govindjee.htm). To honor these students, he has placed their photographs at his web site. For the 2010 Awardees, see http://www.life.illinois.edu/govindjee/photooftheyear.html and Fig. 33.4.

I recommend his excellent web site (http://www.life.illinois.edu/govindjee/) to all the readers of this book as it is a gold mine of information on photosynthesis: historical, educational and research.

Acknowledgement

I thank Julian Eaton-Rye for inviting me to write here the contributions of my friend and colleague Govindjee with whom I have been associated during his retirement years. I am happy to mention that our collaboration will continue. We have continued exciting discussions on the mechanism of excitation energy transfer in photosynthesis. Together, we plan to explore the unique photophysical characteristics of unique mutants of a green alga *Chlamydomonas reinhardtii* as well as of a cyanobacterium *Synechocystis* sp. PCC 6803.

I am thankful to Govindjee for providing me his autobiographical notes that helped me complete this chapter.

References

Blankenship RE (2007) 2007 Awards of the International Society of Photosynthesis Research (ISPR). Photosynth Res 94: 179–181

Clegg RM (2004) Nuts and bolts of excitation energy migration and energy transfer. In: Papageorgiou GC and Govindjee (eds) Chlorophyll *a* Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Vol 19, pp 83–105. Springer, Dordrecht

Eaton-Rye JJ (2007a) Celebrating Govindjee's 50 years in photosynthesis research and his 75th birthday. Photosynth Res 93: 1–5

Eaton-Rye JJ (2007b) Snapshots of the Govindjee lab from the late 1960s to the late 1990s, and beyond. Photosynth Res 94: 153–178

Jajoo A, Guruprasad KN, Bharti S and Mohanty P (2009) International conference "Photosynthesis in the Global Perspective" held in honor of Govindjee, November 27–29, 2008, Indore, India. Photosynth Res 100: 49–55

Rabinowitch E and Govindjee (1965) The role of chlorophyll in photosynthesis. Sci Am 213: 74–83

Rebeiz CA, Benning C, Bohnert H, Hoober JK and Portis AR (2007) Govindjee was honored with the First Lifetime Achievement Award, and Britta Forster and coworkers with the First Annual Paper Prize of the Rebeiz Foundation for Basic Research. Photosynth Res 94: 147–151

Subject Index

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