Advances in Photosynthesis and Respiration

Volume 23

# The Structure and Function of Plastids

Edited by

Robert R. Wise and J. Kenneth Hoober

Springer

# The Structure and Function of Plastids

# Advances in Photosynthesis and Respiration

VOLUME 23



The scope of our series, beginning with volume 11, 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, from the photophysics of reaction centers, through the electrochemistry of intermediate electron transfer, to the physiology of whole orgamisms, and from X-ray crystallography of proteins to the morphology or 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.

*The titles published in this series are listed at the end of this volume and those of forthcoming volumes on the back cover.*

# The Structure and Function of Plastids

*Edited by*

**Robert R. Wise**
*University of Wisconsin, Oshkosh, WI, USA*

and

**J. Kenneth Hoober**
*Arizona State University, Tempe, AZ, USA*

Springer

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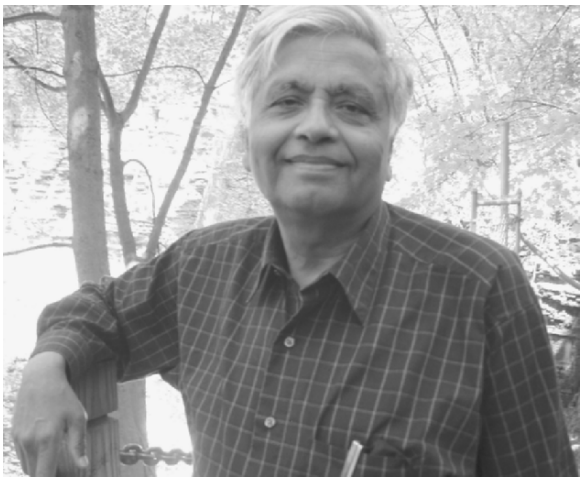
# From the Series Editor

## *Advances in Photosynthesis and Respiration, Volume 23*



I am delighted to announce the publication, in *Advances in Photosynthesis and Respiration* (AIPH) Series, of **The Structure and Function of Plastids**, a book covering the central role of plastids for life on earth. It deals with both the structure and the function of the unique organelles, particularly of chloroplasts. Two distinguished authorities have edited this volume: Robert R. Wise of University of Wisconsin at Oshkosh, Wisconsin, and J. Kenneth Hoober of Arizona State University, Tempe, Arizona. Two of the earlier AIPH volumes have included descriptions of plastids: Volume 7 (The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*, edited by Jean David Rochaix, Michel Goldschmidt-Clermont and Sabeeha Merchant); and Volume 14 (Photosynthesis in Algae, edited by Anthony Larkum, Susan Douglas and John Raven).

The current volume follows the 22 volumes listed below.

### Published Volumes (1994–2005)

- *Volume 1:* ***Molecular Biology of Cyanobacteria*** (28 chapters; 881 pages; 1994; edited by Donald A. Bryant, from USA; ISBN: 0-7923-3222-9);
- *Volume 2:* ***Anoxygenic Photosynthetic Bacteria*** (62 chapters; 1331 pages; 1995; edited by Robert E. Blankenship, Michael T. Madigan and Carl E. Bauer, from USA; ISBN: 0-7923-3682-8);
- *Volume 3:* ***Biophysical Techniques in Photosynthesis*** (24 chapters; 411 pages; 1996; edited by the late Jan Amesz and the late Arnold J. Hoff, from The Netherlands; ISBN: 0-7923-3642-9);
- *Volume 4:* ***Oxygenic Photosynthesis: The Light Reactions*** (34 chapters; 682 pages; 1996; edited by Donald R. Ort and Charles F. Yocum, from USA; ISBN: 0-7923-3683-6);
- *Volume 5:* ***Photosynthesis and the Environment*** (20 chapters; 491 pages; 1996; edited by Neil R. Baker, from UK; ISBN: 0-7923-4316-6);
- *Volume 6:* ***Lipids in Photosynthesis: Structure, Function and Genetics*** (15 chapters; 321 pages; 1998; edited by Paul-André Siegenthaler and Norio Murata, from Switzerland and Japan; ISBN: 0-7923-5173-8);
- *Volume 7:* ***The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*** (36 chapters; 733 pages; 1998; edited by Jean David Rochaix, Michel Goldschmidt-Clermont and Sabeeha Merchant, from Switzerland and USA; ISBN: 0-7923-5174-6);
- *Volume 8:* ***The Photochemistry of Carotenoids*** (20 chapters; 399 pages; 1999; edited by Harry A. Frank, Andrew J. Young, George Britton and Richard J. Cogdell, from USA and UK; ISBN: 0-7923-5942-9);
- *Volume 9:* ***Photosynthesis: Physiology and Metabolism*** (24 chapters; 624 pages; 2000; edited by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer, from UK, USA and Australia; ISBN: 0-7923-6143-1);
- *Volume 10:* ***Photosynthesis: Photobiochemistry and Photobiophysics*** (36 chapters; 763 pages; 2001; authored by Bacon Ke, from USA; ISBN: 0-7923-6334-5);
- *Volume 11:* ***Regulation of Photosynthesis*** (32 chapters; 613 pages; 2001; edited by Eva-Mari Aro

and Bertil Andersson, from Finland and Sweden; ISBN: 0-7923-6332-9);

- *Volume 12:* ***Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism*** (16 chapters; 284 pages; 2002; edited by Christine Foyer and Graham Noctor, from UK and France; 0-7923-6336-1);
- *Volume 13:* ***Light Harvesting Antennas*** (17 chapters; 513 pages; 2003; edited by Beverley Green and William Parson, from Canada and USA; ISBN: 0-7923-6335-3);
- *Volume 14:* ***Photosynthesis in Algae*** (19 chapters; 479 pages; 2003; edited by Anthony Larkum, Susan Douglas and John Raven, from Australia, Canada and UK; ISBN: 0-7923-6333-7);
- *Volume 15:* ***Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers*** (13 chapters; 326 pages; 2004; edited by Davide Zannoni, from Italy; ISBN: 1-4020-2001-5);
- *Volume 16:* ***Respiration in Archaea and Bacteria 2: Diversity of Prokaryotic Respiratory Systems*** (13 chapters; 310 pages; 2004; edited by Davide Zannoni, from Italy; ISBN: 1-4020-2002-3);
- *Volume 17:* ***Plant Mitochondria: From Genome to Function*** (14 chapters; 325 pages; 2004; edited by David A. Day, A. Harvey Millar and James Whelan, from Australia; ISBN: 1-4020-2339-5);
- *Volume 18:* ***Plant Respiration: From Cell to Ecosystem*** (13 chapters; 250 pages; 2005; edited by Hans Lambers, and Miquel Ribas-Carbo, 2005; from Australia and Spain; ISBN: 1-4020-3588-8);
- *Volume 19:* ***Chlorophyll a Fluorescence: A Signature of Photosynthesis*** (31 chapters; 817 pages; 2004; edited by George C. Papageorgiou and Govindjee, from Greece and USA; ISBN: 1-4020-3217-X);
- *Volume 20:* ***Discoveries in Photosynthesis*** (111 chapters; 1210 pages; 2005; edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen, from USA, Canada and Sweden (& UK); ISBN: 1-4020-3323-0);
- *Volume 21:* ***Photoprotection, Photoinhibition, Gene Regulation, and Environment*** (21 chapters; 500 pages; 2005; edited by Barbara Demmig-Adams, William W. Adams III and Autar K. Mattoo, from USA; ISBN: 1-4020-3564-0); and
- *Volume 22:* ***Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase.*** (34 chapters; 820 pages; 2005; edited by Thomas J. Wydrzynski and Kimiyuki Satoh, from Australia and Japan; ISBN: 1-4020-4249-3).

Further information on these books and ordering instructions can be found at <http://www.springeronline.com> under the Book Series "Advances in Photosynthesis and Respiration". Special discounts are available for members of the International Society of Photosynthesis Research, ISPR (<http://www. photosynthesis-research.org/>).

## The Structure and Function of Plastids

*The Structure and Function of Plastids*, Volume 23 in the *Advances in Photosynthesis and Respiration* Series, provides a comprehensive look at the biology of plastids, the multifunctional biosynthetic factories that are unique to plants and algae. Fifty-nine international experts, from 11 countries, have contributed an excellent "Dedication" and 27 chapters that cover all aspects of this large and diverse family of plant and algal organelles. The book is divided into five sections: (I) **Plastid Origin and Development** (5 chapters); (II) **The Plastid Genome and its Interaction with the Nuclear Genome** (5 chapters); (III) **Photosynthetic Metabolism in Plastids** (4 chapters); (IV) **Non-Photosynthetic Metabolism in Plastids** (6 chapters); and (V) **Plastid Differentiation and Response to Environmental Factors** (7 chapters). Each chapter includes an integrated view of plant biology from the standpoint of the plastid. The book is intended for a wide audience, but is specifically designed for advanced undergraduate and graduate students and scientists in the fields of photosynthesis, biochemistry, molecular biology, physiology, and plant biology. This book, edited by Bob Wise and Ken Hoober, is a very important addition to the already published books in the AIPH Series.

*The Structure and Function of Plastids* begins with *a dedication* by Brian Gunning (of Australia) Friederike Koenig (of Germany) and Govindjee (USA) to the early pioneers. This dedication ends by honoring Wilhelm Menke, who had coined the word "thylakoids" in 1961. I provide below some of the names, mentioned in this dedication (arranged here by the year of birth of those mentioned):

- Anthony van Leewenhoeck (1632–1723) described chloroplasts without naming them
- Nehemiah Grew (1641–1712) may have indeed seen chloroplasts

- Franz Julius Ferdinand Meyen (1804–1840) described "chlorophyll granules" (or "corpuscules")
- Hugo von Mohl (1805–1872) provided detailed description of "chlorophyll granules" ("Chlorophyllkörner")
- Nathanael Pringsheim (1823–1894) used the term "Stroma" for the non-green part of "Chlorophyllkörner"
- Eduard Strasburger (1844–1912) used the word "Chloroplast" for chloroplast
- Arthur Meyer (1850–1922) used the term "Grana" and distinguished between "Autoplasten" (what we call chloroplasts); "Chromoplasten" (chromoplasts); "Trophoplasten" (reserve storing plastids); and "Anaplasten" (leucoplasts)
- Constantin Sergeevich Mereschkowsky* (1855–1921) provided a detailed hypothesis of endosymbiosis (* also spelled as Konstantin Sergejewicz Mereschkovsky)
- Andreas Franz Schimper (1856–1901) described three types of plastids ("Chloroplastiden", "Leukoplastiden", and "Chromoplastiden"); stated that "Chloroplastiden" resembled cyanobacteria

Twenty-seven chapters, in order of appearance, are (authors names are in parentheses):

(1) The Diversity of Plastid Form and Function (Robert R. Wise, USA);
(2) Chloroplast Development: Whence and Whither (J. Kenneth Hoober, USA);
(3) Protein Import into Plastids: Who, When, and How? (Ute C. Vothknecht and Jürgen Soll, both from Germany);
(4) Origin and Evolution of Plastids: Genomic View on the Unification and Diversity of Plastids (Naoki Sato, Japan);
(5) The Mechanism of Plastid Division: The Structure and Origin of The Plastid Division Apparatus (Shin-ya Miyagishima and Tsuneyoshi Kuroiwa, USA and Japan);
(6) Expression, Prediction and Function of the Thylakoid Proteome in Higher Plants and Green Algae (Klaas van Wijk, USA);
(7) The Role of Nucleus- and Chloroplast-Encoded Factors in the Synthesis of the Photosynthetic Apparatus (Jean-David Rochaix, Switzerland);
(8) Plastid Transcription: Competition, Regulation, and Promotion by Plastid- and Nuclear-Encoded Polymerases (A. Bruce Cahoon, Yutaka Komine and David B. Stern, all from USA);
(9) Plastid-to-Nucleus Signaling (Åsa Strand, Tatjana Kleine and Joanne Chory, from Sweden and USA);
(10) Trace Metal Utilization in Chloroplasts (Sabeeha Merchant, USA);
(11) Light/Dark Regulation of Chloroplast Metabolism (Shaodong Dai, Kenth Johansson, Hans Eklund and Peter Schürmann, of USA, Switzerland and Sweden);
(12) Chlororespiratory Pathways and their Physiological Significance (Peter J. Nixon and Peter R. Rich, both from UK);
(13) $CO_2$ Concentrating Mechanisms (Sue G. Bartlett, Mautusi Mitra and James V. Moroney, all from USA);
(14) Synthesis, Export, and Partitioning of the End Products of Photosynthesis (Andreas P.M. Weber, USA);
(15) Chlorophyll Synthesis (Robert D. Willows, Australia);
(16) Carotenoids (Abby J. Cuttriss, Joanna L. Mimica, Barry J. Pogson and Crispin A. Howitt, all from Australia);
(17) Lipid Synthesis, Metabolism and Transport (Peter Dörmann, Germany);
(18) Amino Acid Synthesis in Plastids (Muriel Lancien, Peter J. Lea and Ricardo A. Azevedo, from UK and Brazil);
(19) Sulfur Metabolism in Plastids (Elizabeth A.H. Pilon-Smits and Marinus Pilon, both from USA);
(20) Regulation and Role of Calcium Fluxes in the Chloroplast (Carl Hirschie Johnson, Richard Shingles and William F. Ettinger, all from USA);
(21) The Role of Plastids in Ripening Fruits (Florence Bouvier and Bilal Camara, both from France);
(22) Fate and Activities of Plastids During Senescence (Karin Krupinska, Germany);
(23) The Kleptoplast (Mary E. Rumpho, Farahad P. Dastoor, James R. Manhart and Jungho Lee, from USA and Korea);
(24) The Apicoplast (Soledad Funes, Xochitl Pérez-Martínez, Adrián Reyes-Prieto and Diego González-Halphen, all from Mexico);
(25) The Role of Plastids in Gravitropism (Maria Palmieri and John Z. Kiss, both from USA);
(26) Chloroplast Movements in Response to Environmental Signals (Yoshikatsu Sato and Akeo Kadota, both from Japan) and
(27) Oxygen Metabolism and Stress Physiology (Barry A. Logan, USA).

## A List of Selected Books

Volume 20 of the AIPH Series (Discoveries in Photosynthesis, edited by Govindjee, J.T. Beatty, H.Gest and J.F. Allen) contains a recently published time-line on oxygenic photosynthesis covering its many aspects, including research on the functional work on chloroplasts (see Govindjee and D. Krogmann (2004) "Discoveries in oxygenic photosynthesis (1727–2003): a perspective". Photosynth Res **80**: 15–57). In addition, this book contains a historical perspective by Andrew Staehelin: "Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes" (Photosynth Res **76**: 185–196, 2003).

Plastids have been at the heart of plant biology and several books have been written on them. Bob Wise, Ken Hoober and I have selected to list some of these books that have influenced research in the field of *plastids*. They are listed chronologically. From the students' point of view, the book by J.K. Hoober (1984), and by U.C. Biswal, B. Biswal and M.K. Raval (2003) are most suitable in providing a basic background of the field of plastids.

- T.W. Goodwin (ed.) (**1966**) Biochemistry of Chloroplasts, Volume 1. Proceedings of a NATO Advanced Study Institute held at Aberystwyth, UK, August, 1965. Academic Press, London and New York
- T.W. Goodwin (ed.) (**1967**) Biochemistry of Chloroplasts, Volume 2. Proceedings of a NATO Advanced Study Institute held at Aberystwyth, UK, August, 1965. Academic Press, London and New York
- J.T.O. Kirk and R.A.E. Tilney-Bassett (**1967**) The Plastids, their Chemistry, Structure, Growth and Inheritance. WH Freeman and Co., London
- B.E.S. Gunning and M.W. Steer (**1975**) Ultrastructure and the Biology of Plant Cells . Edward Arnold, London
- J. Barber (ed.) (**1976**) The Intact Chloroplast (Topics in Photosynthesis, Vol. 1) Elsevier Scientific Pub. Co., Amsterdam and New York
- G. Akoyunoglou and J-H. Argyroudi-Akoyunoglou (**1978**) Chloroplast Development: Proceedings of the International Symposium on Chloroplast Development held on the Island of Spetsai, Greece, July 9–15, 1978. Elsevier/North-Holland Biomedical Press, Amsterdam and New York
- J.T.O. Kirk and R.A.E. Tilney-Bassett (**1978**) The Plastids, their Chemistry, Structure, Growth, and Inheritance, 2nd edition. Elsevier/North Holland Biomedical Press, Amsterdam and New York
- J. Reinert (**1980**) Chloroplasts (in Results and Problems in Cell Differentiation, Vol. 10). Springer-Verlag, Berlin
- J.A. Schiff and H. Lyman (eds.) (**1982**) On the Origins of Chloroplasts. Elsevier/North-Holland, Amsterdam and New York
- M.A. Tribe and P. Whittaker (**1982**) Chloroplasts and Mitochondria (Institute of Biology's Studies in Biology, No. 31), 2nd edition. Edward Arnold, London
- N.R. Baker and J. Barber (eds.) (**1984**) Chloroplast Biogenesis. Elsevier Science Pub. Co., Amsterdam
- B. Halliwell (**1984**) Chloroplast Metabolism: The Structure and Function of Chloroplasts in Green Plants. Clarendon Press, New York and Oxford University Press, Oxford
- J.K. Hoober (**1984**) Chloroplasts. Plenum Press, New York
- J.R. Ellis (ed.) (**1984**) Chloroplast Biogenesis (Seminar Series/Society for Experimental Biology, No. 21) Cambridge University Press, Cambridge and New York
- J.H. Argyroudi-Akoyunoglou and H. Senger (**1999**) The Chloroplast: From Molecular Biology to Biotechnology. Kluwer Academic Publishers (now Springer), Dordrecht
- U.C. Biswal, B. Biswal and M.K. Raval (**2003**) Chloroplast Biogenesis from Proplastid to Gerontoplast. Kluwer Academic Publishers (now Springer), Dordrecht
- S.G. Moller (ed.) (2004) Plastids (in Annual Plant Reviews, Vol. 13). Blackwell Publishing, Oxford, U.K.

## A Useful Compact Disc (CD)

A very useful CD that should be helpful to students of "Plastids" is*: Plant Cell Biology on CD, Part 1* (ISBN 0-9751682-0-7); it was produced by Brian Gunning, of the Research School of Biological Sciences, Australian National University, Canberra, Australia. It is a two-CD set containing more than 1000 images covering an introduction to plant cells, mitochondria, plastids and peroxisomes, designed as a source of information for students and a resource for teachers. It is probably the largest collection of images of plastids ever assembled for these purposes, including most kinds of light and electron microscopy, diagrams and numerous time-lapse movies, organized in

a user-friendly menu-driven interface. Further details are available at: www.plantcellbiologyonCD.com.

## Future AIPH Books

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

- *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications* (Editors: Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger and Hugo Scheer);
- *Photosystem I: The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase* (Editor: John Golbeck);
- *Biophysical Techniques in Photosynthesis. II* (Editors: Thijs J. Aartsma and Jörg Matysik);
- *Photosynthesis: A Comprehensive Treatise; Physiology, Biochemistry, Biophysics and Molecular Biology, Part 1* (Editors: Julian Eaton-Rye and Baishnab Tripathy); and
- *Photosynthesis: A Comprehensive Treatise; Physiology, Biochemistry, Biophysics and Molecular Biology, Part 2* (Editors: Baishnab Tripathy and Julian Eaton-Rye)

In addition to these contracted books, we are already in touch with prospective Editors for books on the following topics:

- *Molecular Biology of Cyanobacteria. II*
- *Protonation and ATP Synthases*
- *Genomics and Proteomics*
- *Anoxygenic Photosynthetic Bacteria. II*
- *Sulfur Metabolism in Photosynthetic Systems*
- *Global Aspects of Photosynthesis and Respiration*
- *Molecular Biology of Stress*
- *Artificial Photosynthesis*
- *Chloroplast Bioengineering*

**Readers are encouraged to send their suggestions for future volumes (topics, names of future editors, and of future authors) to me by email (gov@uiuc.edu) or fax (1-217-244-7246).**

In view of the interdisciplinary character of research in photosynthesis and respiration, it is my 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 and Biophysics, but also in Bioengineering, Chemistry and Physics.

## Acknowledgments

First of all, I thank Achim Trebst as his emails have been a constant source of inspiration to continue our projects. I take this opportunity to thank Ken Hoober and Bob Wise for their outstanding and painstaking editorial work for this volume. I also thank Brian Gunning and Friederike Koenig for their excellent contributions to the "Dedication", and most importantly to all other 56 authors of volume 23: without their authoritative chapters, there would be no such volume.

I owe Jacco Flipsen and Noeline Gibson (both of Springer) special thanks for their friendly working relation with us that led to the production of this book. Thanks are also due to Jeff Haas (Director of Information Technology, Life Sciences, University of Illinois at Urbana-Champaign, UIUC) and Evan DeLucia (Head, Department of Plant Biology, UIUC) for their support. Larry Orr constantly provides us with guidance regarding the rules and the format of our Series that started in 1994. All the members of my immediate family (my wife Rajni Govindjee; our daughter Anita, her husband Morten Christiansen, and our grand-daughter Sunita; our son Sanjay, his wife Marilyn, and our grandsons Arjun and Rajiv) have been very supportive during the preparation of this and other books in the AIPH Series. I also thank Arvind Sohal (of Techbooks, in New Delhi, India) and André Tournois (of Springer) for their contribution towards the production of this volume.

September 14, 2005

Govindjee
Series Editor, *Advances in Photosynthesis and Respiration*
University of Illinois at Urbana-Champaign,
Department of Plant Biology
Urbana, IL 61801-3707
email: gov@uiuc.edu;
URL: http://www.life.uiuc.edu/govindjee

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# Preface

The very origin of the plastid is clouded in mystery, even though the evidence is clear that the wide diversity of plastids is a historical consequence resulting from a single event, in which an evolving eukaryotic cell captured a photosynthetic prokaryotic cell. The product of this event—all algae and plants, the primary producers in all ecosystems—was one of the major outcomes of evolution. Core metabolic processes such as amino acid and fatty acid synthesis, and the ability to synthesize secondary products such as carotenoids, enzyme cofactors and antioxidants, were provided by the prokaryotic endosymbiont. ATP formation via oxidative phosphorylation was provided by another, earlier endosymbiont. The principal contributions of the eukaryotic nucleus, which was derived from an Archean organism, were the mechanisms of expression of the nuclear genome—nuclear transcription and cytosolic translation. Somehow, this latter organism was able to co-opt the genetic information of the respiratory and photosynthetic prokaryotes for its own use. Thus, the endosymbiotic events, first to produce the mitochondrion and then the plastid, were essential for the development of modern, complex organisms.

Most of the volumes in this series emphasize mechanisms related to photosynthesis or respiration. We all recognize that photosynthesis in the chloroplast is the most fundamental process in biology and provides the major foodstuff on which almost all organisms depend for survival. However, the plastid is, in addition, essential for many other processes, which are highlighted in this volume. For instance, the herbicide glyphosate, although not an inhibitor of photosynthesis, is highly toxic to all photosynthetic organisms. This is because glyphosate inhibits aromatic amino acid synthesis, an essential but non-photosynthetic function of plastids. We considered the ability of the plastid to provide these other, important functions worthy of attention. An astonishing feature of this volume is the phenomenal amount of research that has been done over the past several decades to understand these other, non-photosynthetic plastid functions.

It has been almost 40 years since John T.O. Kirk and Richard A.E. Tilney-Bassett published the first comprehensive treatment of plastid biology: "The Plastids: Their Chemistry, Structure, Growth and Inheritance", 1967, W.H. Freeman. While photosynthesis and chloroplasts (and indeed each of the topics covered in this volume) have received their due attention in the ensuing four decades, no single volume has brought together the amazing variety of plastid types and the remarkable diversity of plastid functions. We felt it was time for a modern synthesis of plastid biology and it was with this goal in mind we selected the topics and authors for this volume. We sought to produce a volume that is comprehensive without being encyclopedic; complete but readable; synthetic with even a touch of thought-provoking speculation, where warranted; a book that both beginning and experienced plant scientists will want to read, not one that will only reluctantly be referred to when absolutely necessary.

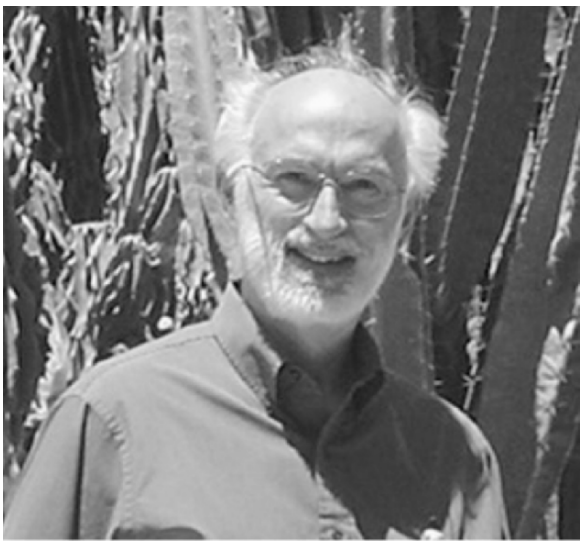
First, and foremost, we thank our 57 co-authors who have given their time and expertise to the writing of the chapters contained herein. Without them this volume would not have been possible. (For details, see the *Table of Contents* and *From the Series Editor*.) We hope that the scope of this volume will provide a framework in which new studies of the plastid can be launched and anyone interested in research in plastid biology would do well to start with the information provided by these authors. We also wish to thank senior series editor Govindjee as his efforts and vision have been instrumental in bringing this project to completion.

Robert R. Wise
Department of Biology
University of Wisconsin, Oshkosh, WI 54901
Telephone: 1-920- 424-3404
Fax: 1-920- 424-1101
Email: wise@uwosh.edu

J. Kenneth Hoober
School of Life Sciences
Arizona State University
Tempe, AZ 85287
Fax: 1-480-965-6899
Email: khoober@asu.edu

**Robert R. Wise**

**Bob Wise** is a professor of plant physiology at the University of Wisconsin at Oshkosh. His research interests are the biochemistry and bioenergetics of plant stress and plant cell ultrastructure. He graduated from the University of Wisconsin at Stevens Point in 1977 with a B.Sc. in Biology. It was there under the direction of Joseph B. Harris he was introduced to the world of plant cell ultrastructure and began a life-long interest in all matters plastidal. Discovering, and devouring, J.T.O. Kirk and R.A.E. Tilney-Bassett's 1967 seminal volume "The Plastids" was a decisive experience in his undergraduate education. In 1986 he earned his Ph.D. from the Botany Department of Duke University, Durham, North Carolina. For his dissertation he studied reactive oxygen species generation during chilling in the light under the direction of the late Aubrey W. Naylor. Postdoctoral work at the University of Illinois at Urbana-Champaign in the laboratory of Donald R. Ort (funded by the United States Department of Agriculture and the McKnight Foundation) included studies of the bioenergetics of chilling stress, the pattern of carbon fixation in leaves during drought stress, and the diurnal regulation of photosynthesis under both laboratory and field conditions. He joined the Biology Department at the University of Wisconsin at Oshkosh in 1993 and has conducted research in and taught plant physiology, plant anatomy and electron microscopy. He very much enjoys teaching at every collegiate level from large, freshman-level biology lectures to small, advanced graduate level seminars. Both undergraduate and graduate students play large roles in his research program. He spent the 2001/2002 academic year on sabbatical in the laboratory of Thomas D. Sharkey in the Botany Department at the University of Wisconsin at Madison investigating the response of photosynthesis to high temperature stress and the central role played by the thylakoid membrane in the sensing and signaling of heat stress in laboratory and field-grown plants. That collaboration continues. Elucidating the proteome of heat-stress-induced plastoglobuli is a current focus of his research program. Further information can be found at his web page (http://www.uwosh.edu/departments/biology/wise/wise.html). He can be reached by e-mail at: wise@uwosh.edu.



**J. Kenneth Hoober**

**Ken Hoober** started his scientific career as an undergraduate in chemistry and then received a Ph.D. degree in 1965 in biochemistry from the University of Michigan in Ann Arbor. His graduate work involved characterization of unique proteins synthesized during differentiation of the epidermis of neonatal rats in the laboratory of Isadore Bernstein. He then spent 15 months as a postdoctoral fellow with Stanley Cohen at Vanderbilt University in Nashville, Tennessee, working on the biochemical effects of epidermal growth factor. During a 2-year postdoctoral fellowship at the Rockefeller University in New York City with Philip Siekevitz and George Palade, he began working on chloroplast development in the green alga *Chlamydomonas reinhardtii*, his first introduction to the chloroplast, photosynthesis and plants. He obtained his first faculty position in 1968 in the Department of Biochemistry at Rutgers Medical School in New Jersey. In 1971 he moved with his chairman, Gerhard Plaut, to the Department of Biochemistry at Temple University School of Medicine in Philadelphia. He rose through the ranks and in 1989 became chairman of the department. An exceptional opportunity in 1987 was the experience working with Diter von Wettstein, Gamini Kannangara and Simon Gough at the Carlsberg Laboratories in Copenhagen, Denmark, during which he contributed to elucidation of the mechanism of glutamate 1-semialdehyde aminotransferase. In 1991, he accepted an offer to become chair of the Department of Botany at Arizona State University in Tempe. Several years later the departmental name was changed to Plant Biology. Perhaps the justification of being offered this position was derived from his monograph *Chloroplasts* that was published by Plenum Press in 1984. Although he knew little about plants, writing this book formed the basis of his knowledge about the plastid. At Arizona State University, he was accepted as a member of the world-renowned Center for the Study of Early Events in Photosynthesis. As a result of interactions with members of the Center, he received a legitimate education in photosynthesis. His research has emphasized assembly of the major light-harvesting complexes as probes for biogenesis of thylakoid membranes. He was one of the first to characterize ribosomes of chloroplasts and describe their differences from cytosolic ribosomes, and was the first to establish that the apoproteins are synthesized on cytosolic ribosomes. He established that in *Chlamydomonas* the apoproteins are initially integrated into the membranes of the chloroplast envelope. The well-established need for chlorophyll *b* was revealed as a requirement for import and retention of the apoproteins in the chloroplast envelope and that, most significantly, chlorophyll *a* and chlorophyll *b* accept different structures in the protein as ligands during assembly of the chlorophyll-protein complexes. In 2004, he retired from the faculty but continued working as a Research Professor. As a scientist, he has cherished the opportunities to travel internationally to conferences and meet many of the other investigators with similar interests, some of whom are authors of chapters in this volume. Further information can be found on his web page (URL: http://sols.asu.edu/faculty/khoober.php). He can be reached by email at: khoober@asu .edu.

# A Dedication to Pioneers of Research on Chloroplast Structure

In his famous paper proposing that plastids had an endosymbiotic origin, Constantin Sergeevich Mereschkowsky (1855–1921) presented a delightful mind picture:

> Let us imagine a palm tree, growing peacefully near a spring, and a lion, hiding in the brush nearby, all of its muscles taut, with bloodthirsty eyes, prepared to jump upon an antelope and to strangle it. The symbiotic theory, and it alone, lays bare the deepest mysteries of this scene, unravels and illuminates the fundamental principle that could bring forth two such utterly different entities as a palm tree and a lion. The palm behaves so peacefully, so passively, because it is a symbiosis, because it contains a plethora of little workers, green slaves (chromatophores) that work for it and nourish it. The lion must nourish itself. Let us imagine each cell of the lion filled with chromatophores, and I have no doubt that it would immediately lie down peacefully next to the palm, feeling full, or needing at most some water with mineral salts.

(Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biol. Centralbl. **25**: 593–604, 1905; annotated English translation by W. Martin and K.V. Kowallik, Eur. J. Phycol. **34**: 287–295, 1999)

## The First Observations

Some historians have taken a discourse by the English physician Nehemiah Grew (1641–1712, Fig. 2, shown later) as a starting point of our knowledge of chloroplast structure. He read his communication *A Discourse on the Colours of Plants* to the Royal Society of London, UK, on May 3, 1677, telling how he extracted the green pigment of leaves with olive oil and noted its different colors when held up to a candle. However, it is another passage in his text that has aroused conjecture (see Fig. 1).

Did he see chloroplasts with the compound microscope that he used for his pioneering work on plant anatomy, and describe them as a "precipitate" in the cells? If he did, he did not elaborate when some five years later he compiled and republished revisions of his earlier books and lectures on anatomy (written between 1671–1677) in the form of his master work *The Anatomy of Plants, with an Idea of a Philosophical History of Plants* (1682, see No. 11 of The Sources of Science, Johnson Reprint Corporation, New York and London, 1965). Although this volume included magnificent drawings, many of which stand up well alongside the most modern scanning electron micrographs in their depiction of cellular patterns, there are no illustrations of intracellular components.

The claim of Grew's contemporary and correspondent, Anthony van Leeuwenhoek (1632–1723, Fig. 2), to be the first to describe chloroplasts (not, of course, using that word) is on more solid ground. He made his own single-lens microscopes in spare time from his trade as a draper and a job as Chamberlain with the town council, and over a period of 50 years starting in August 1673 sent a series of letters from his home in Delft, The Netherlands, to the Royal Society of London. Among his numerous discoveries, his "little animalcules" (the name Protozoa was not coined until 1817) are the best known. Clifford Dobell published a detailed account of van Leeuwenhoek's life and work in 1932, 300 years after his birth, having learned Old Dutch to do so (C. Dobell, *Anthony van Leeuwenhoek*

14. §. I ſuppoſe therefore, That not only *Green*, but all the *Colours* of *Plants*, are a kind of *Precipitate*, reſulting from the concurrence of the *Saline Parts* of the *Aer*, with the *Saline* and *Sulphurious Parts* of the *Plant*; and that the *Subalkaline*, or other like *Saline Part* of the *Aer*, is concurrent with the *Acid* and *Sulphurious Parts* of *Plants*, for the *Production* of their *Verdure*; that is, as they ſtrike altogether into a *Green Precipitate.*

*Fig. 1.* Extract from Chapter 1 of *A Discourse on the Colours of Plants* (from page 271 of *The Anatomy of Plants*).


*Fig. 2.* Nehemiah Grew (left) and Anthony van Leeuwenhoek (right). Source: Mikroskopie: Entwicklungen im 19. Jahrhundert und ihre Anfänge im 17. Jahrhundert at http://www.biologie.uni-hamburg.de/b-online/d01/01f.htm, accessed on June 15, 2005.

*and his Little Animals*, Swets and Zeitlinger, 1932). Letter number 6, dated 7 September, 1674, narrates how van Leeuwenhoek took water samples from the Berkelse Mere and found floating in it "some green streaks, spirally wound serpent-wise, and orderly arranged, after the manner of the copper or tin worms which distillers use to cool their liquors as they distil over. The whole circumference of these streaks was about the thickness of a hair of one's head . . . All consisted of very small green globules joined together: and there were many small green globules as well". There is little doubt that he had resolved the chloroplast of *Spirogyra*, and possibly the glistening starch sheaths around its pyrenoids (described again by Jean-Pierre Vaucher in 1803 and named as such by Friedrich Karl Johann Schmitz (1882, *Die Chromatophoren der Algen*, Max Cohen and Sohn, Bonn). It was the first structure to be recognized within a plant cell.

van Leeuwenhoek's next observation was incidental, but again it is plain that he saw chloroplasts, this time in a higher plant. In September 1678 many people in his home district were stricken with fever, and suspected a red dust that colored their shoes when they walked through grassy meadows, indicating, they thought, "infected, fiery air". van Leeuwenhoek examined the dust, which was probably spores of a rust-fungus, and saw that they came out of the grass and turned red upon exposure to the air ". . . whereas these same globules, when they lie enclosed in the pores are green". van Leeuwenhoek probably used the word "pores" in the same sense as his other correspondent at the Royal Society, Robert Hooke (1635–1703, *Micrographia*, Dover Publications (1961, New York) facsimile reproduction of the 1665 original). Certainly Dobell considered that the "pores" must have been leaf *cells,* and that van Leeuwenhoek had taken green chloroplasts to be precursors of the rust spores.

Long after Dobell's studies, a search of van Leeuwenhoek's letters stored in the Royal Society's strong room in London revealed that several were accompanied by packages of material. One such (letter of 17 October, 1687) was a sample of an algal mat which he had dried in front of his fire. Some of it retained its green color, and he had recorded seeing filaments with green globules *one sixth* [the volume] *of a globule of blood*, i.e., about 4 μm in diameter. When Brian Ford re-examined this historic material with modern techniques he found that the algae *Cladophora*, *Cosmarium*, *Vaucheria* and *Rhizoclonium*, as well as a number of diatoms, were still identifiable (*The Leeuwenhoek Legacy*, Biopress and Farrand Press, Bristol and London, 1991). Again it is apparent that van Leeuwenhoek had seen algal chloroplasts.

## Discovery of Grana and Stroma

Physiology advanced relative to microscopy in the 18th century, and few discoveries relating to the structure of chloroplasts were recorded. Two examples that have come down to us are Bonaventura Corti's (1729–1813) well known observation of green particles streaming around *Chara* cells (1774), and Andrea Comparetti's (1745–1801) study of *green grains in plant cells*, in

which he saw grains of starch (page 11, *Prodromus de fisica vegetabile*, Padua, 1791) (quotation from A. Trécul, cited later).

Ludolph Christian Treviranus (1779–1864), Charles Francois Brisseau de Mirbel (1776–1854), Kurt Polycarp Joachim Sprengel (1766–1833), George Wahlenberg (1781–1851), Johann Heinrich Friedrich Link (1767–1851) (who isolated and chemically analyzed starch grains), Pierre Jean François Turpin (1775–1840), Johann Jacob Paul Moldenhawer, Henri Dutrochet (1776–1847, proponent of the cell theory considerably before Schleiden and Schwann) and François Vincent Raspail (1794–1878, credited with founding the discipline of histochemistry) were among those who described green corpuscles in plants in the early decades of the 19th century. Brief biographies of many of these pioneers are given by Henry Harris in *The Birth of the Cell* (Yale University Press, New Haven, 1999). Even in this early period there were some indications that the "green corpuscles" might be complex structures. Thus Treviranus may have detected particles within chloroplasts (see T.E. Weier, cited below). Also, following his first descriptions in 1807 and 1814, Link wrote that the "green corpuscles are sometimes composite, that is to say, large corpuscles sometimes contain smaller ones . . . " (para 44, *Grundlehren der Kräuterkunde*, Berlin, 1837, translated from A. Gris, cited below).

Link's extraordinary pupil, Franz Julius Ferdinand Meyen (1804–1840) also saw composite green corpuscles. He graduated in medicine in 1826, published books on algae and plant anatomy (*Phytotomie*, Haude and Spener, 1830), then took the advice of Alexander von Humboldt (after whom the famous Humboldt Foundation is named) and voyaged around the world for three years—just ahead of that other voyager, Charles Darwin. On his return to Germany he published a multivolume account of his discoveries, including first descriptions of the *Radiolaria* and still-cited articles on amphibians of South America and Borneo and plants of China and Oahu. He became a Professor in Berlin in 1834 and resumed studies of plant anatomy while carrying on his professorial duties as well as practicing medicine. Between then and his early death in 1840 he published books on plant physiology (3 volumes), secretion, plant geography, cultivated plants, embryology and fruiting in plants, and plant pathology—an astonishing output. In between he gave *Saccharomyces* its name and engaged in fierce arguments with Matthias Jakob Schleiden (1804–1881) on cell theory and the chemist Justus von Liebig (1803–1873) on plant physiology and the chemistry of humus (see P. Werner and F.L. Holmes, *Justus Liebig and the Plant Physiologists*. J. History of Biol. **35**: 421–441, 2002). Set against this frenzied activity his contribution to the world of chloroplasts may seem minor, yet in his book *Phytotomie* he too reported chlorophyll granules or corpuscles. The name "chlorophyll" was by then in use, having been coined in 1818 by the French pharmacists Pierre-Joseph Pelletier (1788–1842) and Joseph Bienaimé Caventou (1795–1877). Of more significance, in his 3-volume *Neues System der Pflanzenphysiologie* (1837; Haude and Spener, Berlin), Meyen described cases in which chlorophyll grains appeared as bodies with dark spots on a light background (reported by T. Elliott Weier in Cytologia **7**: 504–509, 1936 and Bot. Rev. **4**: 497–530, 1938). Further, in respect of *Vallisneria*, he wrote about the existence of a substratum for the green component (reported by Arthur Gris, cited below). His early glimpse of inhomogeneity of chloroplast contents (setting aside starch grains) preceded the now familiar nomenclature of grana and stroma by nearly fifty years.

Hugo von Mohl (1805–1872; Fig. 3) was another medical graduate who turned to natural science and made many contributions to the study of plant cells, including descriptions of cell division and his introduction of the word "protoplasm" (1846, independently of Purkinje's use of the word in 1840). Earlier he had provided detailed descriptions of "Chlorophyllkörner" or "chlorophyll granules" (*Untersuchungen über anatomische Verhältnisse des Chlorophylls*. Dissertation, W. Michler, University of Tübingen, Germany, 1837), and in due course presented them as components of the protoplasm. He extracted the green pigment from them and examined the residue with a variety of stains, concluding that it was a vesicular, proteinaceous material in which the chlorophyll was somehow distributed non-uniformly (H. von Mohl, *Über den Bau des Chlorophylls* Bot. Zeit. **13**: 89–99, 105–115, 1855). It was not clear, however, whether the pigmented spots had something to do with starch deposition or were the result of pathological changes. There followed prolonged debates on heterogeneity versus homogeneity, and if the former, whether the chlorophyll was randomly distributed, or in fibrils, granules or vesicles.

Two contributions in French interrupted the otherwise almost complete domination of the field by German microscopists. Both gave valuable surveys of the earlier literature, and both authors saw minute spots of concentrated pigment in chloroplasts. Jean Baptiste Arthur Gris (1829–1872) (*Recherches microscopiques sur la chlorophylle*, Ann. Sci. Nat. Bot. Ser **IV**, **7**: 179–219, 1857) looked at many species, e.g., the shade orchids *Phajus* (with grains of chlorophyll that are *very*

*Fig. 3.* Hugo von Mohl (top left; from http://www.biologie.uni-hamburg.de/b-online/e01/01f.htm), Nathanael Pringsheim (top right; provided by by E. Höxtermann), Andreas Franz Wilhelm Schimper (bottom left; scanned in from K. Mägdefrau: Geschichte der Botanik, Gustav Fischer Verlag Stuttgart 1973, page 221) and Constantin Sergeevich Mereschkowsky (bottom right; provided by E. Höxtermann).

*granular*) and *Acanthophippium* (with *finer granules* in the chlorophyll grains), and *Colocasia* (with *chlorophyll grains containing granules, some mobile, some immobile*). He decolorized *Phajus* chloroplasts with ether and was still able to see internal granulation, and went on to conclude that chlorophyll grains are *albumino-graisseux* in nature, i.e., lipoprotein in modern parlance. Some of his illustrations (monochrome) are reproduced in Fig. 4. Auguste Adolphe Lucien Trécul (1818–1896) (*Des formations vésiculaires dans les cellules végétales*, Ann. Sci. Nat. Bot. Ser IV, **8**: 20–163, 205–382, 1858) reviewed all known categories of "vesicle" at great length, including chlorophyll and starch grains. He described chlorophyll grains that contain *granular points of stronger color*. Like Gris, he published exquisite drawings of chloroplasts and chromoplasts (but in color); some of the former are reproduced in Fig. 4. Schimper (see later) described Trécul's work as the first thorough study in the field. The current state of knowledge did not allow Gris or Trécul to attach any significance to the deeply pigmented spots they saw in chlorophyll grains, and their work has not received as much credit as the later studies of Schimper and Meyer, both of whom were able to place their observations in a broader context, and who are heavily cited because they introduced new and useful terminology.

Trécul and many others employed the word *vesicle* (vésicule, Bläschen) as a convenient diminutive. Nowadays it implies a small intracellular compartment with a bounding membrane but at first it referred simply

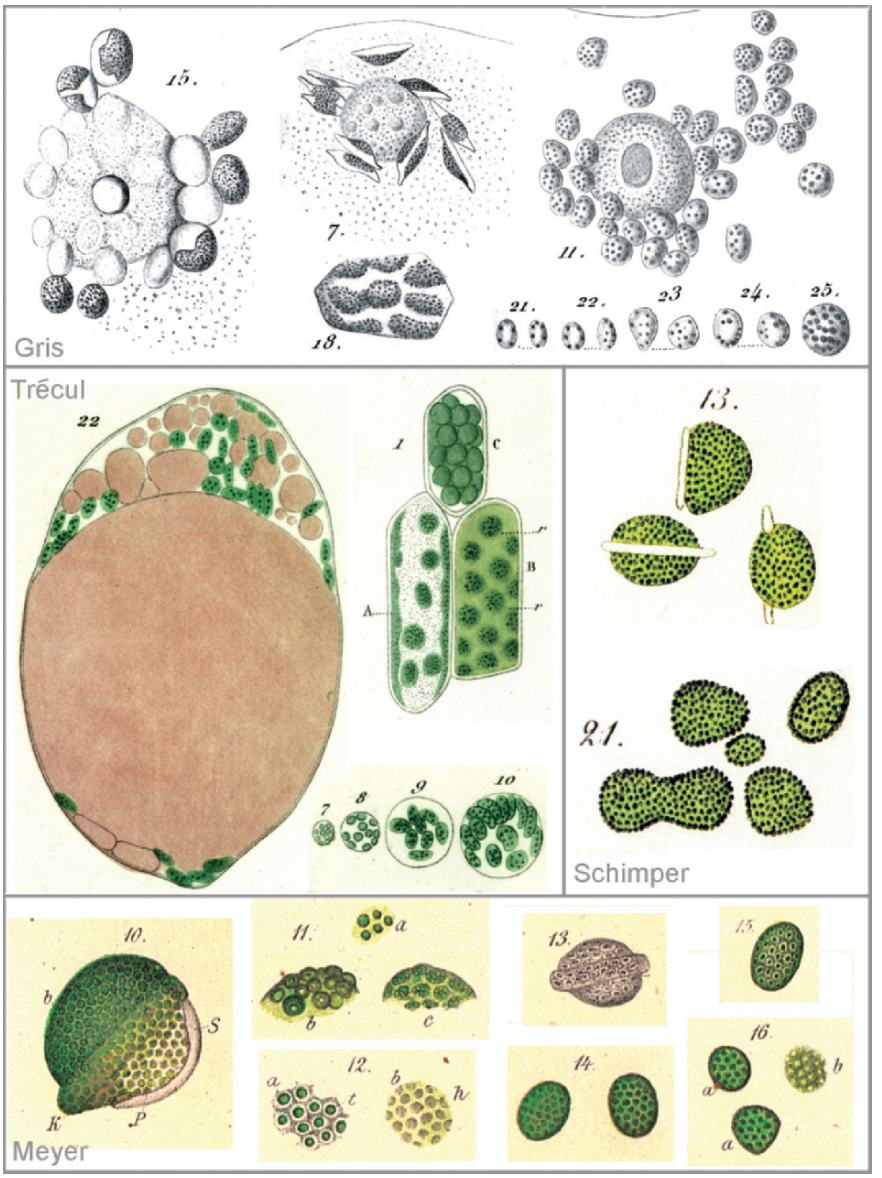



*Fig. 4.* Grana in chloroplasts, 1857–1885. (Top): from A. Gris (1857, see text for details); 15—*Solanum tuberosum* stem cell with nucleus surrounded by "spheres coated with green granular material"; 7—*Phajus grandiflorus*, cell from green bulb; 18—*Hydrangea hortensia* mesophyll; 11—*Vanilla planifolia* leaf cell; 21–25—stages of chloroplast development in *Sempervivum tectorum*. (Center left): from A. Trécul (1858, see text for details); 22—*Solanum nigrum* fruit cell; 1—*Lepanthes cochlearifolia*, stages of development A-B-C, with cell B showing "more granular" green bodies; 7–10—developmental stages of young fruit of *Solanum nigrum* (22). (Center right): from Schimper (1885, see text for details); 13—the orchid *Goodyera*; 21– the moss *Polytrichum*, described as having "prominent grana". (Bottom): from Meyer (1883, see text for details); 10–15 *Acanthophippium*; 16—*Vallisneria*; 10—chloroplast from green tuber; k crystalloid, s starch, p granum (dated 1 April 1882); 11—successive stages of swelling of grana in water; 12—effects of "Chlorallösung" (the clearing agent chloral hydrate) on grana; 13—after extraction with alcohol; 14—chloroplasts from leaf tissue; 15—grana in leaf chloroplast, swollen by uptake of water; 16—a) internal planes of focus, b) surface focus.

to a distinctive, observable unit which could, according to context, be what was later termed a "cell" or else something from which a cell might develop. It was much used by another pioneer of chloroplast biology, Carl Wilhelm von Nägeli (1817–1891), who made extensive studies of starch grains and developed the concept of growth by interpolation of new molecules between existing ones (intussusception). He also detected a distinct surface component of chlorophyll grains and provided the first documentation of

their division (1846), using cells of *Nitella* and other algae. Carl Sanio (1832–1891) extended Nägeli's observation on division of chloroplasts to flowering plants in 1864, though the process had been seen in mosses, hornworts and horsetails earlier. Sanio is better known for elucidating the origin and activity of the cambium; his description (without any illustrations) of chloroplast division appeared more or less as an aside in a study of phloem development (Bot. Zeit. **22**: 193–200, 1864). There are remarkably few, if any, subsequent descriptions of chloroplast division in higher plants until 1936 (Seikan Kusunoki and Yoshio Kawasaki, Cytologia **7**: 530–534, 1936). Division of chloroplasts remains as one of the most fundamental processes in the life of plants, yet for higher plants it has very seldom been observed in the sense of continuous monitoring, though of course presumed stages have been photographed and molecular aspects analyzed.

The great physiologist and historian, of the plant sciences, Julius Sachs (1832–1897) was another who extracted chlorophyll, commenting that it can be removed without altering the dimensions of the ground substance, which remains as a solid, minutely vacuolated body (see Conway Zirkle, *The structure of the chloroplast in certain higher plants*, Amer. J. Bot. **13**: 301–341, 1926). In his *History of Botany 1530–1860* (Russell and Russell, New York, 1890, reissued 1967) Sachs pointed out that many advances in microscope technique were made by the biologists of the mid 19th century, notably von Mohl and von Nägeli, and that those who came after were able to build upon these advances. Thus it was that many key publications appeared in the period 1881–1885, which should have, but did not, settle the questions that had arisen about the distribution of chlorophyll in chloroplasts.

Nathanael Pringsheim (1823–1894, Fig. 3) extended the experiments on extraction of chlorophyll that had been done by Gris, von Mohl and Sachs. He described the pigment-free residue as a spongy or trabecular structure whose meshes are normally occupied by the chlorophyll (*Ueber Lichtwirkung und Chlorophyllfunction in der Pflanze*, Jahrb. Wiss. Bot. **12**: 288–437, 1881). One year later he gave us the word "stroma": "*Ich habe ferner gezeigt, dass die farblose Grundmasse, die ich ein für alle Mal als das Gerüst (**Stroma**) der Chlorophyllkörper bezeichnen will, eine schwammartige Structur besitzt . . .*" (*Ueber Chlorophyllfunction und Lichtwirkung in der Pflanze*, Jahrb. Wiss. Bot. **13**: 377–490, 1882).

Next, Andreas Franz Wilhelm Schimper (1856–1901, Fig. 3), who in his later career became better known as a plant geographer, encapsulated much preceding work by establishing a logical nomenclature based on the Greek word *Plastikos*, meaning formed, or moulded (*Ueber die Entwickelung der Chlorophyllkörner und Farbkörper*, Bot. Zeit. **41**: 105–112, 121–130, 137–146, 153–162, 1883). He wrote: "*Ich werde sie als Plastiden bezeichnen, und zwar nenne ich die Chlorophyllkörper Chloroplastiden, die Stärkebildner und alle hierher gehörigen farblosen Gebilde Leukoplastiden und die Farbkörper Chromoplastiden*". This sentence (page 108) follows an introductory review of preceding observations and in it Schimper names the green types *Chloroplastiden*, the colorless types (usually with starch) *Leukoplastiden*, and the non-green colored types as *Chromoplastiden*. The category of plastid that we now know as amyloplasts was not separated as a subset of Schimper's "Leukoplastiden" until much later. Schimper followed up with a book-sized issue of the Jahrbücher für Wissenschaftliche Botanik, *Untersuchungen über die Chlorophyllkörper und die ihnen homologen Gebilde*, **16**: 1–247, 1885, including five plates, two of which are in color. His Plate 3 is readily available in the form of the frontispiece of J.T.O. Kirk and R.A.E. Tilney-Bassett's comprehensive book *The Plastids* (1967; Freeman, New York). Two of his figures, showing chloroplasts with grana, are reproduced in Fig. 4.

Schimper's other major contribution was to see that large plastids (chloro- and chromo-) develop from small leucoplasts in egg cells and meristematic cells, and in essence to propound the genetic continuity of plastids and the absence of their *de novo* formation in the protoplasm. In these conclusions he concurred with Friedrich Schmitz (cited above in connection with van Leeuwenhoek), who had made continuous observations of chloroplast division and development from spores and zygote stages in algae. In 1893, Andrei Sergeevitch Famintzin (1835–1918), one of the founders of Russian plant physiology, demonstrated that "Chlorophyllkörner" persist as small, shriveled structures in seeds, and that those in the seedlings develop from them, further establishing the uninterrupted continuity of plastids (*Über Chlorophyllkörner der Samen und Keimlinge*, Mélanges biologiques. T. **XIII**. St. Petersburg, 1893). Confirmation that chloroplasts can carry genetic traits awaited the rediscovery of Mendel's results and subsequent work of Erwin Baur (1875–1933) on non-Mendelian inheritance in the *albomarginata* form of *Pelargonium zonale* (Z. Indukt. Abstamm. Vererbungsl. **1**: 330–351, 1909). Moreover Schimper surmised that chloroplasts might be symbionts, though this was merely an aside written in a footnote in his 1883 paper (page 112, see W. Martin

and K.V. Kowallik, Eur. J. Phycol. **34**: 287–295, 1999). The idea was later developed in detail by Constantin Sergeevich Mereschkowsky (1855–1921, Fig. 3) (Biol. Centralblatt **25**: 593–604, 1905—for translation see W. Martin and K.V. Kowallik, cited above and quoted at the very beginning of this *dedication*—and Biol. Centralblatt **30**: 278–303, 321–347, 353–367, 1910).

Knowledge of another class of cellular inclusion, chondriosomes (one of many names for them), was growing during this period, and some of the ideas on genetic continuity of plastids became clouded by uncertainty about the distinctions between mitochondria (as they came to be called) and small leucoplasts in meristematic cells. Conway Zirkle (1895–1972) clarified this situation and distinguished "plastid primordia" from mitochondria (Amer. J. Bot. **14**: 429–445, 1927). Eventually Siegfried Strugger (1906–1961) (Naturwiss. **37**: 166–167, 1950) applied the term "proplastid", signifying a sub-category of leucoplast which can multiply in dividing cells and mature into other forms of plastid when cell progeny leave meristems and differentiate. Pierre Dangeard (1862–1947) expressed the relatedness of members of the plastid family by classifying them as part of the overall "plastidome" (Comptes Rendus Acad. Sci. **169**: 1005–1009, 1919)—an early use of the currently fashionable "-ome" suffix.

In his 1885 publication Schimper described chloroplasts as a colorless stroma with numerous "vacuoles" filled with green, viscous substance. He saw this basic organization in chloroplasts of flowering plants, pteridophytes and bryophytes, and was able to refer to the green components as "grana", this new term having been introduced by Arthur Meyer (1850–1922) in 1883. Meyer's critical review, including many black and white and colored figures on three plates, discussed observations on "Autoplasten" (= chloroplasts); "Chromoplasten" (= chromoplasts); "Trophoplasten" (= reserve-storing plastids); and "Anaplasten" (= leucoplasts) (*Das Chlorophyllkorn in chemischer, morphologischer und biologischer Beziehung. Ein Beitrag zur Kenntnis des Chlorophyllkornes der Angiospermen und seiner Metamorphosen*, pp. 1–91. Leipzig: Arthur Felix). On page 24 (lines 6–10, para 2), in relation to observations on the shade plant *Acanthophippium*, he defines "Grana" as follows: "*Es wäre die Klärung dieses Punktes von Wichtigkeit, weil im Falle der Farblosigkeit der Grundsubstanz die Auffassung gerechtfertigt erschiene, dass die grünen Körner, welche wir der Kürze halber* '***Grana***' *nennen wollen, aus unserem Chlorophyll beständen, und dass dieses in der übrigen Masse des Autoplasten nicht weiter vorkäme*". Here, he gives the green bodies he has seen the name *Grana* "for convenience". His drawings clearly show grana (e.g., Fig. 11–16 on his plate 1, reproduced in Fig. 4). He demonstrated that they are constitutive components of chloroplasts, and not transient products of metabolism.

Schimper's terminology soon became widely accepted, for instance in his widely-used textbook *Das kleine botanische Practicum* (Gustav Fischer, Jena, 1884). Eduard Strasburger lists the three main categories of plastid ("Chromatophoren") under their former and new names, and points out that they are closely related and can change into one another: "*Wir können alle drei Gebilde als Chromatophoren zusammenfassen und weiter die Chlorophyllkörper, Farbkörper und farblosen Stärkebildner als Chloroplasten, Chromoplasten und Leukoplasten unterscheiden. Diese Gebilde sind nahe verwandt und können ineinander übergehen*". His three terms (*Chloroplasten*, *Chromoplasten* and *Leukoplasten*) are close to our modern terminology, but the leucoplasts of that time included categories which we now distinguish as proplastids (see above), amyloplasts and etioplasts (this term was introduced by J.T.O. Kirk and R.A.E. Tilney-Bassett in *The Plastids*, cited above, page 64), with leucoplasts as currently understood being a collective term for a multitude of different sizes and shapes of plastid which have in common the attributes of little or no pigment and occurrence in non-dividing cells, but still with the ability to re-differentiate into other forms of plastid.

## Towards the Modern Era

Despite the clarity with which grana and stroma had been demarcated by 1885, a long period of comparative stagnation ensued. A view of the cell as a unit containing homogeneous colloidal material gained the upper hand, and in textbooks up to 1935, chloroplasts were generally regarded as homogeneous too. Grana were relegated to the status of artefacts arising from preparation methods (for accounts of this period, see Conway Zirkle (Amer. J. Bot. **13**: 301–341, 1926) and Eugene Rabinowitch, *Photosynthesis and Related Processes,* **1:** Chapter 14, 1945, Interscience Publishers, New York).

The climate of opinion concerning grana began to change in the mid-1930s. J. Doutreligne produced the first photomicrographs of grana in chloroplasts in 1935 (*Note sur la structure des chloroplastes*, Proc. Kon. Ned. Akad. Weten. Amst. **38**: 886–896, 1935). It seems strange that it should have taken so long for photomicrography to be employed in this field—its first

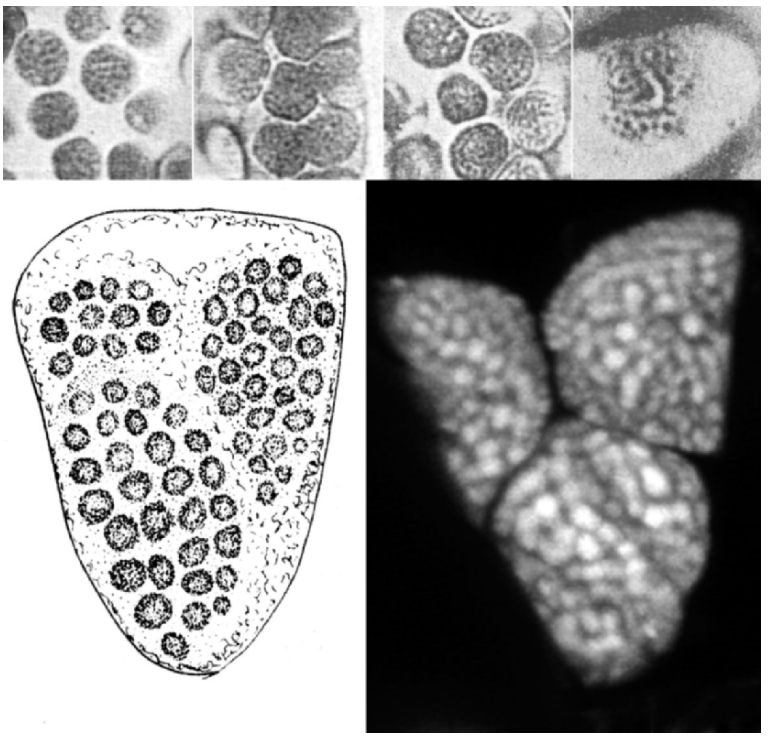


*Fig. 5.* Top row, first 3 photographs: a selection of the first photomicrographs of grana in chloroplasts, from Doutreligne (1935, see text for details); left to right: *Vallisneria*, *Mnium* and *Cabomba*, respectively; top far right photograph: part of a chloroamyloplast from *Pellionia*, with grana displayed in a very thin film over the surface of a starch grain, from Jungers and Doutreligne (1943, see text). These micrographs are copied here at approximately the same magnification as in the original papers, the image of *Pellionia* is at a magnification of 1400x. Lower left: A mesophyll cell of the shade plant *Pellionia* containing three chloroplasts with large grana (Fig. 4 from Weier 1938, see text); Lower right: a confocal fluorescence micrograph of a similar *Pellionia* cell by one of us (BG).

use in tissue anatomy was no less than 90 years earlier (O. Breidbach, *Representation of the microcosm—the claim for objectivity in 19th century scientific microphotography*. J. History Biol. **35**: 221–250, 2002). Doutreligne's images were especially convincing because she used colored filters to vary the contrast between the grana and the stroma, and because they included living photosynthetic tissue of water plants, free of potential damage from preservatives. She published another paper during the war (*Sur la localisation de la chlorophylle*, La Cellule **49**: 407–418, 1943) which included a perceptive footnote saying it is *piquant* to note that the combination of achromatic objective (with low numerical aperture) and eyepiece lenses used by some proponents of homogeneous chloroplasts would have been incapable of resolving objects smaller than 0.8 μm, which could well have contributed to their failure to see grana; she herself used a much better objective lens of numerical aperture 1.32.

In the year after Doutreligne's paper appeared, Emil Heitz (1892–1965, discoverer of heterochromatin) submitted two papers (Ber. Dtsch. Bot. Ges. **54**: 362–368, 1936 and Planta **26**: 134–163, 1937) containing unequivocal micrographs from very many species, and including "face" and "side" views of grana that led him to include the words "oriented chlorophyll-discs" (*Gerichtete Chlorophyllscheiben*) in his titles. He added an Addendum to his Planta paper in which he confirmed the appearance of grana as disc-shaped in living material using fluorescence microscopy. In the same year T.E. Weier (Cytologia **7**: 504–509) compared living and preserved (by various methods) chloroplasts and amyloplasts of the shade plant *Pellionia*, confirming the existence of grana (see Fig. 5). His other paper in 1936 (Amer. J. Bot. **23**: 645–652) contained equally convincing views of grana in beet chloroplasts, but he still allowed that healthy plants could have both grana-containing and homogeneous chloroplasts.

Other approaches had been used before the images described above removed doubts about the reality of grana. Birefringence of chloroplasts was discovered by G.W. Scarth in 1924 and was further investigated by others, including A. Frey-Wyssling and W. Menke, in the 1930s. Infiltration with glycerol abolished

the birefringence, indicating that it arises from structural layering. Menke and H.-J. Küster elaborated by studying dichroism of gold impregnated chloroplasts, inferring the existence of submicroscopic laminae (Protoplasma **30**: 283–290, 1938), and Menke and E. Koydl succeeded with ultra-violet microscopy to visualize them directly (Naturwiss. **27**: 29–30, 1939). Almost concomitantly G. Kausche and H. Ruska obtained the first electron micrographs of whole mounts (Naturwiss. **28**: 303–304, 1940), with further progress having to await the end of the war and development and application of more refined methods of specimen preparation such as vacuum drying and shadow casting (S. Granick and K. Porter, Amer. J. Bot. **34**: 545–555, 1947; E. Steinmann, Exper. Cell Res. **3**: 367–372, 1952), osmic acid fixation and ultra-thin sectioning (G. Palade, cited in Granick (1955, reference below), 1952; J.B. Finean, E. Steinmann and F.S. Sjöstrand, Exper. Cell Res. **5**: 557–559, 1953; E. Steinmann and F.S. Sjöstrand, Exper. Cell Res. **8**: 15-23, 1955), and the advent of glutaraldehyde as a fixative for chloroplasts (B. Gunning, J. Cell Biol. **24**: 79–93, 1965). The architectural details of the membranes in grana were elucidated by W. Wehrmeyer (Planta **62**: 272–293, 1964), J. Heslop-Harrison (Sci. Prog. Oxf. **54**: 519–541, 1966) and D. Paolillo (J. Cell Sci. **6**: 243–255, 1970) using a variety of techniques of electron microscopy and reconstruction. The early literature of electron microscopy of chloroplasts is well covered by S. Granick (Encyclopedia of Plant Physiology **1**: 507–564, 1955, Springer, Berlin), and L.A. Staehelin takes the story forward to the supra-molecular architecture of thylakoids (Photosynth. Res. **76**: 185–196, 2003).

It is appropriate to end this dedication to some of the pioneers of chloroplast structure by honoring Wilhelm Menke, with whom one of us (FK) worked for several years. It is he who, in 1961, coined the term "thylakoids" for "membrane sacs" (*Über die Chloroplasten von* Anthoceros punctatus. Z. Naturforsch. **16b**: 334–336). He wrote: "*Da die Beschreibung der Lamellar-Struktur der Chloroplasten mit Hilfe des bisher verwendeten Begriffs* '*in sich geschlossene Doppellamellen*' *umständlich ist, und weil diese morphologisch und funktionell bedeutsamen Strukturelemente wohl eine eigene Bezeichnung verdienen, werde ich sie in Zukunft Thylakoide nennen*". In English (as translated freely), it is: "Since the term 'self-contained double lamellae' which is currently used to describe the lamellar structure of the chloroplasts is long and complicated and since these morphologically and functionally important structural elements may well deserve a proper term, I will call them in future 'thylakoids', an expression coming from the Greek language meaning sac-like." (Also see W. Menke (1962) *Structure and chemistry of plastids*. Annu. Rev. Plant Physiol. **13**: 27–44; W. Menke (1990) *Retrospective of a botanist*. Photosynth. Res. **25**: 77–82).

## Acknowledgments

We thank Bob Wise and Ken Hoober for inviting us to present this historical dedication and Ekkehard Höxtermann for help with the Pringsheim papers and for portraits of Pringsheim and Mereschkowsky. BG thanks the Plant Cell Biology Group, RSBS, Australian National University for a Visiting Fellowship to enable him to work in the Group in an Emeritus capacity. G thanks the University of Illinois at Urbana-Champaign for library and office facilities.

Brian Gunning (Corresponding author)
Research School of Biological Sciences
Australian National University
Canberra, ACT 2601, Australia
email: brian.gunning@anu.edu.au

Friederike Koenig
Department of Biology and Chemistry
University of Bremen
28334 Bremen, Germany
email: fkoenig@uni-bremen.de

Govindjee
Department of Plant Biology and
Department of Biochemistry
University of Illinois at Urbana-Champaign
Urbana, IL 61801
email: gov@life.uiuc.edu

# Color Plates

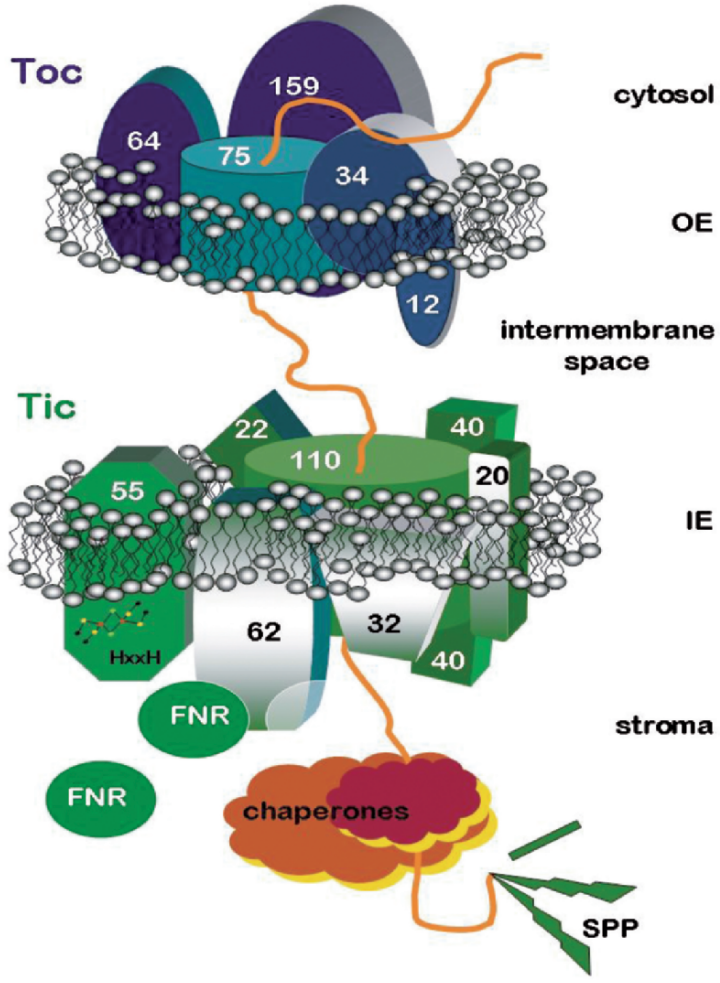



*Fig. 1.* Schematic depiction of the chloroplast protein translocon. The majority of nuclear-encoded plastid proteins are synthesized with an N-terminal cleavable presequence. These proteins translocate into the organelle via two proteinaceous heteromeric protein complexes, the Toc and Tic translocons. Components of these complexes are denoted by their molecular weight. After recognition of the precursor proteins by the receptor proteins of the Toc translocon, the translocation occurs simultaneously across the outer and the inner envelope membrane. Chaperones assist the translocation process at the outside as well as from within the organelle. Once the N-terminus of the precursor protein has entered into the stroma the presequence is cleaved off by a soluble enzyme called the stromal processing peptidase (SPP). (See Chapter 3, p. 55.)

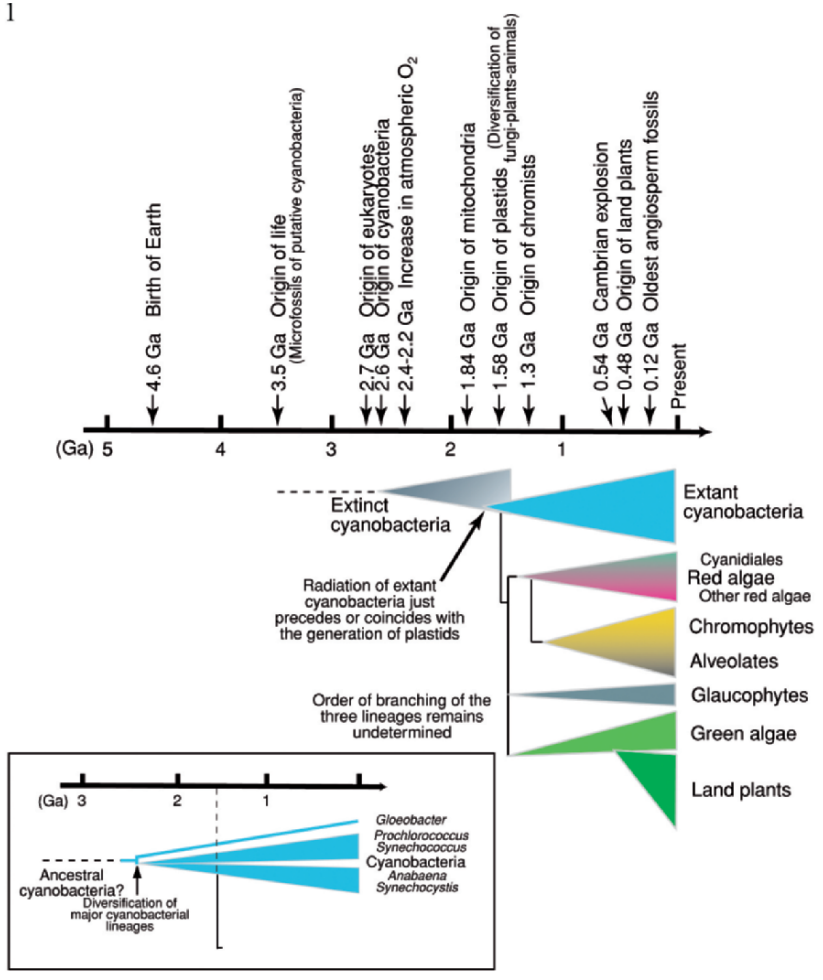



*Fig. 2.* Evolution of cyanobacteria and plastids in the geological time scale. Origin of cyanobacteria dates from 2.6 Ga (billion years ago) according to Hedges *et al.* (2001). One view is that the radiation of extant cyanobacteria just precedes or coincides with the generation of plastids (see text). Shown in the inset is another possibility that plastids diverged from one of the two major cyanobacterial lineages. The origin of plastids is estimated to be 1.58 Ga (Wang *et al.*, 1999). Origin of chromists (and possibly alveolates) was 1.3 Ga according to Yoon *et al.* (2002b). The origin of land plants was 0.48 Ga (Kenrick and Crane, 1997). (See Chapter 4, p. 84.)

Color Plates

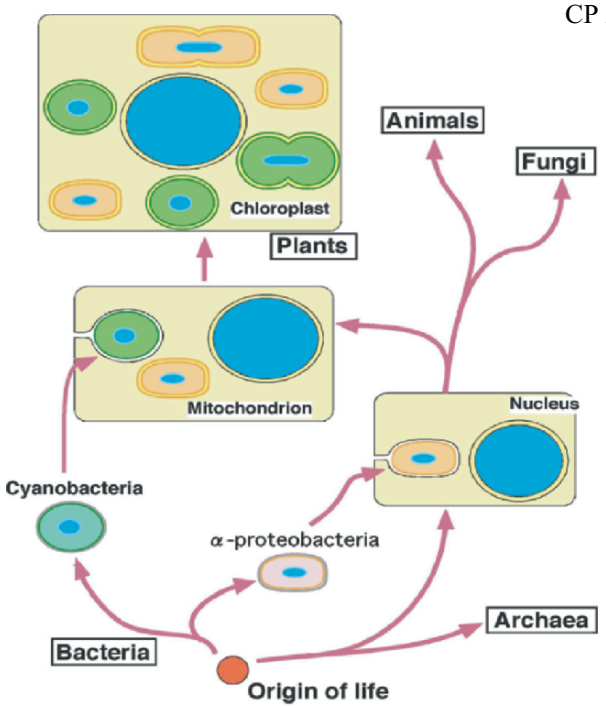



*Fig. 1.* Mitochondria and plastids are the descendants of serial endosymbiotic events. Mitochondria arose first from an ±-proteobacterial ancestor that was acquired by primitive eukaryotic host evolved from archea. Chloroplasts later arose from a cyanobacterial ancestor acquired by a eukaryote in which mitochondria were already established. (See Chapter 5, p. 104.)

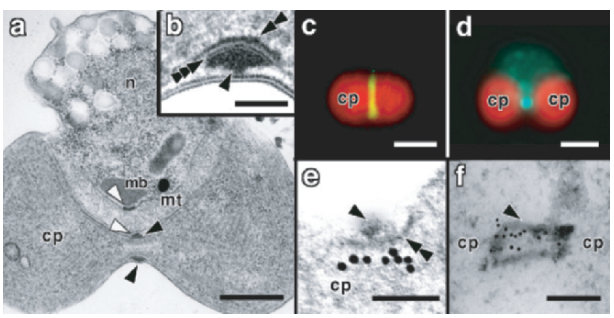



*Fig. 3.* The PD, FtsZ, and dynamin rings in the red alga *C. merolae*. (a) Electron micrographs of a *C. merolae* cell containing a dividing chloroplast and mitochondrion. (b) Magnified cross-section of the PD ring. The PD ring is composed of an outer ring (on the cytosolic side of the outer envelope), a middle ring (in the intermembrane space), and an inner ring (on the stromal side of the inner envelope). The MD ring, structure similar to the PD ring, is also observed at mitochondrial division site. (c, d) Immunofluorescence images of the FtsZ (CmFtsZ2; c) and dynamin (CmDnm2; d) rings during chloroplast division. Bright fluorescence shows localization of each protein and transparent fluorescence is autofluorescence of the chloroplasts. (e, f) Immunoelectron micrographs showing localization of the FtsZ and the dynamin at the chloroplast division site. The FtsZ ring localizes in stroma and faces the inner plastid-dividing ring at the far side from the inner envelope while the dynamin ring localizes between the outer PD ring and the outer envelope. Gold particles indicate location of each protein. Black arrows, arrowheads and double arrowheads indicate the outer, inner, and middle PD rings, respectively. White arrows indicate the MD ring. cp, chloroplast; mb, microbody; mt, mitochondrion; n, nucleus; Bars = 500 nm (a), 50 nm (b, e), 1 µm (c, d), and 100 nm (f). (See Chapter 5, p. 108.)

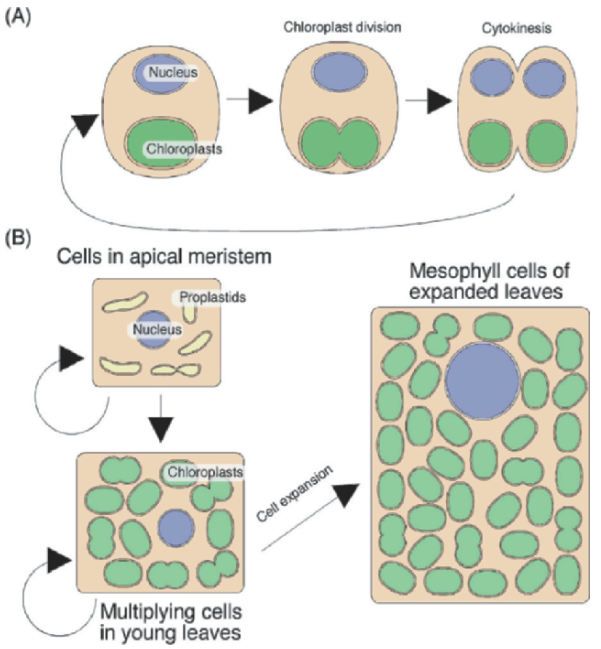



*Fig. 2.* Schematic representation of the timing of chloroplast division relative to the cell cycle. (A) In unicellular algae, which have one chloroplast per cell, chloroplast division occurs once per one cell cycle prior to cytokinesis, although the timing of chloroplast division varies depending on species. (B) In meristematic cells in higher plants, the rate of proplastid and cell multiplication is almost the same, thereby allowing daughter cells to receive nearly the same number of proplastids as the mother cell. After leaf cells start to differentiate, proplastids are converted to chloroplasts. During the early proliferative stage in which mitosis takes place, chloroplast replication keeps pace with cell division even though chloroplasts divide nonsynchronously. During late expanding stage, cell division ceases but chloroplast division continues for two or three more cycles and nuclear DNA replication also continues, resulting in enlarged cell of high ploidy. (See Chapter 5, p. 106.)

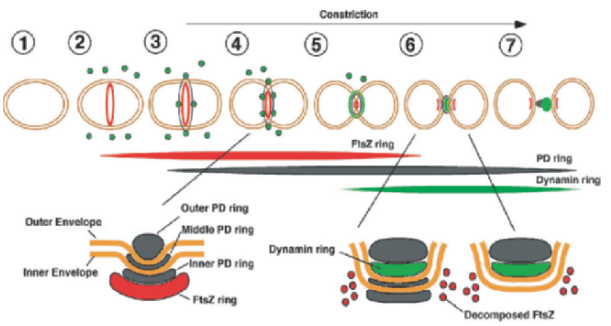



*Fig. 4.* Sequential transition of the plastid division apparatus composed of chloroplast the FtsZ, PD, and dynamin rings. Sequential events during the chloroplast division are illustrated in the upper side. A cross section of the division site is shown on the lower side. Red, FtsZ ring; green, patches or ring of dynamin-related protein; black, PD ring (only the cytosolic outer PD ring is shown in the upper illustration). (See Chapter 5, p. 115.)

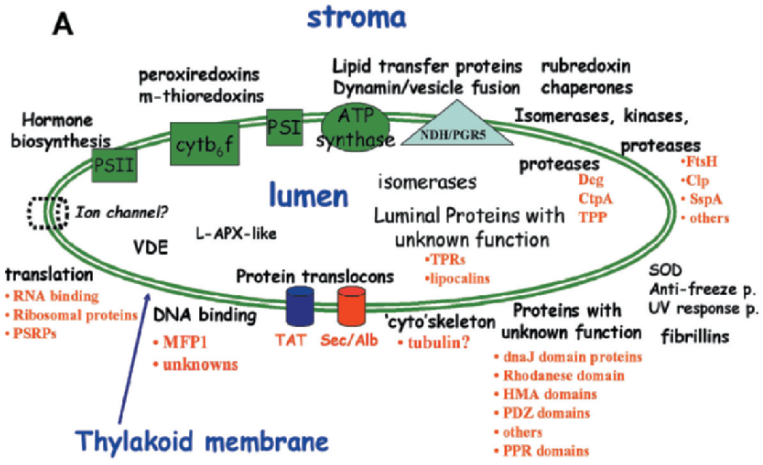



*Fig. 1A.* Functions of the thylakoid proteome. (A) Overview of the thylakoid proteome and its compartmentalization. (See Chapter 6, p. 131.)

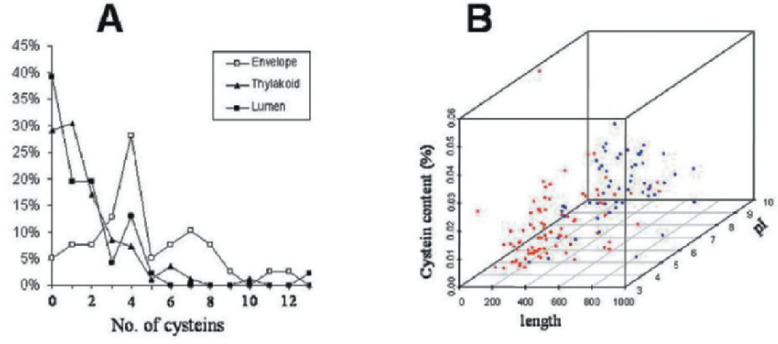



*Fig. 3.* Properties of the thylakoid and envelope proteomes. (A) Frequency distribution of proteins in the three curated proteomes based on the number of cysteines. (B) Rule of thumb to distinguish integral thylakoid membrane proteins from integral inner envelope proteins. Scatter plot of the relative cysteine content, protein length and pI for the curated sets of 61 inner envelope and 110 thylakoid integral membrane proteins. The predicted cTPs were removed prior to calculation of these three parameters. The curation procedure and the list of accession numbers can be found in Sun *et al.* (2004). Envelope proteins are symbolized by blue dots and thylakoid proteins by red dots (adapted from Sun *et al.*, 2004). (See Chapter 6, p. 133.)

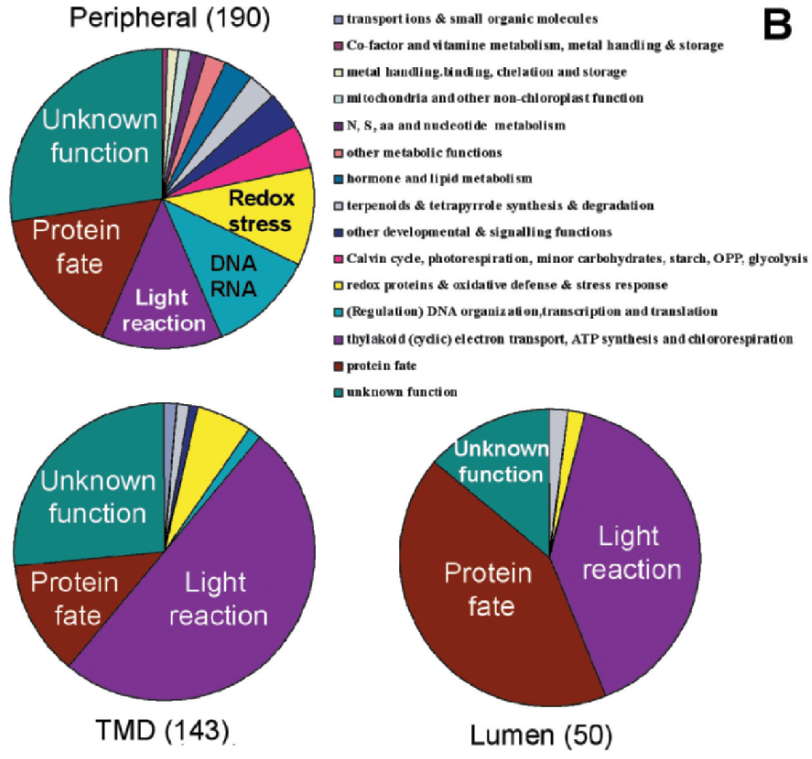



*Fig. 1B.* Functions of the thylakoid proteome. (B) Functional classification of the lumenal, peripheral and integral thylakoid proteome from *Arabidopsis thaliana.* Proteins were identified by large scale proteomics studies or with more 'classical' biochemical tools or forward or reverse genetics. Thylakoid proteins were collectively identified from experimental thylakoid proteomics studies (Peltier *et al.*, 2002, 2004; Schubert *et al.*, 2002; Friso *et al.*, 2004). In addition, the literature was carefully screened for additional thylakoid and envelope proteins. Together this resulted in 384 thylakoid proteins. (See Chapter 6, p. 131.)

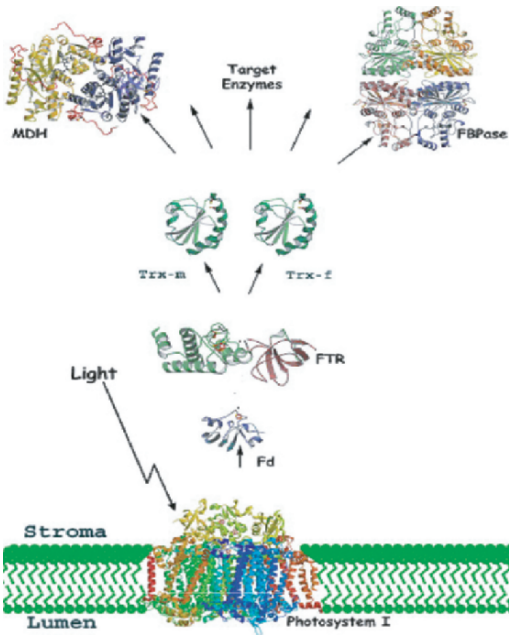



*Fig. 1.* Light activation of chloroplast enzymes by the FTR-system. Photosystem produces reduced ferredoxin upon illumination (bottom). With reduced ferredoxin, ferredoxin:thioredoxin reductase catalyzes the reduction of thioredoxins, which then reduce disulfides in target enzymes, exemplified by MDH and FBPase. (See Chapter 11, p. 222.)

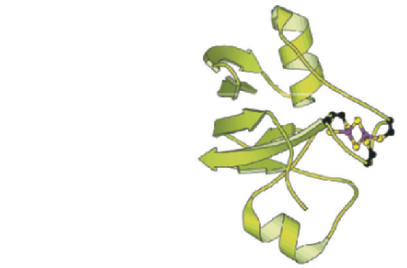


*Fig. 2.* Ferredoxin structure. Plant type ferredoxins contain a [2Fe-2S] center (iron atoms are shown in purple, sulfur atoms in yellow). The *Synechocystis* ferredoxin structure is shown, with the iron-sulfur center to the right. Sulfurs are yellow and irons are purple in all figures. (See Chapter 11, p. 223.)

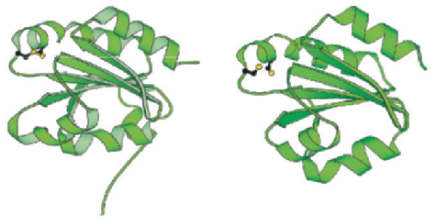


*Fig. 3.* Thioredoxin structure. Thioredoxin *f* and thioredoxin *m* structures. The active site disulfide (iron is purple, sulfur is yellow) in its oxidized form (Trx-*f*, left panel) and its reduced form (Trx-*m*, right panel) is shown in ball and stick representation. (See Chapter 11, p. 224.)

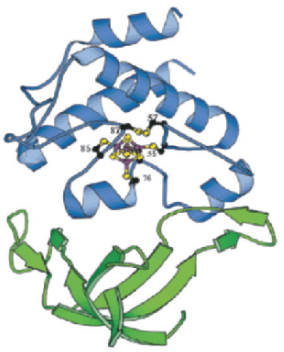


*Fig. 4.* FTR structure. The catalytic subunit (top) contains the iron-sulfur center, with iron atoms coordinated by cysteines 55, 74 76 and 85, and the redox active disulfide bridge, formed between cysteines 57 and 87. The variable subunit is located at the lower part of the molecule (iron is purple, sulfur is yellow, and carbon isblack). (See Chapter 11, p. 225.)

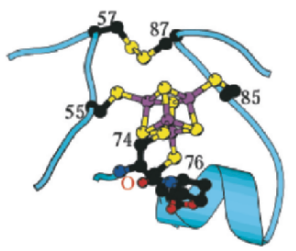



*Fig. 5.* Iron center - disulfide structure. The active site cysteines and Pro75 are shown in ball and stick models. Incoming electrons from ferredoxin can pass through proline and the iron center to the disulfide bridge (iron is purple, sulfur is yellow, carbons are black and oxygens are red). (See Chapter 11, p. 225.)

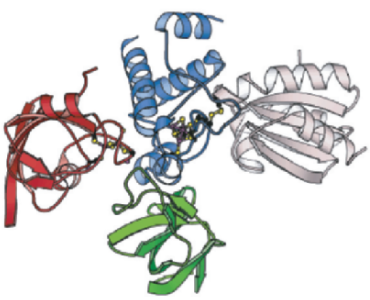


*Fig. 7.* FTR interactions with ferredoxin and thioredoxin. FTR (center) is shown interacting with ferredoxin (to the left) and thioredoxin (to the right). The figure is based on preliminary data on binary complexes with ferredoxin and thioredoxin respectively. (See Chapter 11, p. 228.)

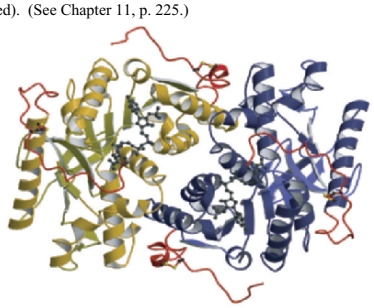


*Fig. 8.* MDH structure. The dimeric NADP-dependent MDH from *Flaveria bidentis* (Carr *et al*., 1999). The two subunits are in lilac and yellow. In red are the N- and C-terminal extensions, specific for all chloroplastic NADP-MDHs. One regulatory disulfide bridge is present in each of the extensions. The N-terminal extensions (top and bottom) sit like wedges between the subunits, thereby locking the domains relative each other. The C-terminal extensions (at the left and right side of the molecule) fold back into the active sites and the disulfides stabilizes this conformation. The very C-termini interact with residues of the active site and with the NADP+ (shown in ball and stick models). (See Chapter 11, p. 230.)

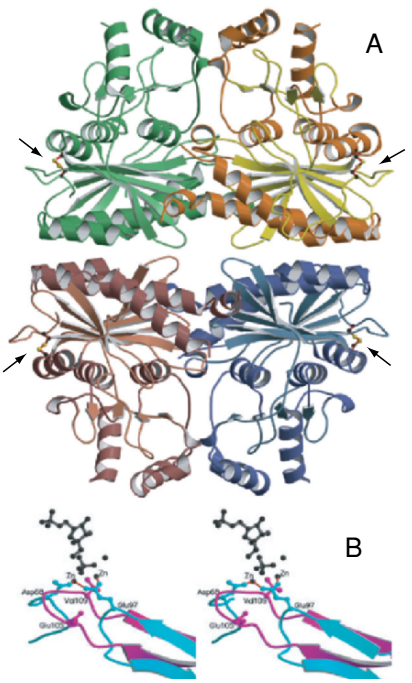


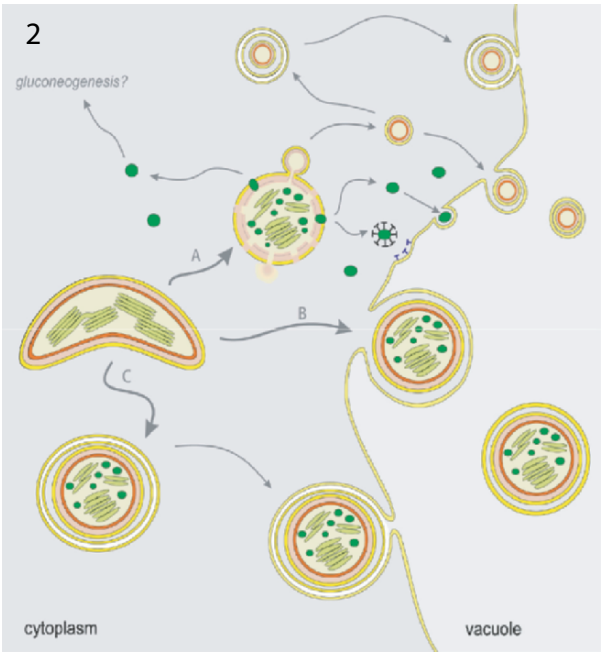

*Fig. 9.* FBPase structure. A. Structure of the oxidized tetrameric pea chloroplastic FBPase (Chiadmi *et al*., 1999). The accessible, regulatory disulfides between Cys153 and Cys173 are shown in ball and stick at the outside of the beta-sheet of each subunit, and are indicated with arrows.

B. Comparison of the active site area of oxidized pea chloroplastic FBPase and non-redox regulated pig enzyme. The catalytic Glu97 of the pig enzyme (in cyan) is together with Asp68 coordinating two zinc ions. In oxidized pea FBPase (in magenta) the movement of ß-strands has pushed Glu105, corresponding to the zinc coordinating Glu97 in the pig FBPase, out of the active site and positioned Val109 near the location of the cation binding site. Through reduction of the pea enzyme the catalytically important Glu105 is positioned in the active site. Fructose-6-phosphate, Pi and Zn2+ are modeled according to the positions in the pig enzyme. (See Chapter 11, p. 232.)

1


zeaxanthin
lutein
11-cis-retinal
β-carotene
lycopene
capsanthin
astaxanthin

2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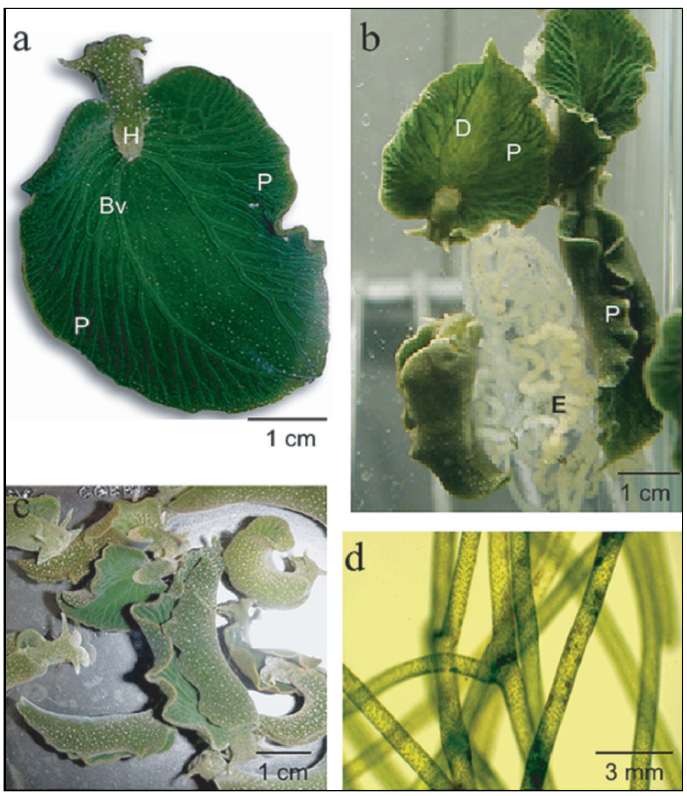
3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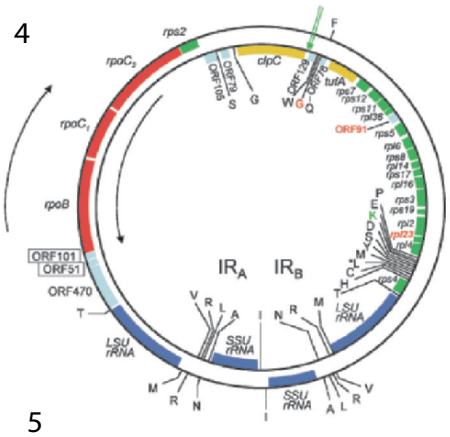
rps2
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ORF470
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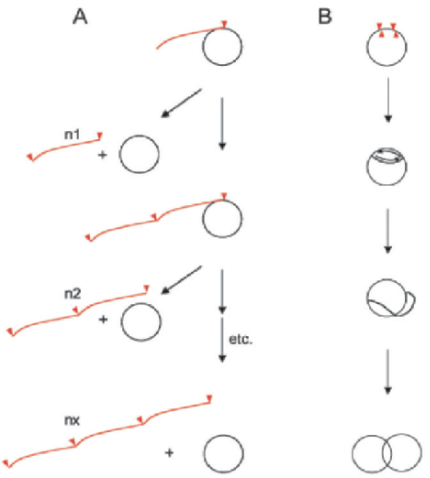
LSU rRNA
SSU rRNA

5


A
B
n1
n2
etc.
nx

Legends for figures in CP 5

*Fig. 1.* Carotenoid diversity. Carotenoids are highly diverse in terms of color and structure. Likewise, they pigment a wide range of bacteria, fungi and plant tissues. The xanthophylls and β-carotene are essential for assembly and function of the photosynthetic apparatus. These yellow pigments, including lutein and zeaxanthin, become apparent in autumn leaves once the green chlorophyll has been degraded. Lutein is the major pigment in marigold flowers, which can range from white to dark-orange because of differences in lutein content. Astaxanthin, complexed with crustacyanin, is responsible for the blue color of lobsters, which shifts to red when the protein is denatured. Capsanthin and capsorubin have unusual cyclopentane rings and are the predominant pigments in red capsicum. Carrot is a source of α- and β-carotene, both of which can be cleaved to form vitamin A (11-*cis*-retinal), and the distinctive red color of ripe tomatoes is due to accumulation of lycopene. (See Chapter 16, p. 317.)

*Fig. 2.* Model of three different pathways by which chloroplasts may be degraded in senescing mesophyll cells. Chloroplasts become smaller and change from an ellipsoid to a round shape. Thylakoids become swollen, and plastoglobuli with thylakoid-derived material accumulate. Senescing chloroplasts may release material into the cytoplasm (A), may be engulfed by the central vacuole (B) or may be included in autophagosomes that may fuse with the vacuole (C). A. Early during senescence vesicles including stromal material are formed. These vesicles are called RCB (rubisco-containing bodies). The vesicles may be engulfed directly by the central vacuole or may give rise to the formation of autophagosomes which are incorporated into the central vacuole by membrane fusion. Later on, during senescence the envelope ruptures and releases globules containing thylakoid-derived material including chlorophyll. The lipids included in the globules may be used for gluconeogenesis. Other components may be further degraded in the cytoplasm. A part of the globules is covered by a coat of proteins resembling clathrin. A receptor-mediated endocytosis by the vacuole may occur. B. A part of the population of chloroplasts is, under certain conditions, engulfed by the central vacuole. The material is degraded by enzymes of the vacuole. C. Another part of the chloroplast population may be surrounded by ER membranes resulting in the formation of autophagosomes. These structures may be incorporated into the vacuole by membrane fusion. (See Chapter 22, p. 440.)

*Fig. 3.* Images of *Elysia chlorotica* and *Vaucheria litorea*. a) Dorsal view of *E. chlorotica* with extended wing-like parapodia and a highly branched blood vascular system emanating from the lighter-colored pericardial mass containing the heart. b) *E. chlorotica* specimens cultured for 6 months in a saltwater aquarium showing parapodia furled and unfurled and non-chlorophyllous eggs. The more ventrally located digestive gland can be better seen in this panel as a lighter colored network that branches off from the stomach (not visible). It is in this extensively branched system that the chloroplasts pass and get phagocytotically absorbed, ultimately distributing the chloroplasts throughout the entire surface of the animal. As a result of the uniform green pigmentation and the two highly branched systems (vascular and digestive), the sea slugs appear much like a dicot leaf. c) Specimens of freshly collected sea slugs demonstrating variation in size and morphology. d) Filaments of coenocytic *V. litorea* cultured in a modified f/2 quarter-strength artificial seawater media. Images 'a', 'c' and 'd' are reprinted with permission of the *Journal of Plant Physiology*. Image 'b' is reprinted with permission of the journal *Zoology*. Bv, blood vessels; D, digestive gland; E, egg; H, heart; P, parapodia. (See Chapter 23, p. 453.)

*Fig. 4.* Comparison of the gene content and arrangement of the plastid DNA among apicomplexa. The gene map of the circular 35 kb plDNA from *P. falciparum* is presented (modified from Wilson *et al*., 1996). The two halves of the inverted repeat region are represented as IRA and IRB. The black arrows indicate the direction of transcription of the genes in the outer and inner strands. The *P. falciparum* plDNA is compared with the *T. gondii* (Gene Bank accession number NC_001799) and *E. tenella* (Gene Bank accession number NC_004823) plastid genomes. Genes specified by red letters are absent in *T. gondii* and *E. tenella*. The tRNAL gene (asterisk) present in the tRNAs cluster is the only gene where an intron was found in *P. falciparum* and *T. gondii*, but it is absent in *E. tenella* In the same cluster, the tRNAK (green character) is encoded in the opposite strand in *T. gondii* and *E. tenella*. ORF101 and ORF51 have a different location in *T. gondii* and *E. tenella*, indicated by the green arrows. ORF 129 is annotated as the rpl11 gene in *T. gondii* and *E. tenella*. (See Chapter 24, p. 481.)

*Fig. 5.* Models for plDNA replication in *Plasmodium* and *Toxoplasma.* A) Rolling circle model for *T. gondii* plDNA. Replication initiates on a circular molecule at or near the center of the inverted repeat (t). After one round of replication, the linear tail can be cut to produce a 35 kb linear monomer, and replication ceases. Processing is not always successful. If processing of the linear molecule fails, replication continues for a further complete round. If processing of the tail is successful, a linear 70 kb dimer is produced and replication ceases. If not, a third round of replication takes place, and so on. By this mechanism a linear molecule of up to 12 copies of the plDNA can be generated. Adapted from Williamson *et al*. (2001). B) D-loop replication model for the circular plDNA from *P. falciparum.* Replication starts from twin D-loop origins (t) present in each half of the plDNA inverted repeat region. Each D loop is generated by unidirectional replication from an origin, resulting in displacement of a single-stranded region. The D loops might expand toward each other and fuse to form a Cairns structure, with replication proceeding bi-directionally around the plastid DNA. Modified from Wilson *et al*. (2003). (See Chapter 24, p. 483.)

CP 6

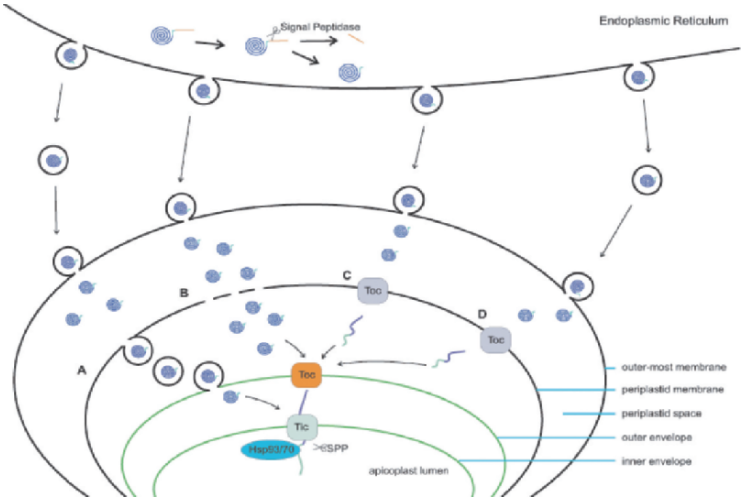



*Fig. 1.* Targeting of apicoplast proteins. Apicoplast proteins are synthesised as preproteins with N-terminal bipartite presequences. The first segment directs the protein to the Endoplasmic Reticulum where is cleaved by a signal peptidase. Later, it is delivered to the outer-most membrane of the apicoplast via vesicles. The periplasmic membrane is crossed via vesicles (A), pores (B), or secondary Toc complexes (C and D). After the precursor protein contacts the outer envelope, conserved Toc and Tic machineries mediate the final transport to the apicoplast lumen where a Stromal Peptidase (SPP) digest the transit peptide. (See Chapter 24, p. 485.)

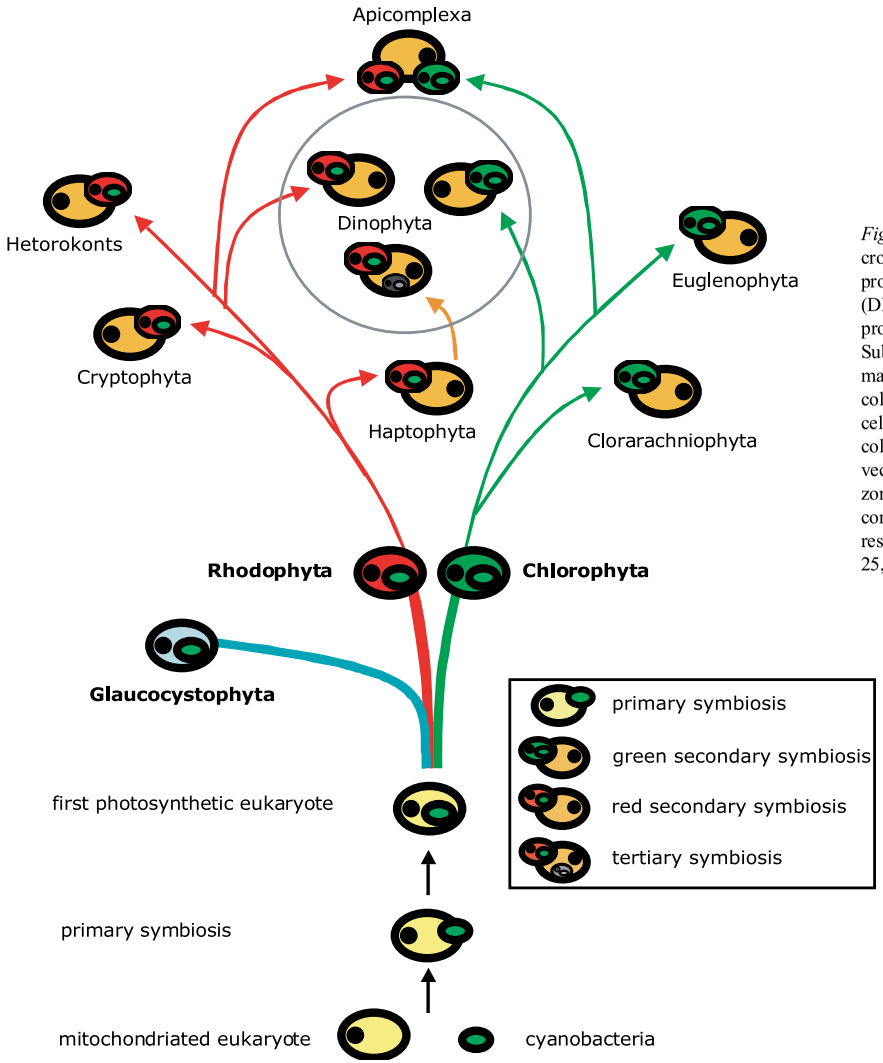



*Fig. 1.* Phylogenetic origins of plastids with a possible origin of apicoplasts. (See Chapter 24, p. 493.)

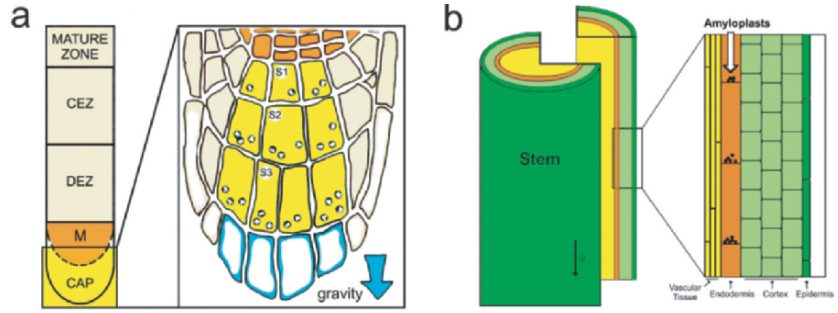



*Fig. 2.* Diagram of a generalized segment of a plant root (a) and stem (b) with an enlarged view of a cross-section from each. (a) A root consists of several growth zones. The meristematic region (M) produces undifferentiated cells that first elongate and then become part of the distal elongation zone (DEZ). As more cells arise from the meristematic region the elongating cells in the DEZ become progressively farther from the meristematic region, and enter the central elongation zone (CEZ). Subsequently, the cells continue to elongate and progress away from the meristem until they enter the maturation zone (mature zone), where they differentiate. Within the root cap, some cells develop into columella cells (yellow), and these are the gravity-sensing cells of the root. In Arabidopsis, the columella cells comprise three layers termed story one, story two and story three (S1, S2, and S3, respectively). The columella cells contain amyloplasts (white on black circles) which sediment in the direction of the gravity vector (arrow). (b) A stem consists of (from the outside in) an epidermal layer (epidermis), a cortical zone (cortex), an endodermal layer or layers (endodermis), and vascular tissue. The endodermal cells contain membrane-bound organelles filled with starch grains (amyloplasts, open arrow) that sediment in response to gravity (g, black arrow). Figure 1a is redrawn from Swatzell and Kiss (2000). (See Chapter 25, p. 510.)

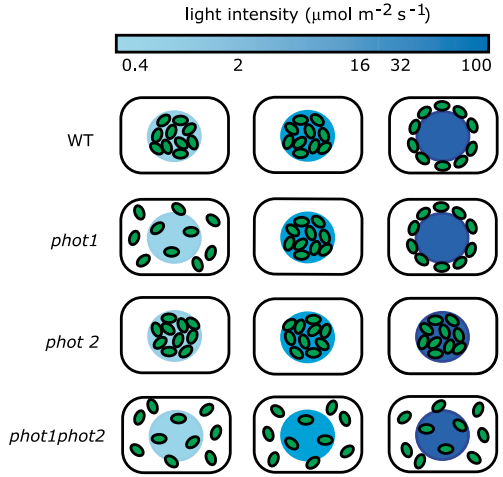



*Fig. 3.* Blue light-induced chloroplast movement in Arabidopsis wild type (WT), single and double phototropin-deficient plants (*phot1, phot2* and *phot1phot2*) as analyzed by microbeam irradiation. The area of microbeam irradiation is indicated in a blue circle of each illustration of mesophyll cells. (See Chapter 26, p. 530.)

# Section I

## Plastid Origin and Development

# Chapter 1

# The Diversity of Plastid Form and Function

Robert R. Wise*
*Department of Biology, University of Wisconsin-Oshkosh, Oshkosh, WI 54901, U.S.A.*

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## Summary

Plastids are semiautonomous organelles found, in one form or another, in practically all plant and algal cells, several taxa of marine mollusks and at least one phylum of parasitic protists. The members of the plastid family play pivotal roles in photosynthesis, amino acid and lipid synthesis, starch and oil storage, fruit and flower coloration, gravity sensing, stomatal functioning, and environmental perception. Plastids arose via an endosymbiotic event in which a protoeukaryotic cell engulfed and retained a photosynthetic bacterium. This polyphyletic event occurred multiple times between roughly 1.5 to 1.6 billion years ago. Although most of the algal genes were transferred to the nuclear genome, plastids have retained a complete protein synthesizing machinery and

*Email: wise@uwosh.edu

*Robert R. Wise and J. Kenneth Hoober (eds.), The Structure and Function of Plastids,* 3–26.

enough information to code for about 100 of their approximately 2,500 proteins; all other plastid proteins are coded for by the nuclear genome and imported from the cytoplasm. Plastids divide via fission prior to cytokinesis and are equally apportioned between the two daughter cells, along with the rest of the cytoplasmic contents.

There are three evolutionary lines of plastids: the glaucophytes, the red lineage and the green lineage. The glaucophyte line of chloroplasts is represented by the *muroplasts*, primitive walled chloroplasts found in the glaucocystophytic algae. *Rhodoplasts* represent the red lineage and are the chloroplasts of the red algae. The green lineage gave rise to the plastids of the green algae and members of the Kingdom Plantae. It is the plant kingdom, with its higher level of tissue complexity, that has fully availed itself of the diversity available in plastid evolution. Seeds, meristematic tissues, and several other tissues contain *proplastids*, the smallest and least complicated member of the plastid family, and the ontological precursor to all other plant plastid types. If development of the meristematic or embryonic tissues is allowed to proceed in the dark, plastid development proceeds to and is arrested at the *etioplast* stage. *Leucoplasts* are colorless (i.e. non-pigmented) plastids, of which three types are generally recognized, amyloplasts, elaioplasts, and proteinoplasts. Proplastids in root tissues typically develop into colorless starch-containing *amyloplasts*. Some amyloplasts, such as those in a potato tuber, function entirely in starch storage. Other amyloplasts, found primarily in the root cap, are said to be "sedimentable" and are intimately involved in gravity perception. *Elaioplasts* play roles in oil storage and metabolism, and are centrally involved in pollen grain maturation. *Proteinoplasts* may be sites of protein storage, but their significance is questionable. Brightly colored *chromoplasts* contain high levels of carotenoids and provide the color to many flowers, fruits and vegetables. *Gerontoplasts* represent a degrading, but still functional, stage in the plastid life cycle found in senescent tissues. Upon ingestion of algal filaments, several groups of sacoglossan sea slugs incorporate algal chloroplasts into cells lining the digestive diverticulae. The slugs turn deep green and are able to live in the absence of added food for at least nine months in the light, but only two weeks in the dark. These purloined plastids have been named *kleptoplasts*. *Apicoplasts* are plastids found in the Apicomplexan group of parasites, of which *Plasmodium falciparum* (the malarial parasite) is the most heavily studied. To date, no specific function has been assigned to the apicoplast although its metabolism has been a major target for anti-malarial drugs.

If plant tissue is exposed to the light during development, the proplastids will multiply, enlarge, turn green and develop into mature, photosynthetically competent *chloroplasts*. Chloroplasts are the most metabolically multitalented of the plastids and are involved in a large number of anabolic pathways, in addition to photosynthesis. Chloroplasts differ in structure and function between $C_3$ and $C_4$ plants and between shade versus sun leaves. Although appearing similar to those in mesophyll cell chloroplasts, *guard cell chloroplasts* function in stomatal opening and closing, and not primarily in photosynthesis. This chapter will briefly review each of the plastid types given above and finish with a discussion of the need for a three-dimensional, mathematical model of thylakoid topology.

## I. Introduction

Plastids are a diverse group of phylogenetically-, ontogenetically- and physiologically-related eukaryotic organelles that play central roles in plant metabolism via the processes of photosynthesis, lipid and amino acid synthesis, and nitrogen and sulfur assimilation (Table 1). They are represented by over a dozen different variants and are present, with very few exceptions, in all algal, moss, fern, gymnosperm and angiosperm cells. Plastids also are found, perhaps surprisingly to some, in a few parasitic worms and a number of marine mollusks. Their study, and the history of their study, is the basis for much of what we now know about plant physiology and biochemistry. The Dedication to this volume by Brian Gunning, Friederike Koenig and Govindjee provides an excellent history of plastid research.

Modern day plastids are the result a series of endosymbiotic events that established the three basic lineages seen in extant organisms–the so-called glaucophytes, red lineage, and green lineage (see Chapter 4). All plastids arose from a common primary endosymbiosis (the incorporation of a photosynthetic prokaryote by a prokaryote host cell), but then secondary (the incorporation of a phototropic (proto?)-eukaryote by a

*Abbreviations:* BSC – leaf vascular bundle sheath cell; ER – endoplasmic reticulum; GCC – guard cell chloroplast; GFP – green fluorescent protein; GS-GOGAT – glutamine synthetase/glutamate synthase; LSCM – laser scanning confocal microscopy; MC – leaf mesophyll cell; OAA – oxaloacetate; OEC – oxygen evolving complex; PAP – plastoglobule-associated proteins; PC – plastocyanin; PEPcase – phospho*enol*pyruvate carboxylase; PG – plastoglobule; 3PGA – 3-phosphoglyceric acid; PLB – prolamellar body; PQ – plastoquinone; PR – chloroplast peripheral reticulum; PSI – photosystem I; PSII – photosystem II; Rubisco – ribulose-1,5-*bis*phosphate carboxylase/oxygenase; TEM – transmission electron microscopy.

*Table 1*. Summary of plastid forms and functions.

| Plastid type | Function(s) | Distinctive features |
|---|---|---|
| Muroplast | Photosynthesis | Walled chloroplast found in Glaucocystophytic algae; wall similar to that found in prokaryotes |
| Rhodoplast | Photosynthesis | Red chloroplast of the Rhodophytes; photosynthetically competent at depths up to 268 m |
| Proplastid | | |
| Germinal | Source of other plastids | Found in egg, meristematic and embryonic cells; source of all other plastids in the plant |
| Nodule | $N_2$ assimilation | Site of the GOGAT cycle in nitrogen-fixing root nodules |
| Etioplast | Transitionary stage | Develops in dark-grown tissue; site of gibberellin synthesis; converts to chloroplast in light |
| Leukoplasts | | |
| Amyloplast | Starch synthesis and storage | Also functions in gravisensing |
| Elaioplast | Oil synthesis and storage | Supplies lipids and oils to excine upon pollen grain maturation |
| Proteinoplast | Protein synthesis and storage? | May not be a functional subcategory; most protein stored in RER-derived seed storage bodies |
| Chromoplast | Fruit and flower coloration | Rich in carotenoids; used to attract pollinators and seed/fruit-dispersing animals |
| Gerontoplast | Catabolism | Controls the dismantling of the photosynthetic apparatus during senescence |
| S-type plastid | Unknown–starch storage? | Found in phloem sieve tubes, relevant in systematics of many plant taxa |
| P-type plastid | Unknown–defense? | Found in phloem sieve tubes; systematically relevant; rupture upon damage to sieve tube cell |
| Kleptoplast | Photosynthesis | Stolen from algal cells by sea slugs; functionally active for lifetime of slug (up to nine months) |
| Apicoplast | Unknown | Found in parasitic worms that cause malaria; drugs that target apicoplast reduce infectivity |
| Chloroplast | | |
| algal | Photosynthesis, etc. | Probably serves many of the same functions as tracheophyte chloroplasts |
| $C_3$ | Photosynthesis, etc. | Also functions in fatty acid, lipid, amino acid and protein synthesis, N and S assimilation |
| $C_4$ | Photosynthesis, etc. | Dimorphic chloroplasts provide a $CO_2$-rich, $O_2$-poor environment for enhanced Rubisco activity |
| Sun/Shade | Photosynthesis, etc. | Dimorphic forms develop under different light conditions in order to optimize photosynthesis |
| Guard cell | Stomatal functioning | Senses light and $CO_2$; signals and metabolically drives opening and closing of stomata |

host cell) or even tertiary (incorporation of a secondary endosymbiont) events produced the diversity seen in the three modern-day lineages (see Chapter 4). Genes contained within the chloroplast itself code for about 100 of the roughly 2,500 proteins found in a typical chloroplast; the other 95% are coded for by the nuclear genome (see Chapter 6). The nuclear-encoded proteins must then be translocated into the plastid (see Chapter 3). A significant and ever increasing amount of data indicates that the genes coding for plastid proteins (be they plastidal or nuclear genes) have multiple origins that involve both the red and green lineages. Horizontal gene transfer, gene duplication, gene loss and perhaps even the recruitment of mitochondrial genes and gene products have all contributed to the biology of extant plastids. Evidence of the prokaryotic origin of plastids can be seen in their genome (see Chapter 7) and the molecular machinery used for transcription and translation (see Chapter 8). Tight communication exists between the plastid and nuclear genomes (see Chapter 9) and, in keeping with their prokaryotic origins, chloroplasts divide via fission (see Chapter 5).

This review will focus almost entirely on the plastids of tracheophytes. Algal chloroplast structure and function (Larkum and Howe, 1997) and evolution (McFadden, 1993; Raven and Allen, 2003; see Chapter 4) have been reviewed elsewhere. In addition, a previous volume in this series (Larkum *et al*., 2003) has provided ample coverage of algal chloroplast biology.

## II. The Plastid Family

### *A. Muroplast (Cyanoplast or Cyanelle)*

Muroplasts are the photosynthetic organelles of Glaucocystophyte algae and are the only members of

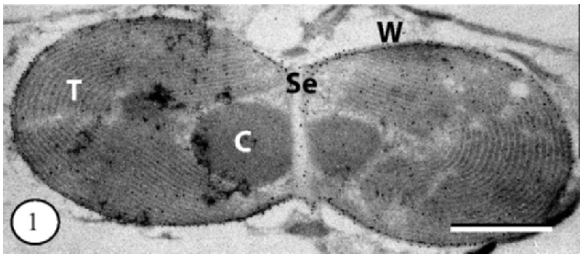



*Fig. 1.* Muroplast. This dividing photosynthetic organelle of the Glaucopytic algae *Griffithsia pacifica* has unstacked, parallel thylakoids (T), a large carboxysome (C) a vestigial peptidoglycan wall (W) and a septum (Se) between the two daughter cells. Scale bar = 5.0 μm. Micrograph courtesy of Dr. Wolfgang Löffelhardt (University of Vienna, Austria).

the so-called "glaucophyte" lineage of plastids (see Chapter 4). The name comes from the Latin "mura" meaning "wall" and was coined by Schenk (1994) to highlight a key feature of muroplasts; namely, they are bound by a vestige of a peptidoglycan prokaryotic cell wall (Fig. 1). "Muroplast" will be used in this review to recognize this unique feature. Originally called "cyanelles", the abandonment of that term has been advocated because, strictly defined, "cyanelle" should be limited to endosymbiotic cyanobacteria (many examples of which may be found in nature; Pascher, 1929), and not applied to a fully evolved cellular organelle, such as the modern-day muroplast (Schenk, 1990). In short, although muroplasts clearly had an endosymbiotic origin (Helmchen *et al.*, 1995; Stiller *et al.*, 2003; see Chapter 4) and retain many primitive features, they have evolved to be totally dependent on the algal host cell and should thus be referred to as muroplasts, not cyanelles (Schenk, 1990). "Cyanoplast" is also in common usage, but muroplast is preferred because the former term could also apply to the plastids in any "cyano-" organism such as the red alga *Cyanidium*, which clearly contains rhodoplasts not cyanoplasts (see Section II.B). In practice, however, many authors continue to use "cyanelle" or "cyanoplast" for historic (Steiner and Löffelhardt, 2002) or other purposes (Iino and Hashimoto, 2003). [To further muddy the nomenclaturic waters, "cyanoplast" (or "anthocyanoplast") has also been used to describe pigmented, anthocyanin-containing, non-membrane-bound, lipid globules found in the vacuoles of some species such as sweet potato (Nozue *et al.*, 1997). Anthocyanin-containing cyanoplasts bear no relationship to the photosynthetic cyanoplasts/cyanelles/muroplasts found in the glaucophytes and with whom they share a name.]

As noted above, Glaucocystophytic muroplasts are bound by a double membrane with a prokaryotic-like, peptidoglycan layer between the two membranes (Kugrens *et al.*, 1999). Internally, muroplasts have unstacked, concentric thylakoid membranes bearing many phycobilisomes and contain a carboxysome (a protein inclusion rich in ribulose-1,5- *bis*phosphate carboxylase/oxygenase; a.k.a. Rubisco); all of these characters are very cyanobacterial and mark the muroplasts as being the most primitive of all plastids. The muroplast genome contains genes for about 150 polypeptides, as well as numerous rRNAs and tRNAs (Löffelhardt *et al.*, 1997); about 1.5 times as many genes as found in the Arabidopsis chloroplast genome and further evidence of the primitive nature of the muroplasts.

In the evolution of plastids, 80 to 90% of the plastid genome has been transferred to the nucleus (see Chapter 4). Therefore, most plastid proteins are translated on cytoplasmic ribosomes and translocated into the organelle either during or after synthesis (see Chapter 3). In addition to gene transfer, the acquisition of the protein translocating machinery is seen as a key development in the endosymbiotic origin of both plastids and mitochondria making the muroplast, with its primitive characters and external wall, an ideal subject for the study of the evolutionary origins of organellar protein uptake machinery (Steiner and Löffelhardt, 2002).

### *B. Rhodoplast*

Rhodoplasts are the photosynthetic plastids found in the red algae (Phylum Rhodopyhta) and represent the "red lineage" of plastids (Chapter 4). Technically chloroplasts, because they do contain chlorophyll *a*, rhodoplasts are red to purple, depending on the water depth, which influences the light environment and phycocyanin or phycoerythrin pigment composition (MacColl and Gaurd-Friar, 1987; Cunningham *et al*., 1989).

Rhodoplast morphology varies from stellate to cup-shaped to ovoid and rhodoplasts may or may not contain a pyrenoid (Broadwater and Scott, 1994). They are bound by a double membrane and contain unstacked thylakoids studded with phycobilisomes (Fig. 2; see also Fig. 4a, Chapter 5). Phycobilisomes are supermolecular aggregates of pigmented phycobiliproteins and linker proteins (MacColl and Gaurd-Friar, 1987). Phycobilisomes funnel the majority of their absorbed light energy to PSII whereas PSI is served by Chl *a*- and zeaxanthin-containing light harvesting complexes (Wolfe *et al.*, 1994; Grabowski *et al.*, 2001).

The Rhodophyta do not contain chlorophylls *b* (as is found in the Chlorophyta) or *c* (as found in the Chromophyta; Fawley and Grossman, 1986). Absorption of

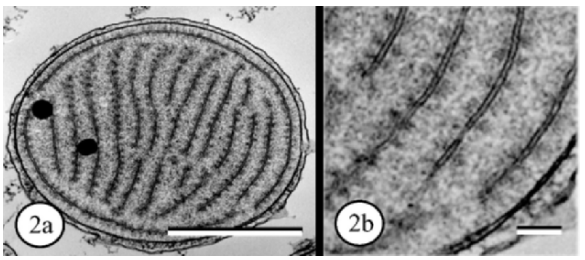



*Fig. 2.* Rhodoplast. 2a. The plastid is on a cytoplasmic strand across the vacuole, so the outermost membrane visible is that of the vacuole. The two membranes of the chloroplast envelope have little separation. Inward and parallel to the chloroplast envelope is a single peripheral thylakoid, which surrounds numerous plate-like thylakoids. Scale bar = 1.0 μm. 2b. All the thylakoids have phycobilisomes. Scale bar = 0.1μm. Micrographs courtesy of Dr. Curt Pueschel (State University of New York at Binghamton, USA).

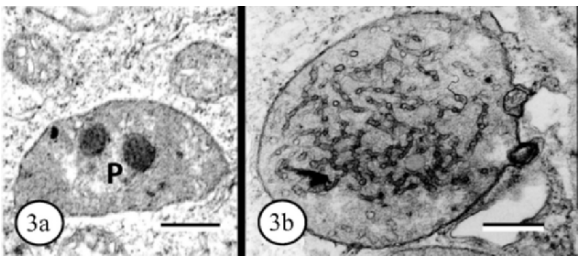



*Fig. 3.* Proplastids. 3a. Proplastids from sweet potato ( *Ipomea batatas*) root tip. Note the lack of internal structure and two apparent protein inclusions (P). 3b. Proplastid from a developing mangrove ( *Rhizophora mangle*) embryo with numerous unorganized tubules. Scale bars = 0.5 μm.

blue light by the red pigments found in rhodoplasts allow some marine species of the Rhodophyta to grow at depths of 268 m where the available light may be as little as 0.0005% of full sun (Littler *et al.*, 1985). Members of the Rhodophyta, therefore, are the deepest dwelling photosynthetic organisms.

The Rhodophyta Rubisco holoenzyme is the typical "Form 1" type (i.e. it has eight large and eight small subunits, $L_8S_8$, as is found in the Chlorophytes) but it differs from the Rubisco of green lineage plastids in that both subunits are encoded by the plastid genome (Li and Cattolico, 1987; Steinmüller *et al.*, 1983). Another unusual red algal feature is that they synthesize a unique storage starch called floridean, which is similar in structure to amylopectin. Floridean is also different from the true starch found in Chlorophytes in that it accumulates in the cytoplasm, not in the plastid (McCracken and Cain, 1981).

### *C. Proplastid (Eoplast)*

Proplastids are generally small (~1 μm, Lancer *et al.*, 1976) and undifferentiated (Marinos, 1967) with a poorly defined internal membrane system consisting of only a few tubules (Whatley, 1977; Fig. 3). The tubules are often seen to connect to the inner membrane of the proplastid envelope (c.f. Kirk and Tilney-Bassett, 1967, Fig. I.36) but such connections are lost early in development and are rarely (never?) seen in mature plastids of any variety. In developing chloroplasts, whether the tubules represent a site for incipient thylakoid formation or are more closely and mechanistically related to chloroplast envelope vesicles (see Chapter 3) or the peripheral reticulum (see Section III.D, below) has not been determined.

All proplastids have a similar, simple structure (which is the basis for their common name) but the specificity of the term "proplastid" has not been well defined in the literature. As a consequence, it may encompass several different plastid types with significantly different functions. Thompson and Whatley (1980) referred to proplastids in meristematic tissues as "eoplasts", but the name has not been widely adopted and the use of "proplastids" persists. For purposes of this review, two types of proplastids will be considered, *germinal proplastids* (Thompson and Whatley's "eoplast") and *nodule proplastids*.

Germinal proplastids are found in meristematic and embryonic tissues and cells in tissue culture and they are the developmental precursors to all other plastid types (Pyke, 1999). Initially, they were originally thought to be metabolically unimportant and to merely serve as a source for plastid production through fission. However, recent studies have indicated they may contribute directly to the metabolism of meristematic tissues. For instance, developing seeds and meristems are generally high in gibberellic acid, and contain many proplastids. Aach *et al.* (1997) have found that *ent*-kaurene synthase, a key enzyme in the gibberellin biosynthetic pathway, co-localizes with proplastids in developing wheat tissues throughout the life of the plant. The identification of this key role for proplastids in meristematic tissues suggests they play a larger part in tissue development that was once envisioned.

Nodule proplastids have a similar appearance in the electron microscope to germinal proplastids, but have been linked to a vital role in the biochemistry of nitrogen fixation. In biological nitrogen fixation, $N_2$ is reduced to ammonia by symbiotic bacteria found in root nodules. The resulting ammonia (which is highly toxic and must be removed) is incorporated into glutamine in the cytosol of the nodule cell. The glutamine

is then taken up by nodule proplastids where it enters the glutamine synthetase/glutamate synthase (GS-GOGAT) cycle. The GS-GOGAT cycle is linked to the production of glycine, serine, aspartate, asparagine, α-ketoglutaric acid and several purines (Boland and Schubert, 1983; Ferguson, 1998). In short, the nodule proplastid plays a major role in incorporating fixed nitrogen into a large number of biologically important nitrogenous compounds–a task quite different from that of a germinal proplastid.

The only thing germinal proplastids and nodule proplastids appear to have in common is their simple, undifferentiated structure. Given their disparate locations and unique functions, they should probably be recognized as different plastid types as they no doubt have unique proteomes and metabolomes.

## *D. Etioplast*

Etioplasts are plastids in shoot tissues that have been developmentally arrested during the proplastid to chloroplast transition due to absence of light or to extremely low light conditions (Harsanyi *et al.*, 2005; Domanskii *et al.*, 2003; Fig. 4). The term "etiolated" comes from the French *étioler* (meaning straw) and was coined by Erasmus Darwin in 1791 to describe the white color and straw-like appearance of celery grown in the dark. Kirk and Tilney-Bassett (1967) first applied the *etio-* prefix to etioplasts to distinguish them from their developmental precursors, the undifferentiated germinal proplastids. Etioplasts, therefore, are the plastids found in white, light-deprived stem and leaf tissue (but not roots; etioplasts do not form in dark-grown root cells-Newcomb, 1967).

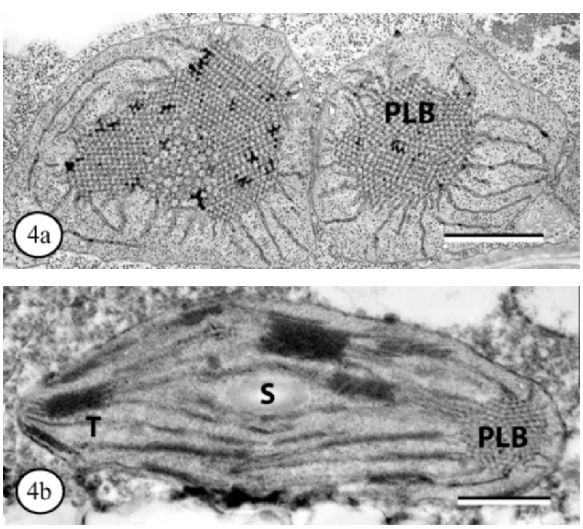



*Fig. 4.* Etioplasts. 4a. Two etioplasts from dark-grown oat (*Avena sativa*) showing large prolamellar bodies (PLB) Micrograph courtesy of Dr. Brian Gunning (Australian National University, Australia) (from "Plant Cell Biology on CD", ©B. Gunning, 2004). 4b. Proplastid to chloroplast transition showing the presence of starch (S), developing thylakoid membranes (T) and a degenerating prolamellar body in the same plastid. Scale bars = 1.0 μm.

Structural studies have demonstrated that the etioplast interior is dominated by a complicated structure called a prolamellar body (PLB) composed of symmetrically arranged, tetrahedrally-branched tubules (Menke, 1962; Kirk and Tilney-Bassett, 1967; Gunning, 2001). Gunning (2004) performed an extensive analysis of PLB structure and reported a number of commonalities and variations in their structure. Plastoglobuli and ribosomes may be found in the interior of a PLB, but whether they play a role in etioplast function, or have just been trapped by the developing PLB is not known (Gunning, 2004). PLBs develop in the dark, require carotenoids for stabilization (Park *et al.*, 2002), contain protochlorophyllide *a* (the precursor to chlorophyll *a*; see Chapter 15) and are converted into thylakoid membranes upon exposure to light. An etioplast typically has a single PLB.

The etioplast has been used extensively as a valuable system to study chloroplast development (Baker and Butler, 1976; Leech, 1984) because etioplast formation is easily induced in the dark and disruption of PLB structure, chlorophyll synthesis, and thylakoid development all begin very soon after exposure to light (Krishna *et al.*, 1999). PLBs themselves cannot perform photochemistry. However, upon transfer to light conditions photosysem I (PSI) activity can be measured within 15 minutes, photosystem II (PSII) within 2 hours and water-splitting, proton pumping and ATP formation within 2 to 3 hr (Wellburn and Hampp, 1979). The assembly of the various photochemical complexes upon the etioplast to chloroplast transition is a controlled, step-wise process (Lebkuecher *et al.*, 1999) and the main reason for the use of etioplast as a research tool.

Once thought to be a laboratory contrivance not found in nature, etioplasts have recently been reported in the head of white cabbage (Solymosi *et al.*, 2004). Starting from the innermost leaves and moving outward, the leaves contained stable (lasting for up to several months) populations of proplastids, etioplasts, or chloroplasts. Intermediary leaves contained chlorophyll precursors (inner layers) and chlorophyll (outer layers). This presents the possibility that these long-lived plastids may play a role in the ongoing metabolism

of the developing cabbage leaves, and are not merely a "resting stage" found in dark-grown tissues.

## *E. Leucoplasts (Leukoplasts)*

Leucoplast (sometimes spelled "leukoplast", *e.g.* Negm *et al.*, 1995) is the term used to categorize colorless, non-pigment-containing plastids; "leuco" meaning white. There are three generally recognized types; starch storing (amyloplasts), oil and lipid storing (elaioplasts) and protein storing (proteinoplast). The structure and function of amyloplasts and elaioplasts are well understood. Proteinoplasts are less well defined.

### *1. Amyloplast*

Because the complete starch biosynthetic pathway is confined to plastids (Yu *et al.*, 1998; see Chapter 14), it should come as no surprise that plants use plastids as starch synthesizing and starch storage organelles; such plastids are called amyloplasts, from the Greek "αμυλ−ον" meaning starch. In fact, all starch in any living plant cell is contained within a double-membrane plastid of one form or another. Amyloplasts are distinguished by the presence of one to many large starch grains and a minimal internal membrane system (Fig. 5).

Plastids synthesize both transitory starch (that made during the day and temporarily stored in the chloroplast until nightfall) and storage starch (starch synthesized and stored in amyloplasts of fruits, seeds, tubers and stems). The vast majority of research on starch synthesis and breakdown has been conducted on the storage starch of cereal grains (James *et al.*, 2003), although recent attention has begun to focus on transitory starch metabolism in photoautotrophic tissues as well (Zeeman *et al.*, 2004).

Because starch grains are denser than water, amyloplasts will sediment at 1x *g* forces on a minute time scale. This property is intimately linked to the well-known role of amyloplasts in graviperception. In brief, amyloplasts respond to gravistimulation by sedimenting in the direction of the gravitational vector (i.e. down). This brings the plastids in contact with the cytoskeleton and this information is used to polarize various growth and development processes. Other cellular components such as the vacuole, endoplasmic reticulum, or plasma membrane may also interact with sedimenting amyloplasts and be involved in the signal sensing and transduction. See Palmieri and Kiss (Chapter 25) for a complete treatment of the role of amyloplasts in gravisensing.

In common with nodule proplastids (see above), amyloplasts in some species, notably alfalfa, contain key enzymes of the GS-GOGAT cycle and are intimately involved in nitrogen assimilation (Trepp, 1999). Whether these should be called "starch-containing proplastids" or "proamyloplasts" may well be a semantic, and not a mechanistic, debate. However, it may be of interest to determine if nodule amyloplasts are biochemically and metabolically distinct from nodule proplastids.

### *2. Elaioplast (Elioplast)*

Elaioplasts are oil-containing leucoplasts. Their name is derived from "elaiov" (ελαιο − ν), the ancient Greek word for olive, and is sometimes spelled "elioplast." Elaioplasts are usually small and round, and their internal structure is dominated by the presence of numerous oil droplets (Fig. 6).

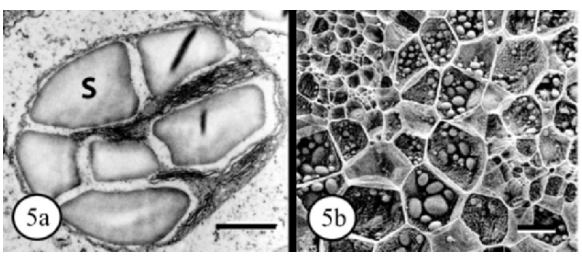



*Fig. 5.* Amyloplasts. 5a. Amyloplast from a strawberry receptacle at anthesis showing copious amounts of starch in the form of granules. Scale bar = 1.0 μm. Micrograph courtesy of Dr. Michael Knee, Ohio State University, USA. 5b. A low magnification SEM view of starch granules in a potato tuber. Scale bar = 20 μm. Micrograph courtesy of Dr. Brian Gunning (Australian National University, Australia) (from "Plant Cell Biology on CD", ©B. Gunning, 2004).

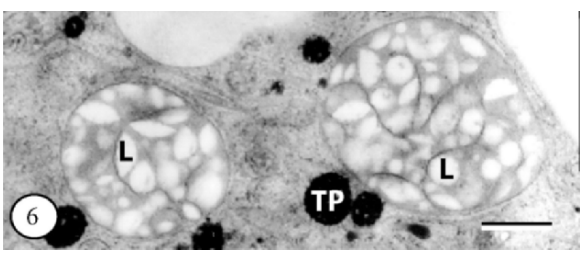



*Fig. 6.* Elaioplasts from *Brassica napus* tapetal tissue. Tissue was fixed using high-pressure-freezing and freeze substitution, therefore the numerous lipid droplets (L) appear clear. Several tapetosomes (Tp) are seen in the cytoplasm. Scale bar = 1.0 μm. Micrograph courtesy of Dr. Denis Murphy (University of Glamorgan, UK).

Plastids are the location of fatty acid synthesis in plant cells and also the site of the so-called "prokaryotic pathway" of lipid synthesis (see Chapter 17). The "eukaryotic pathway" synthesizes lipids in the endoplasmic reticulum using plastid-derived fatty acids (Roughan and Slack, 1982). The two types of lipids show significant differences in fatty acid composition and position and, obviously, were named because of their similarity to lipids found to predominate in prokaryotic or eukaryotic cells. The lipids in elaioplasts occur in droplets composed of prokaryotic-like triacylglycerols and sterol esters, with monogalactosyldiacylglycerol being the polar lipid found in the highest amount (Hernandez-Pinzon *et al.*, 1999). Elaioplasts also contain a number of proteins, with plastoglobule-associated proteins (PAP) such as fibrillin predominating (Wu *et al.*, 1997; Hernandez-Pinzon *et al.*, 1999).

Elaioplasts have been studied most intensively in regard to their important role in pollen maturation (Pacini *et al*., 1992; Ting *et al.*, 1998; Ross *et al.*, 2000; Hsieh *et al*., 2004). Pollen grains develop within the loculus of the anther and are nurtured by a single cell layer called the tapetum. Tapetal cells contain both elaioplasts and tapetosomes (oil and protein bodies that originate from the ER), both of which contribute molecules to the pollen coat at the final stage of pollen maturation (Hernandez-Pinzon *et al.*, 1999). In that final stage, the tapetal cells lyse and release their contents into the loculus where the elaioplasts and tapetosomes are degraded. The elaioplast sterol lipids coat the outside of the pollen grain, whereas the PAPs are degraded and do not appear in the coat (Hernandez-Pinzon *et al.*, 1999). Tapetosomes contribute mainly proteins to the pollen coat–oleosins and oleosin degradation products. So the two organelles, elaioplasts and tapetosomes, work in concert to produce the lipid/protein coat covering the pollen grain surface.

Many plant cells also store oils in separate, non-plastid organelles called oleosomes, which are derived from the rough endoplasmic reticulum and found primarily in seeds (Yatsu *et al.*, 1971). Although perhaps difficult to prove experimentally, oleosomes may be used primarily for longer-term oil storage while elaioplasts function in shorter-term oil synthesis and storage. Their tissue localization (seed storage tissues in the case of oleosomes and actively growing tapetal tissues in the case of elaioplasts) may be evidence of this.

### *3. Proteinoplast (Proteoplast)*

Although all plastids are high in protein content, proteinoplasts (or "proteoplasts") were identified as plastids containing especially large and visible (in both the light and electron microscope) protein inclusions (Newcomb, 1967; Bain, 1968; Esau, 1975; Hurkman and Kennedy, 1976; Casadoro *et al.*, 1977; Thompson and Whatley, 1980). The inclusions can be crystalline or amorphous and are typically enclosed by a membrane.

Textbook authors tend to state with confidence that proteinoplasts are a site of protein storage within the plant cell, as was suggested early on (Price and Thomson, 1967), and probably by analogy to the better-studied starch-storing amyloplasts and oil-storing elaioplasts. Indeed, there is a certain attractiveness to the symmetry of giving each of the three major macromolecules (protein, starch and oils) their own storage plastid. However, a close scrutiny of the literature reveals a picture of the proteinoplast that is much less than definitive. Kirk and Tilney-Basset offer more questions than answers regarding proteinoplast function in their seminal 1967 volume on plastids. Later, in 1980, Thomson and Whatley provided a comprehensive review of the literature and were not able to say with certainty that all "proteinoplasts" indeed function solely as protein storage reservoirs or even if all plastids that contain protein inclusions are true proteinoplasts.

The question of whether proteinoplasts represent a biochemically and metabolically distinct plastid type may have faded from the scientific eye. In fact, a search of electronic bibliographic data bases (Agricola and Science Citation Index) produced no "proteinoplast" or "proteoplast" papers in the last 25 years. This is not to say that plant cells do not store protein. There is a massive literature on storage proteins (particularly in seeds); storage proteins are known to occur in the cytoplasm as seed storage bodies. Their synthesis in the endoplasmic reticulum (ER) and storage in the vacuole have been extensively studied (Herman and Larkins, 1999). However, whether or not a protein-containing plastid variant plays a central role in the anabolism, catabolism and/or storage of protein in any plant tissue remains an open question. The exception may be the well-studied phloem p-type plastids described below in section II.H.

### *F. Chromoplast*

Chromoplasts are brightly colored plastids found in fruits, flowers, leaves and even some roots (see Chapter 21, for a complete review). "Chromo-", of course, comes from the Latin and Greek words for color, and chromoplasts indeed contain large concentrations of red, yellow or orange carotenoids (Juneau

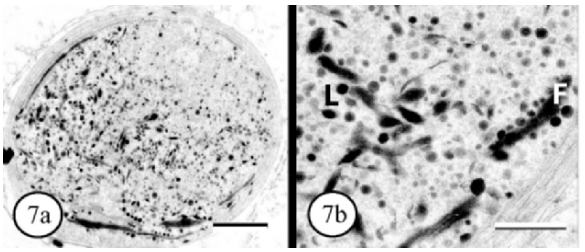



*Fig. 7.* Chromoplasts from fully ripe *Arum italicum* fruits. 7a. A single chromoplast seen filled with lipid droplets and fibrils. Scale bar = 1.0 μm. 7b. Close up of lipid droplets (L) and fibrils (F). Scale bar = 0.1 μm. Micrographs courtesy of Dr. Fasulo Palmira (University of Ferrara, Italy).

*et al.,* 2002). Chromoplasts are generally thought to serve primarily as visual attractors for pollinators and fruit dispersing animals and a brightly colored and highly nutritious attractor molecule such as a carotenoid would be ample reward for any herbivore.

Chromoplasts have an interior dominated by carotenoid/lipid droplets (Fig. 7) and large supramolecular structures called fibrils (Deruère *et al.*, 1994). Fibrils have a carotenoid core, a layer of lipid, and an outer layer composed primarily of a 35 kDa protein called fibrillin. In addition to providing a structure with which to hold the large amounts of carotenoids that accumulate during fruit ripening, fibrils also may serve as a sink to contain excess or toxic lipids produced during fruit ripening (Deruère *et al.*, 1994).

In ripening fruit, chromoplasts most often develop from chloroplasts (Bouvier *et al.*, 1998) although they may arise directly from proplastids in other tissues (Ljubesic, 1972). In the fruit of at least one arum, the developmental pathway is from amyloplast to chloroplast to chromoplast (Bonora *et al.*, 2000). Chromoplasts can also de-differentiate and revert back to chloroplasts under the proper storage and lighting conditions (Devide and Ljubesic, 1974).

Carotenoids such as β-carotene (the orange of carrots) and lycopene (the red of tomatoes) are a significant source of antioxidants in the human diet (Yeum and Russell, 2002). Therefore the enzymology of carotenoid synthesis is an aspect of chromoplast biology that has received considerable attention (Cunningham and Gantt, 1998), particularly during the chloroplast to chromoplast transition (Lawrence *et al.*, 1997; Bruno and Wetzel, 2004).

## G. Gerontoplast

The gerontoplast ("γερoντ" is Greek for "old man") is a biochemically and metabolically distinct plastid

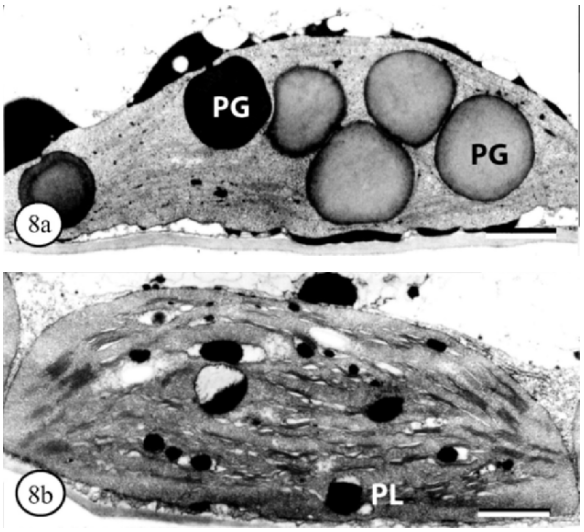



*Fig. 8.* Gerontoplasts from senescing tobacco (*Nicotiana tabacum*) leaves. 8a. A gerontoplast from a yellow leaf with several large plastoglobuli (PG). 8b. A plastid containing protein-lipid extrusion bodies (PL). Micrographs courtesy of Dr. Joseph B. Harris (University of Wisconsin at Stevens Point, USA). Scale bar = 1.0 μm.

variant that develops from a chloroplast during the senescence of foliar tissue (Harris and Arnott, 1973; Parthier, 1988). Gerontoplast formation and fate are extensively reviewed in this volume by Krupinska (Chapter 22) and as such will only be briefly discussed here.

Foliar senescence is characterized by a decline in whole leaf gas exchange and protein levels, leaf yellowing, and the chloroplast-to-gerontoplast transition. Ultrastructurally, gerontoplast development is seen primarily as a progressive unstacking of grana, a loss of thylakoid membranes and a massive accumulation of plastoglobuli (PG; Harris, 1981; Fig. 8a). The plastoglobuli are thought to contain the lipid-soluble degradation products from the thylakoid membranes (Matile, 1992). Other lipid-protein inclusions are often seen in and being exuded from the stroma (Fig. 8b). See Krupinska (Chapter 22) for a full discussion of the nature and fate of these inclusions.

In spite of the thylakoid and stromal changes, most reports state that the gerontoplast envelope remains intact and gerontoplast numbers per cell are stable throughout senescence (Martinoia *et al.*, 1983). Other studies show a decline in gerontoplast number, particularly at the later stages of senescence (Yamasaki *et al.*, 1996).

The function of the gerontoplast, in association with other organelles in an aging cell, is clearly to affect a controlled dismantling of the photosynthetic apparatus during senescence. This control is needed for two

reasons. First, chloroplasts contain approximately 75% of the total leaf protein (Peoples and Dalling, 1988) with the majority of the protein being present in the thylakoid-bound chlorophyll binding proteins and the stromal-localized Rubisco. Therefore, catabolism and remobilization of that protein provides a significant source of amino acids and nitrogen for other plant organs. [It is important to note that chlorophyll breakdown does not result in the liberation and recycling of the pyrrole nitrogen (see Chapter 15), as the final chlorophyll catabolites, and their nitrogen, are deposited in the vacuole; Hörtensteiner and Feller, 2002]. Second, chlorophyll, and its primary degradation products, are extremely photodynamic and potentially toxic molecules (Hörtensteiner, 2004) that must be safely removed to protect the efficient degradation and export of leaf protein.

Not all chloroplasts in a leaf senesce at the same rate. For instance, guard cell chloroplasts are the last plastids in a leaf to degrade during senescence (Ozuna *et al.*, 1985) apparently to allow for the stomatal control of leaf gas exchange until the bitter end.

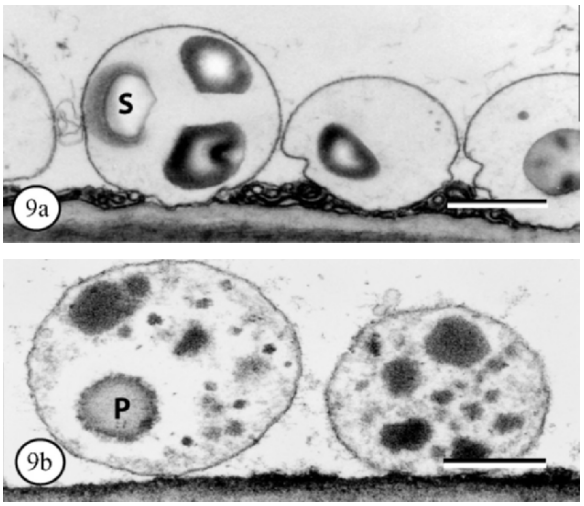



*Fig. 9.* Plastids from phloem sieve tube elements. 9a. S-type plastids from *Lycopersicon esculentum*. 9b. P-type plastids from *Vicia faba*. Scale bar = 1.0 μm. Micrographs courtesy of Dr. Aart van Bel (Justus-Liebig-Universität Giessen, GDR) and Dr. Katrin Ehlers (Institute für Allgemeine Botanik und Pflanzenphysiologie, GDR).

### *H. S-Type and P-Type Plastids in Phloem Sieve Elements*

Phloem tissue is responsible for the transport of photosynthate from source tissues (leaves or other photosynthetic organs) to sink tissues (roots, stem and developing seeds and fruit). Phloem tissue contains four distinct cell types: phloem fibers, phloem parenchyma, phloem companion cells and phloem sieve elements. Long distance transport occurs in the phloem sieve elements while the other phloem cells are involved in support and protection (fibers) or loading and unloading of photosynthate from the sieve elements (parenchyma and companions cells). See van Bel (2003) for a recent and thorough review of phloem structure and function.

The ultrastructure of phloem sieve elements has been the subject of study for decades and the specializations for transport are striking. Sieve elements are anucleate and also lack the large central vacuole that is the hallmark of most other plant cells. The cytoplasm is much reduced and occupies the periphery of the cell in a zone called the parietal layer. Mitochondria, ER, cytoplasmic protein inclusions (called P-protein crystalloids) and plastids are kept in the parietal layer by being anchored to the plasma membrane (Ehlers *et al.*, 2000). Sieve elements are connected end-to-end via perforated sieve plates and form continuous tubes extending from source to sink tissues. Phloem sap flow can therefore proceed practically unimpeded by interfering organelles.

Behnke (1991) characterized phloem plastids as either s-type (starch containing; Fig. 9a) or p-type (protein containing and, in some species, starch containing as well; Fig. 9b). He also noted the taxonomic distribution and value of the various sieve element plastid types. Of 382 dicot families investigated, s-type plastids were found in 320, p-type plastids were found in 48 and 14 families had species with either s-type or p-type plastids (Behnke, 1991). Although the taxonomic value of s- and p-plastid is obvious, the physiological significance of this distribution remains to be established.

The function of sieve element plastids is not fully understood (van Bel, 2003) but they may play a role in the response of phloem to injury (Barclay *et al.*, 1977), particularly the p-type plastids (Knoblauch and van Bel, 1998). Upon mechanical damage, the sieve plates of phloem sieve elements become occluded with protein and cellular debris, thus halting all transport. This would play a large role in a successful defense against a phloem-feeding insect or to prevent the spread of pathogenic bacteria, fungi or virus particles. The severity of the damage influences the severity of the response. A mild stimulus (penetration with a 0.1 μm diameter electrode tip for instance) results in a rapid expansion of the p-protein crystalloids, which then plug the sieve plates. A more severe stimulus (penetration with 1.0 mm diameter electrode tip) disrupts the

p-protein crystalloids and causes an immediate explosion of the p-type plastids as well (Knoblauch *et al*., 1999). The protein contents of both organelles are found at the occlusion site (Knoblauch and van Bel, 1998). Therefore, one function of the p-plastids may be to work in concert with the p-protein crystalloids in the response to severe damage.

S- and p-type sieve element plastids, obviously, may also serve as starch and protein storage reserves, but the presence of starch or protein anabolic or catabolic pathways has not been demonstrated in either organelle (van Bel, 2003).

### *I. Kleptoplast*

Coined in 1990 by Clark and colleagues, the term "kleptoplast" refers to chloroplasts that have been stolen from algal cells and taken up by cells of a host animal ("κλεπτο" from the Greek for "thief") and describes a relationship between animals and algae that was recognized as early as 1904 (see Chapter 23 for a full treatment of this fascinating relationship). Kleptoplasty is fairly common in ciliates, foraminifera and sacoglossan mollusks with the relationship between kleptoplasts and the sacoglossan sea slug *Elysia chlorotica* (phylum Mollusca, subclass Opisthobranchia) being the most advanced and the most studied (Marín and Ros, 2004). In this latter case, the sea slugs feed on algae (usually *Vaucheria litorea*), digest the algal cells and retain only the chloroplasts within the cytoplasm of cells lining the digestive tract. The animals turn bright green and look remarkably like a healthy plant leaf. Kleptoplasts retain their outer and inner boundary membranes and have an ultrastructure seemingly unaltered from that of the natural state (see Fig. 3 Chapter 23).

Kleptoplasts in *Elysia chlorotica* have been shown to retain their photosynthetic capacity after ingestion, in some instances for up to several months. Animals bearing a full complement of kleptoplasts can survive in the light with no external food source for their entire nine-month life span and successfully reproduce whereas animals left in the dark soon die (Rumpho *et al.*, 2001). The basis for kleptoplast longevity and how kleptoplasts are integrated into the host metabolism are areas of active research (see Chapter 23).

### *J. Apicoplast*

Apicoplasts are plastids found in small, parasitic worms of the Apicomplexan family (Phylum Aveolata), most notably *Plasmodium falciparum*, the malarial parasite (see Chapter 24 for a thorough review of apicoplast biology). They were initially described as small cytoplasmic bodies, based on their appearance in the electron microscope (see Fig. 1 Chapter 24). It was not until sequencing of the Apicomplexan genome that genes of certain plastid origin were found and later localized to the apicoplast. The apicoplast genome contains evidence of both red and green algal ancestry, and the story of its origin(s) is continuing to unfold today.

Of primary interest is apicoplast function. What are plastids doing in a parasitic worm? No clear answer is yet available, but specific pharmacological treatments that target apicoplast metabolism are known to inhibit the ability of adult worms to become infective (see Chapter 24). Given that malaria takes more than two million lives a year worldwide (Greenwood and Mutabingwa, 2002) and that drug-resistant *Plasmodium* strains are beginning to appear (Olliaro, 2001), there is considerable interest in gaining a better understanding of apicoplast metabolism and the role this seemingly misplaced organelle plays in the life cycle of the malarial parasite.

## III. Chloroplasts and their Specializations

The chloroplast is the much-studied photosynthetic organelle of algal and plant cells and has been the subject of numerous thorough tomes (Barber, 1976; Reinert, 1980; Hoober, 1984; Halliwell, 1984; Argyroudi-Akoyunoglou and Senger, 1999). Chapters in the present volume and other volumes in this series also cover various aspects of chloroplast biology. As such, chloroplasts will receive only a cursory treatment in this chapter. The reader is referred to Hoober (Chapter 2) for coverage of the basics of photosynthesis.

### *A. Chloroplast Functions Other than Photosynthesis*

Photosynthesis, the light-dependent synthesis of low-molecular-weight, reduced carbon compounds from atmospheric carbon dioxide, is the major, but not only, function of chloroplasts (Neuhaus and Emes, 2000). In addition to carbon reduction (see Chapter 14), chloroplasts are also the site of carbon oxidation via photorespiration (Ogren, 1984), chlorophyll synthesis (see Chapter 15), carotenoid, α-tocopherol (vitamin E), plastoquinone and phylloquinone (vitamin K) synthesis (see Chapter 16), fatty acid and lipid synthesis (see Chapter 17), nitrogen assimilation and amino acid synthesis (see Chapter 18), sulfur metabolism (see

Chapter 19), oxygen metabolism (see Chapter 27), and a even series of oxidative reactions called chlororespiration (see Chapter 12). Apparently, evolutionary processes have taken advantage of the biosynthetic power and flexibility of plastids, and selected them to be, in many ways, the multifunctional anabolic factories of plant cells.

### *B. Chloroplasts in Plants with the $C_3$ Photosynthetic Pathway*

Ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) is the primary carboxylating enzyme in $C_3$ plants and the first stable form of fixed carbon is the three-carbon acid phosphoglycerate (hence the name $C_3$). Rubisco is found in the $C_3$ chloroplast and is probably the most abundant protein on the planet.

When viewed in the light or electron microscope, $C_3$ chloroplasts are plano-convex discs approximately 5 to 10 μm in diameter (Fig. 10a, c). The protein-rich, semi-gel, aqueous chloroplast interior is called the stroma, and is where the enzymes of the Calvin-Benson cycle are found and in which the green thylakoid membranes are suspended. Thylakoids are divided into two types. Those appressed to another thylakoid in a stack called a granum (from "granule") are "appressed" or "granal" thylakoids. Those that found singly and are not part of a granum are "non-appressed", "stromal", or "agranal" thylakoids (Fig. 10b, d). The stroma may also contain plastoglobuli, which are lipid/protein particles found in the stroma whose number and size increase during senescence (Fig. 8; see Chapter 22). Plastoglobuli are seen in non-senescent chloroplasts as well (Fig. 13a). Recent work in the author's laboratory indicates that plastoglobuli in cotton chloroplasts contain about a dozen proteins, the identities of which are currently being elucidated (K. Olsen and R. Wise, unpublished data).

Chloroplasts are enclosed by a double-membrane envelope composed of an outer and an inner membrane (Fig. 10b). A common misconception seen in most biology texts is that the double-membrane envelope of the chloroplast is similar to the double-membrane of the mitochondrion. This is conceptually incorrect. It is true that the outer mitochondrial membrane and the outer chloroplast envelope membrane are analogous in that both are rather minimal barriers between the cytoplasm and the organellar interior (Salomon *et al.*, 1990; Sardiello *et al.*, 2003; see Chapter 14). However, the inner mitochondrial membrane and the inner chloroplast envelope membrane are very different in structure and function. The inner mitochondrial membrane is the site of oxidative phosphorylation,

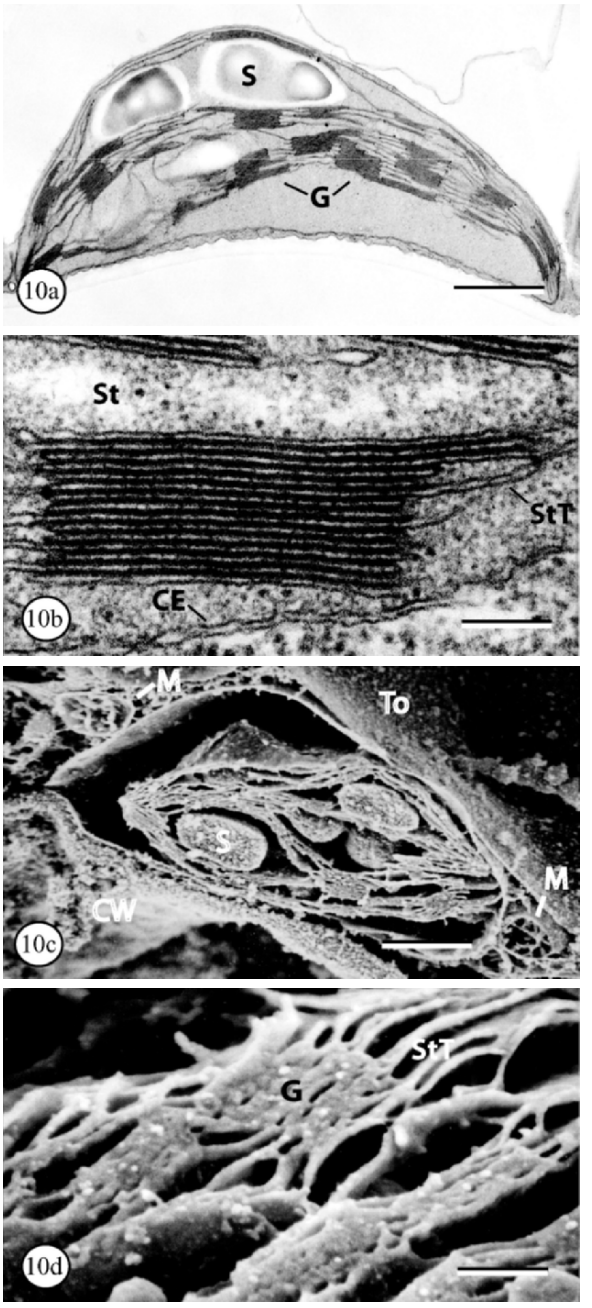



*Fig. 10.* Chloroplasts from spinach (*Spinacea oleracea*), a $C_3$ plant. 10a. A transmission electron micrograph of an entire choloroplast in thin section showing grana (G) and starch grains (S). Scale bar = 1.0 μm. 10b. High magnification view of a single granum in thin section showing the stroma (St) and a stromal thylakoid (StT). The two membranes of the chloroplast envelope (CE) are also visible. Scale bar = 0.1μm. Micrograph courtesy of Dr. Joseph B. Harris, University of Wisconsin at Stevens Point, USA). 10c. A low voltage, high resolution scanning electron micrograph of a pea ( *Pisum sativum*) chloroplast containing several starch grains (S). Also visible are mitochondria (M), the tonoplast (To) and a cell wall (CW). Scale bar = 1.0 μm. 10d. High magnification view of a several grana (G) and stromal thylakoids (StT). Scale bar = 0.1 μm.

while the inner chloroplast envelope membrane controls metabolite transport (see Chapter 14) and synthesizes fatty acids, lipids, carotenoids, and prenyl lipids (see Chapter 16, Chapter 17). The inner chloroplast envelope membrane has no corresponding structure in the mitochondrion, making the thylakoid membrane (the site of photophosphorylation) the operative counterpart to the inner mitochondrial membrane. Therefore, when textbooks make statements such as, "Like chloroplasts, (mitochondria) both have an outer membrane and an inner membrane" (Raikhel and Chrispeels, 2000) they run the risk of misleading the reader into believing that the two organelles are bounded by homologous or analogous double-membraned structures, which they clearly are not.

### *C. Dimorphic Chloroplasts in Plants with the $C_4$ Photosynthetic Pathway*

$C_4$ photosynthesis has two spatially-separated carbon fixation steps; a primary fixation and a secondary fixation. Phospho *enol*pyruvate carboxylase (PEPcase) is the primary carboxylating enzyme in $C_4$ plants. Its substrates are PEP and $CO_2$ and its product is the four carbon acid oxaloacetate (OAA), the first stable form of fixed carbon in $C_4$ photosynthesis and the source of the name "$C_4$." The OAA is decarboxylated and the resulting $CO_2$ is secondarily fixed by Rubisco. There are three basic forms of $C_4$ photosynthesis that differ in the type of four-carbon acid (malate or aspartate) transported between the two fixation steps and the site of decarboxylation (Siedow and Day, 2000). The $C_4$ photosynthetic pathway has arisen at least 45 times over evolutionary time (Sage, 2004) and the details of the pathway sketched above vary somewhat from species to species. That detail will not be discussed here.

PEPcase (the site of primary $CO_2$ assimilation) and Rubisco (the site of secondary $CO_2$ fixation) are spatially separated in $C_4$ plants. Classic $C_4$ plants have a Kranz-type leaf anatomy in which the vascular bundle is separated from the leaf mesophyll cells by a layer of cells called the bundle sheath (Laetsch, 1974). PEPcase

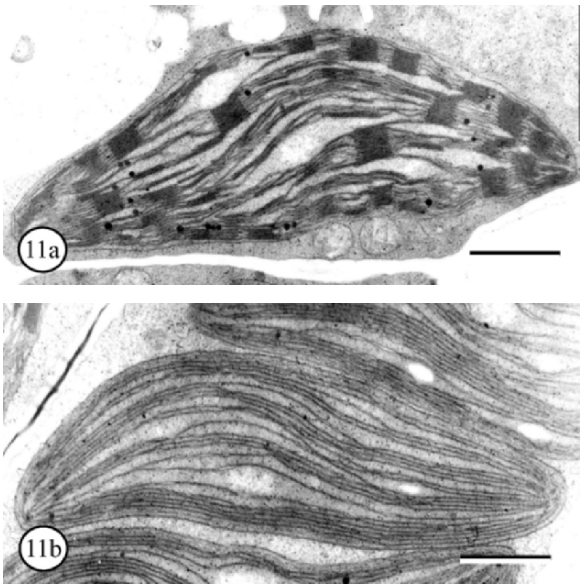



*Fig. 11.* Chloroplasts from a $C_4$ plant (*Zea mays*). 11a. Mesophyll cell chloroplast. 11b. Bundle sheath cell chloroplast. Note the lack of grana in the BSC chloroplast in 11b. Scale bar = 1.0 μm for both.

is found in the cytoplasm of the leaf mesophyll cells (MC) while Rubisco is in the bundle sheath cell (BSC) chloroplasts. MC chloroplasts contain grana with both PSI and PSII but lack Rubisco and are starch-deficient (Fig. 11a). BSC chloroplasts are agranal and lack PSII but have Rubisco and are laden with starch at the end of the photoperiod (Fig. 11b; see also Table 2). These dimorphic chloroplasts play vital and unique roles in $C_4$ photosynthesis (Fig. 12).

In classic, Kranz-type $C_4$ photosynthesis, the four-carbon acid generated by PEPcase diffuses through plasmodesmata from the mesophyll cell cytoplasm to the bundle sheath cell and is decarboxylated in either the BSC cytoplasm, mitochondrion or chloroplast (depending on $C_4$ type). Regardless of which form the carbon takes, pyruvate and $CO_2$ are generated upon decarboxylation. The pyruvate diffuses back to the mesophyll cell, enters the MC chloroplast and is phosphorylated to PEP. The PEP then feeds the MC

*Table 2.* Characteristics of the dimorphic chloroplasts of $C_4$ plants.

| Mesophyll cell | Bundle sheath cell |
|---|---|
| Granal chloroplasts contain both PSII and PSI | Agranal chloroplasts only contain PSI |
| $O_2$ evolution and whole chain e-transport | No $O_2$ evolution and no whole chain e-transport |
| Thylakoids generate both ATP and NADPH | Thylakoids only generate ATP |
| Chloroplasts reduce 3PGA to triose-$PO_4$ | 3PGA transported to MCC for reduction |
| Chloroplasts phosphorylate pyruvate to PEP; PEP used in cytoplasm for 1°C fixation | Calvin-Benson cycle intermediates regenerated using triose-$PO_4$ from MCC |
| No starch | Starch present at end of light cycle |

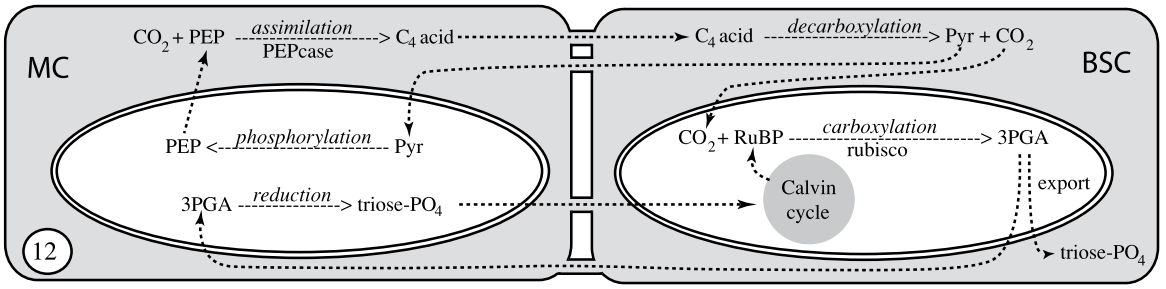
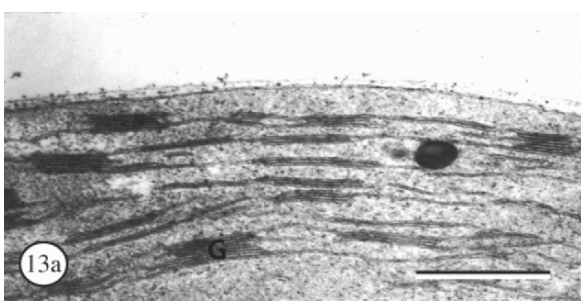



*Fig. 12.* Schematic of the role of dimorphic chloroplasts in $C_4$ photosynthesis. Carbon dioxide is initially fixed in the mesophyll cell (MC) on the left into a $C_4$ acid which is transported to an adjacent bundle sheath cell (BSC) for decarboxylation. The resulting pyruvate is returned to the MC chloroplast for phosphorylation to PEP. The $CO_2$ is fixed by Rubisco in the anoxic BSC chloroplast which, due to a lack of PSII, cannot reduce the resulting 3PGA to the level of a sugar. Therefore, the 3PGA is transported to the MC chloroplast for reduction to a triose-phosphate which can be return to the BSC chloroplast where it enters the Calvin-Benson cycle.

cytoplasmic PEPcase. The $CO_2$ released upon decarboxylation in the BSC is fixed into 3-phosphoglyceric acid (3PGA) in the Rubisco-containing BSC chloroplasts. Thus, one advantage of shuttling carbon into bundle sheath cells is that it is a means to concentrate carbon dioxide at the site of $CO_2$ fixation by the relatively inefficient Rubisco (see Chapter 13). Another equally important advantage is that the carbon is concentrated in a compartment that is essentially oxygen free, because BSC chloroplasts lack PSII and are hence non-oxygenic. It is obvious, however, that BSC chloroplasts also lack the whole-chain electron flow needed to reduce 3PGA to the level of a triose, the ultimate product of the Calvin-Benson cycle. Therefore, the 3PGA is shuttled from the BSC chloroplast to the MC chloroplast where it is reduced (using the NADPH from whole-chain electron transport) before returning to the BSC chloroplast for use in carbon export, starch synthesis or regenerating Calvin-Benson cycle intermediates. Although PSII-deficient BSC chloroplasts are incapable of evolving oxygen and reducing $NADP^+$, but they are fully capable of generating the proton gradient (via PSI cyclic electron flow) needed to supply the ATP required for the Calvin-Benson cycle. So the MC chloroplasts supply the electron flow needed for carbon reduction and the ATP used to regenerate PEP, while the BSC chloroplasts perform carbon fixation and Calvin-Benson cycle activity in a $CO_2$-sufficient and $O_2$-deficient environment. Photorespiration (i.e. the energy-wasting consumption of oxygen by Rubisco) is therefore completely eliminated in $C_4$ plants.



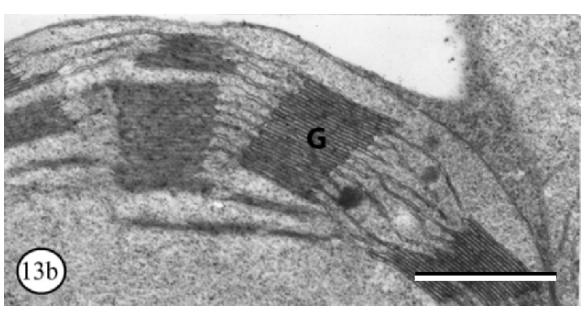




*Fig. 13.* Spinach ( *Spinacea oleracea*) chloroplasts from a high-light-grown leaf (13a) and a low-light-grown leaf (13b). Note the much larger grana in the low-light-grown leaf. Micrographs courtesy of Dr. Ichiro Terashima (Osaka University, Japan). Scale bar = 0.5 μm.

Not all $C_4$ plants show the extreme anatomical differences seen in Kranz anatomy. In some, the cell types show no anatomical differences (although they are biochemically distinct) and in others, called single-cell $C_4$ plants, the two stages of $C_4$ photosynthesis are at the opposite ends of the same cell (Edwards *et al.*, 2004). Regardless of the anatomy of the $C_4$ type, the presence of dimorphic chloroplasts is obviously critical to $C_4$ photosynthesis as even single-cell $C_4$ species have both granal and agranal chloroplasts in the same cell (Voznesenskaya *et al.*, 2004).

## *D. Sun and Shade Chloroplasts*

Leaves, and the chloroplasts therein, are exposed to diverse and fluctuating light environments. Some plants

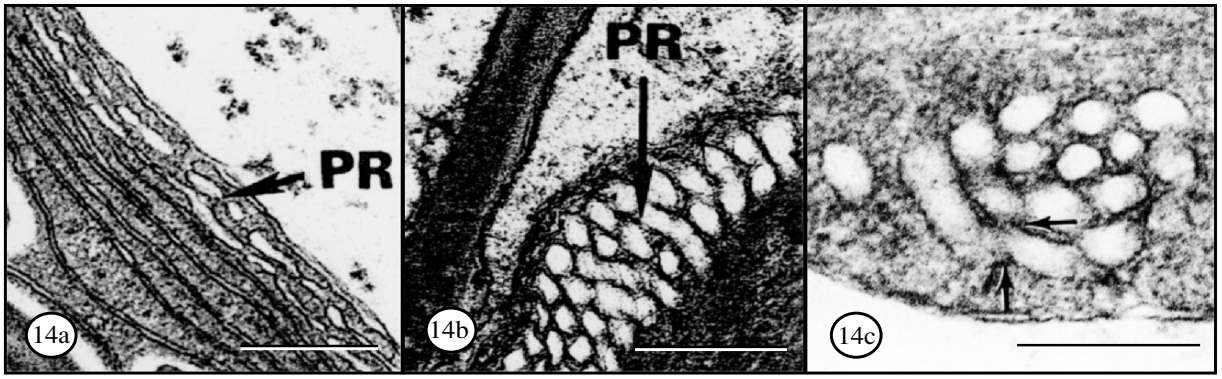



*Fig. 14.* Chloroplast peripherial reticulum. 14a. PR in the plastids of *Zea mays*, a $C_4$ plant, showing an extensive double row of tubules. Micrograph from Gracen *et al.* (1972) with permission. 14b. PR in plastids of the $C_4$ plant *Eragrostis curvula* demonstrating large groups of tubules. Micrograph from Gracen *et al*. (1972) with permission. 14c. PR in plastids of *Cyphomandra betacea* showing individual vesicles attached to the chloroplast inner envelope membrane. Micrograph from Wise and Harris (1984), with permission. Scale bar = 0.5 μm in each panel.

are adapted to high light growth conditions, while others thrive only in the shade. In addition to occupying sun or shade light environments, leaves may also be exposed to variable light conditions on a seasonal, diurnal, minute or even second time scale. Rapid changes in irradiance due to cloud cover or sun flecks can attenuate as much as 90% of the incoming irradiance in a matter of seconds and just as quickly return (Pearcy, 1990). Therefore, chloroplasts have evolved to be able to adjust to changes in light quantity and quality on time scales ranging from seasons to seconds.

Sun chloroplasts are found in leaves exposed to high light during development; shade chloroplasts develop in the shade (Björkman and Holmgren, 1963), demonstrating that chloroplast development is under tight control by the light environment (Oquist *et al.*, 1992). As compared to shade chloroplasts, sun chloroplasts have fewer grana, fewer thylakoids per granum, less total thylakoid area (Fig. 13), smaller light-harvesting antennae, and higher activities of enzymes of carbon reduction (Lichtenthaler *et al.*, 1981; Anderson *et al.*, 1988). Therefore, sun chloroplasts are adapted to take advantage of light-sufficient, $CO_2$-deficient conditions while shade chloroplasts are adapted to perform optimally under light-deficient, $CO_2$-sufficient conditions.

There are both spatial and temporal components to sun/shade chloroplast development. First recognized as a season-long, developmental phenomenon (Björkman and Holmgren, 1963) sun/shade differences show up wherever and whenever chloroplasts are exposed to different light conditions. The classic spatial example is the differences seen in chloroplasts found at the periphery (high light) or interior (shade) of a single tree crown (Eschrich *et al.*, 1989). However, Terashima and Inoue (1985a,b) found sun/shade differences in ultrastructure and biochemistry within a single spinach leaf, depending on distance from the adaxial (irradiated) leaf surface. It does not take a season-long developmental time scale for sun/shade chloroplasts to develop, In fact, granal numbers and granal areas increase after only a 10 minute shade treatment; the effect is fully reversible with 10 minutes of high light (Rozak *et al.*, 2000).

## *E. The Chloroplast Peripheral Reticulum*

The chloroplast peripheral reticulum (PR) is a system of anastomosing tubules and saccules lying along and frequently attached to the inner membrane of the chloroplast envelope. It was first reported in 1967 by Shumway and Weier in maize chloroplasts and has since been found in numerous other species (Wise and Harris, 1984). The latter authors recognized three morphological types of PR. The most complex form appears as distinct single or double rows of tubules in mesophyll cell chloroplasts of $C_4$ plants (Laetsch, 1968; Sprey and Laetsch, 1978; Fig. 14a). A second type appears in both $C_3$ and $C_4$ plants and consists of large groupings of tubules (Fig. 14b) ranging from small, single vesicles to large elaborate rows of tubules or sacs (Gracen *et al*., 1972). A third possible PR variety is represented by individual vesicles or saccules attached in many places to the chloroplast inner envelope membrane (Fig. 14c).

Because the inner membrane is the site of control over metabolite transport in to and out of the chloroplast (Heldt and Saur, 1971; see Chapter 14), the chloroplast

PR is usually considered to be an adaptation for increased transport via increased surface area. This certainly makes sense for the large, elaborate networks seen in many plants. However, the small, individual vesicles may represent transport vesicles from the inner envelope membrane to the thylakoid as discussed by Vothknecht and Soll (Chapter 3).

PR often increases in chloroplasts exposed to stresses such as chilling (Kratsch and Wise, 1998; Kutik *et al*., 2004), drought (Utrillas and Alegre, 1997), and *Fusarium* infection (Achor *et al*., 1993). Whether this serves to increase metabolite transport or is merely a non-specific response to biotic and abiotic stress is not known.

The chloroplast PR should not be confused with an association between the chloroplast and endoplasmic reticulum seen in many algae called the chloroplast endoplasmic reticulum (Gibbs, 1981).

### *F. Amoeboid Plastids*

Amoeboid plastids have been defined as any plastid that has protrusions of the envelope. They were first recognized by early light microscopists (Senn, 1908 in Thomson and Whatley, 1980; Weier, 1938; Esau, 1944) but their exact function, or if indeed their unique shape represents any specific function, is not clear. Electron microscopical studies have found amoeboid plastids in developing leaves (Dvorak and Stokrova, 1993) and roots (Gardner *et al.*, 1989) and in leaves and tepals of members of the Amaryllidaceae (Scepánková and Hudák, 2003). They are also common in the leaves of chilling stressed tissues (Wise *et al.*, 1983; Musser *et al.*, 1984).

Thomson and Whatley (1980) suggested that amoeboid plastids might represent a transitionary stage between the proplastid and chloroplast. However, no one has demonstrated a definite role for amoeboid plastids in any tissue or why environmental stress might cause their formation. Their relationship with the plastid stromules discussed below is also an enigma.

### *G. Plastid Stromules*

Recently, exciting new experiments using green-fluorescent-protein- (GFP) labeled stromal proteins and laser scanning confocal microscopy (LSCM) have been able to show dynamic extensions of the plastid envelope that have been named plastid tubules (Shiina *et al.*, 2000) or plastid stromules (stroma-filled tubules; Köhler and Hanson, 2000; Pyke and Howells, 2002) through which stromal contents can be exchanged from one plastid to another. Their existence had been noted for several years (see Köhler and Hanson, 2000 for a review of the early literature), but it was not until researchers were able to generate transgenic plants containing GFP fusion proteins that stromules could be studied in detail. Stromules have been visulaized extending from proplastids, chloroplasts, etioplasts, leucoplasts, amyloplasts, and chromoplasts (Natesan *et al.*, 2005).

Plastid stromules are up to 220 μm long and 0.4 to 0.8 μm in diameter (Natesan *et al.*, 2005). As a point of reference, mature chloroplasts are typically 5–10 μm in diameter. Stromules are rare on plastids in green tissues and abundant in roots and petals (Köhler and Hanson, 2004) and in other non-green tissues (Waters *et al.*, 2004). Arimura *et al.* (2001) found them to be common on plastids in epidermal cells of several plant species. Plastids in tobacco tissue culture cells contain two distinct tubule morphologies (Köhler and Hanson, 2000). Most cells contained plastids surrounding the nucleus with long, "octopus" or "millipede-like" stromules that extended throughout the cell. Other cells contained plastids distributed throughout the cell that bore short stromules. The functional significance of these two different stromule morphologies is not presently known.

Stromule dynamics are directed by the actin cytoskeleton and require ATP-dependent myosin activity (Gray *et al.*, 2001). Microtubules also play a role as tubulin inhibitors affect the formation and movement of stromules (Kwok and Hanson, 2003). Neither microfilaments nor microtubules are needed for GFP-labeled protein exchange between chloroplasts once they have been connected by a stromule (Kwok and Hanson, 2003).

Stromules may serve two purposes. First, because they can connect one plastid to another they allow the exchange of metabolites (Köhler and Hanson, 2000; Gray *et al.*, 2001, Natesan *et al*., 2005) or perhaps even plasmids (as suggested in Knoblauch *et al.*, 1999) between two plastids. Alternatively, many, if not most, stromules may just be blind sacs that increase the area of chloroplast envelope available for metabolite transfer between the plastid and cytoplasm (Waters *et al.*, 2004, Natesan *et al*., 2005).

Whether or not the protrusions of amoeboid plastids and plastid stromules are related in any fashion is not clear. Amoeboid plastid protrusions seen in the transmission electron microscope (TEM) are generally quite large, much larger than the thin plastid stromules that have been reported in numerous LSCM studies. It is possible that the fixation and thin-sectioning required for TEM might obscure or miss the very long plastid

stromules, leading electron microscopists to only report the larger protrusions. Or perhaps relatively slow-acting EM fixatives might not be able to capture the rapidly changing stromules. Alternatively, GFP labeling and LSCM might not pick up on the large, but relatively anonymous amoeboid protrusions against a sea of dynamic stromules. It is also possible the two phenomena have no direct relationship at all.

### *H. Chloroplasts within Stomatal Guard Cells*

Guard cells control the exchange of gases between the leaf and atmosphere by opening or closing the stomatal aperture. In the process of stomatal opening, a signal to open is sensed by the guard cell chloroplasts and that information is used to direct the synthesis (or release) of cytoplasmic solutes that induce a large drop in cell water potential. This causes an influx of water, which inflates the guard cells and leads to stomatal opening. Closure involves the reverse flux of water. The nature of the signal (blue light, red light, $CO_2$), the signal perception mechanism, the signal transduction route, the osmolyte involved (sucrose, potassium, malate) and the role of ABA in causing stomatal closure have all received considerable attention (Assmann, 1999; Schroeder *et al.*, 2001; Zeiger *et al.*, 2002; Tallman, 2004) and will not be covered here.

Guard cell chloroplasts (GCC) are similar in appearance to $C_3$ mesophyll cell chloroplasts, but typically have fewer thylakoids (Lascève *et al.*, 1997). They contain a fully functional electron transport chain with the usual photosynthetic pigments (Zeiger *et al.*, 1980) and both photosystems (Outlaw *et al.*, 1981). They also contain the zeaxanthin/violaxanthin, energy quenching cycle (Srivastava and Zeiger, 1995). Guard cell chloroplasts are capable of whole chain electron transport (Fitzsimons and Weyers, 1983), photophosphorylation (Shimazaki and Zeiger, 1985), carbon fixation (Gotow *et al.*, 1988) and starch synthesis. In short, guard cell chloroplasts are fully able to conduct all aspects of photosynthetic carbon fixation.

Curiously, however, guard cell chloroplasts probably contribute virtually nothing towards the carbon needs of the plant. They are used, instead, as light and $CO_2$ sensors, signaling and probably driving the opening and closing of stomata, and their physiology has been a matter of debate for decades. Zeiger *et al.* (2002) highlight the long-term controversy surrounding guard cell chloroplast function and conclude that much of the disagreement comes from the fact that guard cell chloroplast metabolism is very plastic. Under various conditions and at various times of the day, the GCC response to environmental conditions can change, even reversing itself entirely. Growth and experimental conditions (greenhouse vs. growth chamber) can have a large impact on guard cell chloroplast sensitivity to both $CO_2$ (Frechilla *et al.*, 2002) and light (Frechilla *et al.*, 2004) and this variability has been proposed to be the source of much of the conflict in experimental results (Zeiger *et al.*, 2002). The mechanism and function of GCC is an area of active research.

### *I. The Three-Dimensional Structure of Chloroplasts–The Need for a Model*

The transmission electron microscope was developed in 1931 and early models became commercially available on a very limited basis in about 1938 (Hawkes, 1972; Rasmussen, 1997). Chloroplasts were one of the first biological specimens to be examined in this revolutionary instrument (Kausche and Ruska, 1940; Algera *et al.*, 1947; Granick and Porter, 1947) and plant scientists have spent the ensuing 60 years attempting to describe the complicated three-dimensional structure of the thylakoid membrane system, with some success. Staehelin (2003) has written an excellent review of the history of and progress in chloroplast ultrastructure research and while the three-dimensional structure of overall thylakoid membrane system in the $C_3$ chloroplast may well have been elucidated (Staehelin, 2003), the relationship between individual granal and stromal thylakoids has yet to be described and modeled. This is a difference with a large distinction as a mathematical, topological model of thylakoid 3-D structure would be a very useful tool in photosynthesis research.

None of the schemes of thylakoid three-dimensional structure have been able to do for the thylakoid-based light reactions what several well-established mathematical models have done for the reactions of photosynthetic carbon metabolism. Tenhunen *et al.* (1976) and Farquhar *et al.* (1980) proposed robust models of carbon fixation nearly 30 years ago. Those models sought to explain the measured rate of carbon uptake by a leaf and included terms for atmospheric carbon dioxide and oxygen concentrations, stomatal conductance, the diffusivity of oxygen and carbon dioxide through both air and water, Rubisco carboxylation activity, and Rubisco oxygenation activity. Later refinements included algorithms for adjusting each of the above parameters as a function of air and leaf temperature (Bernacchi *et al.*, 2002). By manipulating and/or measuring the various parameters, a researcher can ask specific questions about biochemical limitations to whole-leaf gas exchange in both laboratory and field settings. For

example, in a recent study, we were able to establish that photosynthesis in field-grown cotton plants is limited by electron transport capacity during the hottest times of the day (Wise *et al.*, 2004). Therefore, by utilizing mathematical models of carbon fixation, a tremendous amount of biochemical and bioenergetic information can be extracted from simple measurements of whole leaf gas exchange under defined conditions.

The pathway of photosynthetic electron transport in the thylakoid membrane is well known (see Chapter 2). In brief, electrons are extracted from water at the oxygen evolving complex (OEC) and then travel through the various components of PSII, into the plastoquinone (PQ) pool, through the cytochrome $b_6/f$ complex and on to plastocyanin (PC) before they enter PSI and eventually reduce $NADP^+$ to the level of NADPH. Alternative roues of electron flow such as Q loops within the $b_6/f$ complex (Kurisu *et al.*, 2003), cyclic electron flow around PSI (see Chapter 12) and oxygen reduction by PSI (see Chapter 27) are also well described. PQ and PC mediate intersystem electron transport. Upon reduction by PSII, plastoquinol diffuses through the plane of the membrane to the cytochrome $b_6/f$ complex where it is oxidized. Likewise, plastocyanin shuttles reducing equivalents from Cyt $b_6/f$ to PSI by diffusing within the aqueous lumenal space.

Thylakoid membrane architecture is similarly well understood. Years of research have shown that the majority of PSII complexes are localized to the appressed thylakoid regions, PSI and the ATP synthase complex are restricted to the unappressed regions, and cytochrome $b_6/f$ is equally distributed in both (reviewed in Staehelin and van der Staay, 1996). This lateral heterogeneity of the photosynthetic complexes, and indeed thylakoid appression itself, is maintained by attractive forces between the light harvesting antenna protein complexes surrounding PSII (Ryrie *et al.*, 1980).

Hence, the need for PQ and PC diffusion immediately becomes clear—the photosystems are not adjacent neighbors and the total distance between any one OEC and the final site of $NADP^+$ reduction might be substantial, on the order of a hundred nanometers or more (Dekker and Boekma, 2005). This is quite unlike the situation in mitochondrial electron transport in which it is becoming clear that the various protein complexes in the energy-transducing, inner membrane form large super complexes no more than 40 or 50 nm in diameter (Dudkina *et al.*, 2005, Frey *et al.*, 2002). Not only is the photosynthetic electron transport chain potentially quite long, it is also not of fixed length. Varying light conditions can rapidly induce a phenomenon known as a state transition in which the distribution of PSII and PSI, and the degree of thylakoid appression, change in concert (Bennett, 1991). State transitions redistribute and equalize absorbed light energy between the two photosystems, thus balancing their relative turnover rates (Allen, 1992). State changes also lead to measurable differences in grana size and thylakoids per granum in as little as 10 minutes (Rozak *et al.*, 2000). Because PSII is restricted primarily to the appressed granal thylakoids and PSI to the unappressed stromal thylakoids, changes in stacking will alter the diffusional distance between the photosystems. Even though the diffusional coefficients of both PQ (Blackwell *et al.*, 1994) and PC (Haehnel *et al.*, 1989) are known, their diffusional distances under low light, high light, state 1 or state 2 conditions vary and therefore we are unable to include a term for PQ or PC diffusion in any calculations of whole chain electron transport. In short, we know *how fast* the intersystem carriers can move, but we don't know *how far* they have to move to support whole chain electron transport in a three-dimensional and ever changing thylakoid membrane.

A mathematical, topological model of thylakoid three-dimensional structure would allow bioenergeticists to ask novel questions about whole-chain and intersystem electron transport, similar to the sorts of questions made answerable with models of whole-leaf carbon exchange (Farquhar *et al.*, 2001) and whole-leaf electron transport (Buckley and Farquhar, 2004). Cell biology imaging techniques continue to advance and a combination of electron tomography (as suggested by Wildman *et al.*, 2004), low-voltage, high-resolution scanning electron microscopy (Mannella *et al.*, 1994; Wise, 1996; Fig. 10b, d), and confocal microscopy of living chloroplasts would provide a reasonable approach to generating the physical data needed to develop and test a topological model of thylakoid architecture.

Note added in proof: Shimoni *et al.* (2005) recently used electon tomographic techniques to visualize the three-dimensional architecture of a single, dark-adapted granum from a lettuce chloroplast. These data provide a much needed basis for a deeper understanding of the relationship between the structure and function of the photosynthetic light reactions.

## IV. Concluding Remarks

Plastids have been at the center of plant research for over 100 years and have been shown to be truly remarkable organelles. Interest in plastid biology extends well beyond photosynthetic studies. Plastid diversity

and biosynthetic flexibility have been noted throughout this chapter and this volume. Active areas of interest include: the endosymbiotic origin and subsequent evolution of plastids, the signaling between and interaction of the plastid genome with the nuclear genome, protein import into plastids, the light regulation of gene expression and chloroplast development, the various plastid-based anabolic pathways, the photoregulation of chloroplast metabolism, and the bioenergetics of light absorption, electron transfer and ATP synthesis.

The dynamic nature of plastids is something at which to marvel. Plastid development starts with a proplastid and can lead to any one of almost a dozen different mature plastid types ranging in color from white to red to green. Plastids have even found themselves a home in the gut of marine mollusks and the cytoplasm of parasitic worms. The flux of energy through the photosynthetic pathway goes from zero at predawn to a daily maximum at the sun's zenith and then back to zero at dusk. No other organelle has such a huge range of metabolic activity over the course of a day and in the face of environmental pressures such as heat, sunflecks, drought, and cold. Plastids have to respond to their environment on various time scales. Sun/shade chloroplast development, chloroplast movements, state transitions, thylakoid stacking changes, and the violaxanthin/zeaxanthin cycle are all evidence of the inherent plasticity of plastids. No other family of organelles in the plant cell, indeed perhaps in all of eukaryotes, is as flexible and dynamic. Other chapters in this volume will focus on and highlight the various members in this extraordinary family of eukaryotic organelles.

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# Chapter 2

# Chloroplast Development: Whence and Whither

J. Kenneth Hoober*
*School of Life Sciences, and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287, U.S.A.*

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## Summary

The history of the chloroplast is a remarkable story, with an improbable beginning. Yet were it not for the chloroplast, many forms of life would not exist and we would not have the opportunity to look back on its history. The chloroplast provides much of the nutritional base on which animals survive. Its ability to perform photosynthesis is foundational for life on the surface of the earth. Although the plastid varies dramatically in structure and function, the organelle has a monophyletic origin. Much effort has been spent on research to understand the evolution of the plastid and development of its characteristic features. The chloroplasts in green algae and plants have been studied most. Biochemical processes that describe development of the organelle have been extensively studied, and most of the regulatory mechanisms initiated by, and in response to, exposure to light are known in outline form. Although much of the biochemical pathways in the chloroplast do not involve chlorophyll directly, the pigment plays a key role in development of the organelle and through photosynthesis captures the energy in light to drive biosynthetic reactions. Assembly of photosynthetic complexes and of the thylakoid membrane in which these complexes reside involves specific interactions between chlorophyll and proteins. The mechanism of these interactions, and the chemical features that provide their specificity, are beginning to become clear. The surprisingly dramatic differences in the functions of the individual chlorophyll species are determined by seemingly simple chemical modifications. This chapter concludes with a brief summary of the photosynthetic processes that occur within the chloroplast.

*Author for correspondence, email: khoober@asu.edu

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## I. Introduction

The photosynthetic capability of plants sustains the biosphere, and consequently much effort has been directed toward understanding this process. The reactions of photosynthesis occur in the chloroplast, an organelle of great interest. However, as demonstrated by the chapters in this volume, the plastid is a remarkable organelle that is of fundamental important for other functions as well, in particular, the transformation of carbohydrates produced by photosynthesis into other essential compounds. Metabolism in the chloroplast is the major path for entrance of nitrogen and sulfur into eukaryotic cells and it provides the "essential" amino acids and fatty acids necessary for animal life. Among other activities, the isoprenoid compounds that animals require for vision and good health are derived from precursors synthesized in the chloroplast. These functions, which are critically important for animal life, are described in subsequent chapters. This chapter will provide a general overview of the characteristics of plastid development and, in brief, a summary of photosynthetic activities.

## II. Brief Review of Plastid Evolution

### *A. Origin of the Plastid*

A schematic representation of the relationships between organisms that contain plastids is presented in Fig. 1. Eukaryotic cells emerged from the archaebacterial lineage about 2,700 million years ago (MYA) (Hedges *et al.*, 2001). The endosymbiotic event by which a eukaryotic cell entrapped an $\alpha$-proteobacterial ancestor, which led subsequently to mitochondria, occurred about 1,900 MYA (Hedges *et al.*, 2004). Phylogenetic evidence suggests, however, that photosynthesis emerged within the purple bacterial lineage somewhat earlier than 3,500 MYA (Xiong *et al.*, 2000). Two analyses, using a molecular timescale, closely agree that the event that caused divergence of plants and animals, i.e., the endosymbiotic union of the eukaryotic host cell with a photosynthetic, prokaryotic, ancestral cyanobacterium that led to plastids, occurred about 1,600 MYA (Hedges *et al.*, 2004; Yoon *et al.*, 2004). These analyses have shown that all plastids, regardless of the eukaryotic organism in which they reside, have a monophyletic lineage (Bhattacharya and Medlin, 1998; Yoon *et al.*, 2002). The over-arching and surprising conclusion that emerges from studies of the evolution of plastids is that the endosymbiotic event that led to this organelle occurred only one time, with rapid reduction of the organelle genome through either gene loss or migration of most of the endosymbiont DNA to the nucleus (Martin *et al.*, 1998; Palmer, 2000; Timmis *et al.*, 2004). Yoon *et al.* (2004) date the earliest divergence of plastid-containing organisms, which led to the glaucophyte lineage, at 1,558 MYA. The split into red and green algae occurred about 1,500 MYA (Yoon *et al.*, 2004). A subsequent divergence in the green lineage about 1,200 MYA led eventually to land plants, which first appeared 432 to 476 MYA, followed by seed plants 355 to 370 MYA. The split between gymnosperms and angiosperms occurred 290 to 320 MYA, and that between monocots and eudicots 90 to 130 MYA (Yoon *et al.*, 2004). The precision of these estimates was made possible by the wealth of genome sequences that has accumulated over the past decade.

Although drastically reduced in size, the genome of plastids retained the circular DNA structure of the prokaryotic genome. In addition to genes for ribosomal and transfer RNA, the information in algal plastid genomes range from 20 to 200 sequences that encode proteins. A total of 274 protein-coding genes have been identified in sequenced plastid genomes, but only 44 of these exist in all genomes. Approximately half of those absent from the plastid in at least one lineage were detected as homologs in the nucleus (Martin *et al.*, 1998, 2002). Diversification of algal species occurred to some extent prior to complete reduction of the plastid genome, but the similarity of the plastid genome among the late arriving angiosperms indicates that the reduction in genetic content by gene transfer to the nucleus was essentially complete prior to their emergence. However, an assay for transfer of a marker gene, encoding the antibiotic-resistance enzyme neomycin phosphotransferase, from plastid to nucleus to produce tobacco seedlings able to grow on kanamycin, revealed that this process still occurs at a significant rate, with a frequency of one plastid marker sequence detected in about 16,000 pollen (nucleus) grains (Huang *et al.*, 2003). Another analysis of transfer of the antibiotic-resistance marker from plastid to nucleus by selecting for cells in leaf

*Abbreviations:* Chl – chlorophyll; D – Debye; LHC – light-harvesting complex; LHCP – apoprotein of light-harvesting complex; MYA – million years ago; pN – picoNewton; PSI – photosystem I; PSII – photosystem II; TIC – translocon on inner membrane of chloroplast envelope; TOC – translocon on outer membrane of chloroplast envelope; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase; Pchlide – protochlorophyllide; POR – NADPH:protochlorophyllide oxidoreductase.

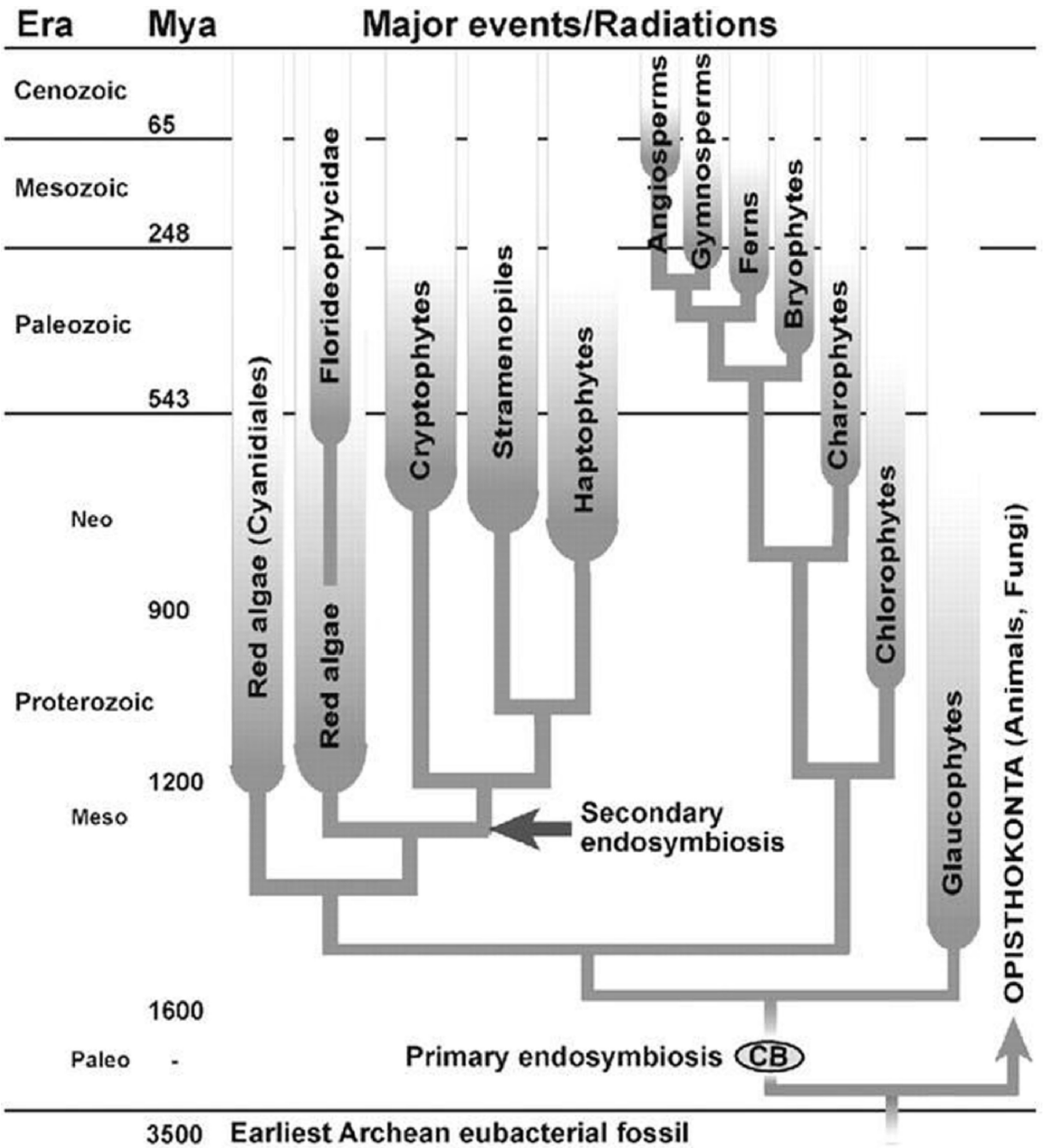


*Fig. 1.* Overview of the evolutionary relationships and divergence times for the red, green, glaucophyte and chromist algae. The origin of the algal tree is indicated by the primary endosymbiosis of a cyanobacterium (CB). Also shown is the position of the secondary endosymbiosis of a red alga. The scheme shows the progression of the green algal lineage to the land plants. The outgroup for the plastid-containing organisms is the Opisthokonta, which provided the animal and fungal lineages. (From Bhattacharya and Medlin, 2004. Used by permission of Oxford University Press.)

tissue of tobacco capable of regenerating plants in the presence of the antibiotic gave a much lower frequency of about 1 in 5 million cells (Stegemann *et al.*, 2003).

Transfer of DNA from plastid to nucleus is often thought to have occurred in gene-sized fragments. As the complete nucleotide sequences of genomes of several plants are now available, it has became clear that

large segments, in some cases entire plastid genomes, were integrated into the nuclear genome. Nearly entire copies of the chloroplast genome occurs in the nucleus of rice (*Oryza sativa*) (Richly and Leister, 2004), with 99.77% identity with the DNA in the plastid. This high degree of identity indicates that the transfer of DNA from plastid to nucleus occurred relatively recently, possibly about 150,000 years ago. Nuclear DNA of other plants, such as *Arabidopsis*, contains shorter fragments that still account for 19% of the plastid genome DNA with high degrees of identity.

The plastid genomes of current land plants encode, over a wide range of species, 75 to 80 proteins (Timmis *et al.*, 2004). This number is in stark contrast to the number of polypeptides in present-day chloroplasts, estimated as 3,500 to 4,000 (Soll and Schleiff, 2004). About 19% of the current *Arabidopsis* nuclear genome is of prokaryotic (mostly cyanobacterial) origin, with *Nostoc* the most similar cyanobacterial organism (Martin *et al.*, 2002; Richly and Leister, 2004). Although chloroplasts contain up to 100 copies of the residual chloroplast genome, the number of *different* genes retained in the chloroplast genome is only a few percent of that in a modern-day cyanobacterium. Because genes encoding most of the plastid proteins are now expressed in the nucleus, an elaborate system is required to transport these proteins back into the plastid after synthesis in the cytosol (Soll and Schleiff, 2004). Mitochondria were already present in cells that engulfed a cyanobacterial ancestor of the plastid, and the import mechanism for chloroplasts may have been borrowed from that already in place for mitochondria, with sufficient modification to afford appropriate direction of protein traffic (Dyall *et al.*, 2004). (see Chapter 3 in this volume for a full description of the import apparatus).

Table 1 shows the functional categories of protein-encoding genes in *Arabidopsis* that are of cyanobacterial origin. The endosymbiotic event that led to development of the plastid not only brought genes for photosynthetic activities into the eukaryotic cell but also all the metabolic processes of the previously free-living, cyanobacterial cell. Core metabolic pathways in eukaryotic cells, such as glycolysis and amino acid and fatty acid synthesis, were provided by the prokaryotic endosymbiont. Although many of the nuclear genes encode products that are returned to the plastid, others provided a rich resource for evolution of new functions (Martin *et al.*, 2002). Protein synthesis in the plastid is clearly of cyanobacterial origin, with 70S-type ribosomes and prokaryotic-type amino acyl-tRNA synthetases, initiation factors and elongation factors (Hoober and Blobel, 1969; Harris *et al.*, 1994; Zerges, 2000). The differences in antibiotic sensitivity between the plastid ribosomes and the 80S-type ribosomes in the cytosol provided an exceptional opportunity to use these specific inhibitors as research tools to study chloroplast development and gene expression. The plastid also contains a prokaryotic-type RNA polymerase, with subunits encoded by the chloroplast genome (Maliga, 1998). This polymerase recognizes promoter sequences that are similar to consensus promoters in bacterial cells. The sigma factors that help the polymerase identify promoter sequences, however, are encoded in nuclear genes (Tan and Troxler, 1999; Allison, 2000). A second plastid-localized RNA polymerase, with similarities to bacteriophage $T_7$ polymerase, is encoded by nuclear genes (Maliga, 1998; Liere *et al.*, 2004). (see Chapter 8 in this volume for a description of the RNA polymerases). A remnant of a respiratory pathway, homologous to the mitochondrial NADH dehydrogenase, is present on thylakoid membranes and seems to play a role in electron transport from ferredoxin to plastoquinone for the cyclic pathway around photosystem I (PSI). Therefore, this complex in the chloroplast functions as a ferredoxin-plastoquinone reductase (Munekage *et al.*, 2004) rather than a ferredoxin-NADP reductase as required for assimilation of $CO_2$. Autotrophic pathways for synthesis of amino acids, carbohydrates, isoprenoids, fatty acids and lipids, which are localized in the plastid, were introduced from the endosymbiont (Martin *et al.*, 2002).

*Table 1.* Functional categories for *Arabidopsis* proteins of cyanobacterial origin (adapted from Martin *et al.*, 2002).

| Functional category | Number* |
|---|---|
| Biosynthesis and metabolism | 562 |
| Energy transduction | 93 |
| Cell growth and division | 31 |
| Transcription | 54 |
| Protein synthesis | 68 |
| Protein destination | 63 |
| Transport facilitation | 35 |
| Intracellular transport | 12 |
| Cellular biogenesis | 38 |
| Signal transduction | 189 |
| Cellular response | 137 |
| Ionic homeostasis | 5 |
| Cell organization | 71 |

*Number of proteins per category among 1,700 identified.

## B. Plastid Divergence

The chloroplast is the final stage of an extensive process of structural evolution of photosynthetic systems.

Simple expansion of the plasma membrane in photosynthetic bacteria to form photosynthetic domains (Drews, 1996) is the most primitive means to expand photosynthetic capacity. Elaboration of a separate and distinct membrane system in cyanobacteria led to the highly developed thylakoid system in chloroplasts. Cyanobacteria contain only chlorophyll (Chl) *a*, whereas plants, along with their most closely related algal ancestors in the genus Charales, and the eukaryotic green algae that belong to the phylum Chlorophyta, contain Chl *b* in addition to Chl *a* (see Fig. 2 for structures). In the green algae and plants, Chl *b* is found essentially exclusively in light-harvesting complexes (LHCs). Chl *b* is also found in prokaryotic species that are grouped as prochlorophytes (*Prochloron, Prochlorothrix* and *Prochlorococcus*), which have recently been included within the Cyanophyta. Sequence homology in the genes encoding Chl *a* oxygenase, the enzyme that catalyzes oxygenation in vitro of Chlide *a* to make Chlide *b*

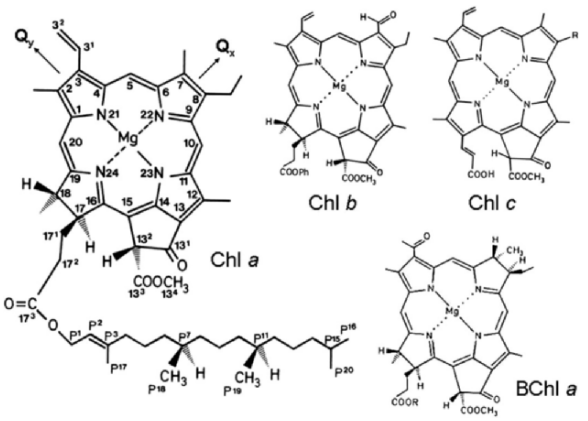


*Fig. 2.* Structures of the major chlorophylls. Except for the oxidation of the 7-methyl group in Chl *a* to the formyl group in Chl *b*, Chls *a* and *b* are identical, including the 20-carbon isoprene alcohol, phytol, esterified to the carboxyl group at position $17^3$. This carboxyl group remains 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 $\pi$ system to the free carboxyl group. The electronegative formyl oxygen in Chl *b* and the carboxyl group on Chl *c* cause redistribution of electron density towards the periphery of the molecule along the $Q_x$ axis, which lowers the $Q_y$ transition dipole and shifts the absorbance maxima towards shorter, higher energy wavelengths. Bacteriochlorophyll *a* has the double bond between carbons 7 and 8 reduced and the 3-vinyl group oxidized to an acetyl group. Consequently, these modifications dramatically strengthen the dipole in the $Q_y$ direction and shift the long-wavelength absorbance maximum into the infra-red region of the spectrum. (R = isoprenoid side chain, usually phytol).

(the "-ide" ending signifies absence of the esterified isoprene alcohol) (Oster *et al.*, 2000), indicate that these genes have a monophyletic lineage (Tomitani *et al.*, 1999), with a gene in *Prochlorococcus* as perhaps the most ancient (Hess *et al.*, 2001). It is not clear whether the oxygenase reaction occurs in vivo at the level of Chlide *a* or Chl *a*. During rapid synthesis of Chl, a substantial pool of Chlides was not detected. Moreover, the esterified alcohol, initially incorporated as geranylgeraniol, had the same pattern of reduction to the phytol moiety in both Chls (Maloney *et al.*, 1989), which suggests that the oxygenation occurred at the level of the Chls in *Chlamydomonas*. Chl *a* oxygenase homologs in the prokaryotic *Prochloron* and *Prochlorothrix* are clearly evolutionarily related to the gene in green algae and plants. This finding led Tomitani *et al.* (1999) to suggest that the ancestral prokaryotic endosymbiont introduced phycobilisomes and a prochlorophyte-type LHC that contained Chl *b* into the nascent plastid. Subsequently, members of the prokaryotic species apparently diverged to produce the prochlorophytes that retained Chl *b* and the modern cyanobacterial species that lost the ability to make Chl *b* but retained phycobilisomes. Likewise, the ancestral photosynthetic eukaryotes diverged into the red algae that lack Chl *b* and the major Chl *a/b*-containing light-harvesting complexes but retained the phycobilisomes of cyanobacteria and the green algae that lost the ability to make phycobiliproteins but gained the source of the abundant family of Chl *a/b*-binding proteins that provide the light-harvesting antenna. The plant-type light-harvesting Chl *a/b*-binding proteins (LHCPs) are not present in cyanobacteria and developed initially in the green algal lineage, although cyanobacteria do contain small, LHCP-like polypeptides that may have been the precursors of LHCPs (Dolganov *et al.*, 1995; Funk and Vermaas, 1999). These small polypeptides, which span a membrane only one time, may have been the evolutionary precursor, via genetic fusion, to form Chl-binding proteins that span thylakoid membranes three or four times (Durnford *et al.*, 1999).

Many other species of algae originated as products of a "secondary" endosymbiotic event, in which an entire eukaryotic red algal cell was engulfed by a non-photosynthetic eukaryotic cell about 1,274 MYA, with subsequent radiation of the Chromista lineage, which includes cryptophytes, haptophytes and stramenopiles (McFadden, 2001; Raven and Allen, 2003; Yoon *et al.*, 2004; McFadden and van Dooren, 2004; Nisbet *et al.*, 2004). Whereas the plastid was maintained nearly in its original form, the remainder of the first algal cell was drastically reduced in volume. Consequently, plastids

in these latter algae usually are separated from the secondary host's cytosol by four membranes, the two original chloroplast envelope membranes, the derivative of the original host algal cell membrane, and the endocytic membrane of the secondary host. A residual nucleus, called a "nucleomorph", of the original host is still found in the group of algae referred to as the cryptomonads (Nisbet *et al.*, 2004). The relic nucleus occurs in the "periplastidal compartment" between the membranes of the plastid envelope and the outer two membranes, which corresponds to the initial host's cytosol. These latter two membranes are designated the "chloroplast endoplasmic reticulum". Algal products of secondary endosymbiosis that contain Chl *b*, such as euglenophytes that have three membranes surrounding the plastid and chlorarachniophytes, are likely the result of engulfment of a green alga by a nonphotosynthetic host cell (Bhattacharya and Medlin, 1998). Such secondary endosymbiosis has provided the majority of algal biodiversity (Yoon *et al.*, 2002). Even more remarkable are products of a third, "tertiary" endosymbiosis of either cryptophyte or chromophyte organisms that led to different species among the dinoflagellates (Stoebe and Maier, 2002). Although diversity of the algae is extensive, the plastids themselves all derive from a single, i.e., monophyletic, origin (Fig. 1).

Algal species that resulted from secondary endosymbiotic events, and thus have four membranes around the plastid, modified late steps in the tetrapyrrole pathway to produce Chl *c* (see Fig. 2 for structure). Although Chl *b* is found in a few species among these organisms (Goss *et al.*, 2000), Chl *c* usually replaces Chl *b* in LHCs, whose apoproteins nevertheless are homologous to those in the green algae (Larkum and Howe, 1997; Durnford *et al.*, 1999). The presence of these accessory Chls in LHCs, and the usual complete absence of one when the other is present, suggests significant differences in the synthesis of these Chls in comparison with Chl *a*. The mechanism of synthesis of Chl *c* is not known, but the molecule itself has several remarkable features. The C17-C18 double bond of ring D remains unreduced, as in Pchlide *a*, and a double bond is introduced in the propionyl sidechain to generate the *trans*-acrylate group. In addition, the acrylate carboxyl group remains unesterified, in contrast to all other Chls. The consequence of all three of these structural modifications is extension of conjugation of the porphyrin ring $\pi$ electron system to the electronegative carboxyl group (Dougherty *et al.*, 1970). Thus, the carboxyl group is able to influence the Lewis acid strength of the central $Mg^{2+}$ in a fashion similar to that of the 7-formyl group of Chl *b*, along the $Q_x$ vector of the molecule. These results suggest that Chls *b* and *c* play important roles in assembly of LHCs that cannot be provided by Chl *a*.

## III. Development of the Chloroplast

### *A. Structural Changes*

Most plant cells contain plastids. Many algal cells contain a single chloroplast (Fig. 3). In contrast, mature cells of a plant leaf may contain between 100 and 150 plastids. Differentiation of tissues within a developing plant is accompanied by differentiation of plastids into chloroplasts in leaves, leucoplasts and starch-filled amyloplasts in root tissues, and carotenoid-rich chromoplasts in flowers and fruits. Chloroplasts are usually lens-shaped structures 1 to

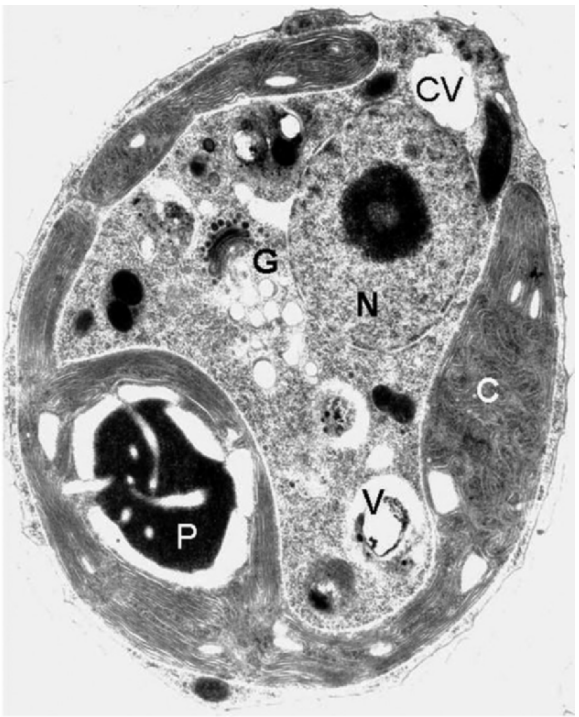


*Fig. 3.* Ultrastructure of the green alga, *Chlamydomonas reinhardtii*. The cell contains one large chloroplast (C) filled with thylakoid membranes. Within the chloroplast, a condensed form of Rubisco is surrounded by starch grains, a structure referred to as the pyrenoid body (P). Also shown are the nucleus (N), Golgi apparatus (G), vacuole (V) similar in function to lysosomes, and a contractile vacuole (CV) that functions in osmotic balance. The long axis of the cell is usually 8 to 10 μm in length. (Micrograph courtesy of Hyoungshin Park)

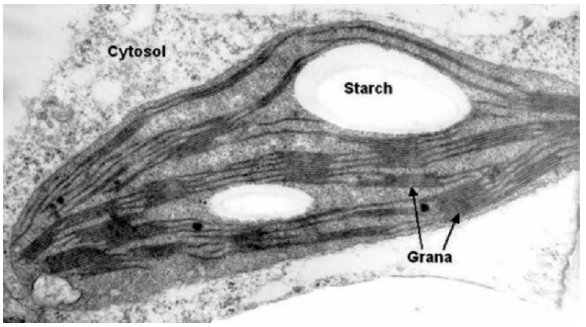


*Fig. 4.* Electron micrograph of a chloroplast in a young leaf of tobacco. In contrast to algal cells, thylakoid membranes in higher plant chloroplasts are generally differentiated into stacks of appressed membranes (grana) that are connected by segments of single thylakoids, called stromal lamellae, which extend into the grana. Rubisco is dispersed throughout the higher plant chloroplast. The long axis of the chloroplast is typically about 6 μm in length. (Micrograph courtesy of Hyoungshin Park)

3 μm on their short axis and 5 to 8 μm on their long axis (Fig. 4) (Staehelin, 2003). The number of chloroplasts per cell is closely correlated with the size of the cell (Pyke and Leech, 1992). Chloroplasts divide by binary fission in concert with expansion in cell size, until the cell reaches maturity. Thereafter, chloroplasts are no longer capable of division. Plastid division has similarities to division of bacterial cells, including involvement of cytoskeletal proteins such as FtsZ, which forms constriction rings at the mid-section of the chloroplast (Osteryoung and Nunnari, 2003). Mutants defective in chloroplast division are phenotypically normal, except that leaf cells contain only 2 or a few very large chloroplasts (Robertson *et al.*, 1995) that nevertheless occupy approximately the same volume as the numerous chloroplasts in wild-type plants and provide similar photosynthetic capacity.

At the time of seed germination, plastids exist as small, 1-μm diameter vesicles called "proplastids". Between 10 and 14 proplastids occur in each meristematic cell (Mullet, 1988; Pyke and Leech, 1992). These organelles are surrounded by a two-membrane envelope, which contains specific transport systems through which proteins and metabolites pass. The outer membrane contains pores that are less discriminatory than the more specific transport systems that reside in the inner membrane (Ferro *et al.*, 2002; Soll and Schleiff, 2004). As observed in cryofixed tissues, the envelope membranes are tightly appressed (Sluiman and Lokhorst, 1988; Nishizawa and Mori, 1989; Park *et al.*, 1999), such that transport complexes on the outer (TOC) and inner (TIC) membranes likely interact to provide continuous passageways. Current evidence indicates that the outer membrane of the chloroplast envelope is more similar to the outer bacterial membrane than the endocytic membrane that presumably enclosed the engulfed bacterial cell. If the progenitor of the chloroplast was similar to modern cyanobacteria, which are "Gram-negative" organisms that have a cell wall sandwiched between two membranes surrounding the cell, then evolution of the plastid envelope also involved loss of the cell wall. Only in glaucophytes, such as *Cyanophora*, in which the plastids are referred to as "cyanelles", is a peptidoglycan residue of the cyanobacterial cell wall retained between membranes of the envelope (Pflanzagl *et al.*, 1996; Kugrens *et al.*, 1999).

Morphogenesis of plant seedlings in response to light involves almost all tissues of the plant. New cells are produced in the meristematic tissue, which in the more ancient monocot species occurs at the base of the leaf, whereas cell division occurs at the growing tips in the more recently emerged dicot species. Light-grown, dicot seedlings have short hypocotyls and expanded, open cotyledons. Differentiation of cell-types occurs in rapid fashion. Along with the dramatic macroscopic changes in the plant, the most striking cellular changes are disappearance of the energy storage forms in the seeds and the concomitant development of the chloroplast, which is accompanied by high levels of expression of photosynthetic genes. This developmental pattern in the light, known as "photomorphogenesis", differs markedly from that when seedlings are grown in darkness. Germination of seeds and growth of seedlings in the dark follow a pattern known as "skotomorphogenesis" or "etiolation", in which the hypocotyls of dicots are much elongated and the cotyledons remain closed and undeveloped. The plastids in the cotyledons of seedlings grown in the dark become etioplasts, in which a tubular network of membrane material, the "prolamellar body", develops over time until it occupies nearly half the organelle

volume. This process of development of the prolamellar body of the etioplast is dramatically displayed in monocot species, such as barley or wheat, in which age and consequently etioplast development corresponds to distance from base to the leaf tip, with the older cells at the tip. Etioplasts are rapidly transformed into chloroplasts when exposed to light. Synthesis of Chl, which requires light in most plants, is the hallmark of chloroplast development, which is often referred to as "greening" or "de-etiolation". Expression of nuclear and plastid photosynthetic genes is strongly regulated as the chloroplast develops the thylakoid membrane system.

The most dramatic events during chloroplast development, aside from the increase in size and number, are appearance and expansion of the thylakoid membrane and assembly of the energy transduction processes contained within this membrane. In seedlings that are exposed early to light, initial thylakoid membranes form in the proplastid by invagination of the inner membrane (reviewed by von Wettstein, 2001). Invagination of the envelope inner membrane was also documented with rapidly greening cells of the alga *Chlamydomonas reinhardtii* (Hoober *et al.*, 1991) and also with cryofixed, developing chloroplasts in rice seedlings (Bourett *et al.*, 1999). Capture of these structures requires rapid fixation (Fig. 5). Thus, photosynthetic domains are expelled as vesicles from the envelope by a series of accessory proteins involved in vesicle traffic (see also chapter 3 in this volume). The vesicles then fuse to form and expand the developing thylakoid system. A mutant of *Arabidopsis thaliana,* deficient in an activity designated vesicle-inducing plastid protein 1 (VIPP1), is unable to induce vesicle formation from the plastid envelope and consequently does not make thylakoid membranes (Kroll *et al.*, 2001). VIPP1 forms high molecular mass complexes at the inner envelope membrane (Aseeva *et al.*, 2004), where it apparently initiates vesicle formation. Formation of vesicles has been observed in plant leaves treated with inhibitors of vesicle fusion and also by application of specific inhibitors of vesicle traffic to isolated chloroplasts (Westphal *et al.*, 2001, 2003). These results provide direct evidence of vesicles emerging from the envelope as the source of material for thylakoid membranes. An analysis of the genome of *Arabidopsis* identified several genes that were predicted to encode chloroplast proteins involved in vesicular traffic (Andersson and Stina, 2004).

The three-dimensional arrangement of membranes in the mature chloroplast of plants has been established by electron microscopy. The thylakoid membrane, separate from the inner envelope membrane, is differentiated into cylindrical stacks of "appressed" membranes, designated "grana", that are interconnected with "unappressed", stromal membranes (Fig. 4). The highly elaborated, folded membrane system encloses a single, continuous lumen (Mustárdy and Garab, 2003), which is an important compartment for the process of photosynthesis. This arrangement seems to maximize efficiency of the overall process. Thylakoid membranes in algal cells are less differentiated, and in many cases are appressed over much of their surface (Fig. 3).

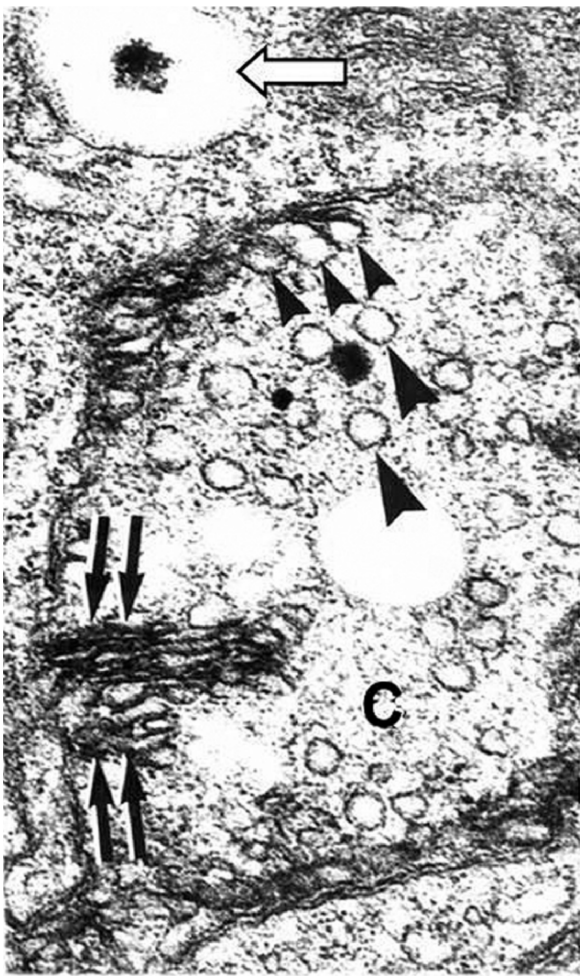


*Fig. 5.* Section of the chloroplast from a dark-grown cell of *C. reinhardtii y*1 exposed to light for 15 min. Extensive regions of the chloroplast envelope are associated with newly formed membranes (double arrows). Numerous vesicles (large arrowheads) in the chloroplast stroma (C) are similar in morphology to membranes emanating from the envelope (small arrowheads). A vacuole containing a small polyphosphate granule is indicated by the open arrow. (Adapted from Hoober *et al.*, 1991)

### *B. Regulation of Gene Expression in Chloroplast Development*

It has long been known that light plays a key role in chloroplast development by up-regulating expression of a large number of nuclear and plastid genes. Current evidence supports a complex mechanism of transcriptional control that involves many proteins, some of which represss and others that activate transcription. Light receptors for these responses include phytochromes (at least five types), cryptochromes or blue-light receptors, and UV-B receptors (Wei and Deng, 1996). Responses to light are normally initiated by two principle pathways, photoisomerization of phytochrome or the response of cryptochromes to blue light (Christie and Briggs, 2001; Fankhauser, 2001). Expression of a "photogene" in the chloroplast genome was discovered by Bedbrook *et al.,* (1978), which was shown to be the *PsbA* gene that encodes protein D1 in the reaction center of photosystem II. Expression of nuclear genes encoding apoproteins of LHCs and the small subunit of Rubisco were found to be stimulated by light (see Hoober, 1987, for a summary of early work on regulation of gene expression by light). Much of the regulation by light was linked to photoisomerization of phytochrome from $P_r$ to the $P_{fr}$ form (Nagy and Schäfer, 2002). $P_{fr}$ is the photomorphogenically active form and is competent for transport into the nucleus. Its subsequent mechanism of action is not clear, but isomerization of phytochrome regulates its interactions with other proteins involved in regulation of gene expression by light.

Mutations in genes encoding regulatory factors revealed much of the mechanisms of regulation of gene expression by light. Although light is normally required for plant and chloroplast development, most of the developmental processes can occur in the dark in such mutant strains. Identification of mutations that allow an extensive photomorphogenetic type of development of the plant, including the chloroplasts, in complete darkness supported the hypothesis that expression of genes is repressed in the dark. The initial discovery of de-etiolated (*det*) phenotype mutations (Chory and Peto, 1990; Chory, 1993), which developed a partial "light-induced" phenotype in the dark, led to an intensive analysis of the role of phytohormones in regulation of chloroplast development. The nuclear *DET2* gene was found to encode a steroid 5$\alpha$-reductase in the pathway of synthesis of the steroid hormone brassinolide. Mutation revealed a second essential enzyme in this pathway, encoded by the gene *CPD*, that is a member of the cytochrome P450 monooxygenase family (see review by Nemhauser and Chory, 2004). Another cytochrome P450 enzyme, CYP72B1, was discovered that inactivated brassinolide by additional hydroxylation of the sidechain (Neff *et al.*, 1999; Turk *et al.*, 2003). Brassinolide binds to cell-surface receptor kinases, the proteins BRI1 and BAK1, which have serine/threonine kinase activity. The substrates of this activity have not yet been identified. Activities of two downstream proteins, BES1 and BZR1, are regulated by a cytoplasmic protein kinase, BIN2. BES1 and BZR1 are phosphorylated by BIN2, and in this form are degraded. Brassinolide induces dephosphorylation and thus facilitates accumulation of these proteins in the nucleus, the extent to which was correlated with characteristics of the etiolated phenotype such as cell elongation and long hypocotyls in the dark (H Wang *et al.*, 2001; Z-Y Wang and He, 2004; He *et al.*, 2005).

Without promotion of the etiolated phenotype in mutant strains unable to synthesize brassinolides, the de-etiolation phenotype emerged in the dark. In these plants, the plastid gained a stage of development similar in ultrastructure to a chloroplast at an early stage of greening in the light, but without Chl. Chory *et al.* (1994) also found that treatment of wild-type *Arabidopsis* seedlings with cytokinins evoked characteristics similar to those of *det1* mutant plants in regard to morphology, plastid development and expression in the dark of normally light-induced genes. In wild-type seedlings, the etiolated phenotype imposed by brassinolide is normally overcome by activation of phytochrome. The long hypocotyl phenotype in phytochrome-deficient plants, caused by mutation in the *phyB* locus, was suppressed by overexpression of the *CYP72B1* gene that encodes the degradative cytochrome P450 enzyme. The resulting increased monooxygenase activity enhanced metabolic inactivation of brassinolide, which suggested that phytochrome B and brassinolide have antagonistic effects. The effects of light intensity and light quality on phytochrome determine the degree of the brassinolide response, which provides additional evidence for a direct relationship between phytochrome and brassinolide activities (Nemhauser *et al.*, 2003; Nemhauser and Chory, 2004).

An extensive, pleiotropic set of genes was discovered that controlled many aspects of plant—and plastid—development (Wei and Deng, 1996; Serino and Deng, 2003). Their multiple names led to the designation of this set as the *COP/DET/FUS* genes, because mutations in these genes were involved with constitutive photomorphogenic (*cop*), de-etiolation (*det*) and often caused a purple coloration of seeds and seedlings as the result of inappropriate accumulation of anthocyanin

pigments (*fusca* mutants) (Castle and Meinke, 1994). In mutant plants, the normal prolamellar body of the etioplast does not form. Rather, the plastid develops a few rudimentary membranes and appears ultrastructurally as a chloroplast at an early stage of normal, light-induced development. In the light, plastids in root tissue of the mutants also begin inappropriate development typical of chloroplasts in leaves, complete with accumulation of Chl and thylakoid membranes (Kwok *et al.*, 1996; Wei and Deng, 1996). These results indicate that the normal repression of genes in the dark, and in root tissue, is inactivated in these mutants. Interestingly, plants with null mutations in these genes are lethal.

Studies of the *COP* genes brought to light the COP9 signalosome, a multiprotein complex that is central to photomorphogenesis in plants (Serino and Deng, 2003). This complex is composed of eight subunits, all of which are required for its assembly. The COP9 signalosome is the master repressor of photomorphogenesis and other developmental processes. The complex acts along with additional proteins, such as COP1, COP10 and DET1, to cause degradation of positive regulators of photomorphogenesis. In the dark, COP1 accumulates in the nucleus and interacts with the transcriptional factor HY5. The critical activity of COP1 seems to be ligation of ubiquitin to HY5, which marks HY5 for degradation by the 26S proteosome, which also requires COP10 and DET1 (Dieterle *et al.*, 2003). In the light, COP1 is transferred to the cytosol and inactivated, which allows the amount of HY5 in the nucleus to increase up to 20-fold and thereby enhance gene expression (Osterlund *et al.*, 2000). Mutations in *COP1* and *DET1* genes have the analogous effect on expression of light-inducible genes by allowing accumulation of HY5 in the dark.

Blue light receptors, the cryptochromes, also regulate seedling de-etiolation (Christie and Briggs, 2001). Cryptochromes (CRYs) are members of a large blue-light-absorbing chromoprotein family that includes DNA photolyase, although cryptochromes do not have DNA repair activity (Liscum *et al.*, 2003). CRY1 and CRY2 interact with COP1, which in the dark regulates degradation of transcription factors in conjunction with the COP9 signalosome (H. Wang *et al.*, 2001). When exposed to blue light, CRY suppresses association of COP1 with COP10 and the COP9 signalosome, thereby allowing accumulation of HY5 and expression of light-inducible genes (Liscum *et al.*, 2003). The discovery of a mutation in an allele of the *COP1* gene during a phytochrome A-dependent screen further suggests that COP1 is downstream of the regulatory pathways of both phytochrome and cryptochromes (Dieterle *et al.*, 2003).

A pair of light-responsive *cis* elements was discovered upstream of the light-inducible promoters of the *LHCB* genes that mediate the action of phytochrome (Degenhardt and Tobin, 1996). Mutations in these sequences increased expression of *LHCB* genes in the dark to an extent that stimulation of transcription by phytochrome was no longer observed, which suggested that binding of *trans*-acting factors to these elements repress expression in the dark. Kenigsbuch and Tobin (1995) discovered a protein, designated CA-1, that binds to these regulatory elements in the promoter of *LHCB* genes. (These genes are also called *CAB* because of an earlier designation of the gene products as Chl *a/b*-binding proteins). This activity was not detected in *det1* mutants, which develop the morphology of light-grown plants in the dark. A protein designated CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) binds to different elements in the promoter region of *CAB* genes and is required for induction of these genes by phytochrome action and for their circadian rhythm of expression (Z-Y Wang and Tobin, 1998). Two additional *cis* elements were identified that are involved in regulation by DET1. One, a dark-response element (DtRE), is required for dark and root-specific repression of *CAB2* transcription (Maxwell *et al.*, 2003). DET1 represses the *CAB2* promoter by regulating the binding of two factors, CAB2 DET-associated factor 1 and CCA1 to the DtRE. Interaction of HY5 and the *CAB2* upstream factor 1 (CUF-1) with DET1 involves a second regulatory element to achieve activation of expression in the light.

A group of pigment-deficient mutants of *Arabidopsis* was isolated and given the designation *CHLOROPLAST BIOGENESIS* (*clb)* genes (de la Luz Gutiérrez-Nava *et al.*, 2004). Development of the *clb* mutants was arrested at an early stage, with plastids remaining similar to proplastids that contain membrane vesicles and a few rudimentary thylakoid membranes. These mutants were severely deficient in Chl. One of the genes, *CLB4,* encodes 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, which catalyzes the next to last step in synthesis of the isoprenoid precursor in plastids, methyl-erythritol 4-phosphate. A dramatic illustration of the requirement of carotenoids for formation of the prolamellar body and for chloroplast development was isolation of a mutant of *Arabidopsis* that lacked the ability to isomerize poly-*cis*-carotenoids to all-*trans*-carotenoids. This reaction is necessary near the end of the pathway to provide the precursor for the cyclization reactions that form all the major carotenoid

end-products, in particular α-carotene, β-carotene and their oxidized products, the xanthophylls (Park *et al.*, 2002). Such mutations emphasize an important role of carotenoids in chloroplast development. Not only are carotenoids required for thylakoid membrane formation, they are also essential for assembly of the prolamellar body in dark-grown *Arabidopis* plants (see Chapter 16 in this volume.)

## C. Role of Chlorophyll in Chloroplast Development

### 1. Synthesis of Chlorophyll

In contemporary plant and algal cells, with few exceptions, tetrapyrroles are synthesized exclusively in plastids. Along with most prokaryotic cells, plastids use the abundant amino acid glutamate as the primary precursor of 5-aminolevulinate, the first committed precursor for tetrapyrrole synthesis (Vavilin and Vermaas, 2002; Eckhardt *et al.*, 2004). Plastids produce both final products, chlorophylls and hemes, by insertion of $Mg^{2+}$ or $Fe^{2+}$, respectively, into protoporphyrin IX (see Chapter 15 in this volume). In contrast to most single-celled photosynthetic organisms, which are able to synthesize Chl and the photosynthetic apparatus in the dark, plants require absorption of light energy by the substrate, protochlorophyllide (Pchlide), for conversion of Pchlide *a* to Chlide *a*. This reaction involves the stereochemical reduction of a double bond between C17 and C18 in ring D (see Fig. 2). This reaction is catalyzed by NADPH: Pchlide oxidoreductase (POR) and is the only step in Chl synthesis that requires light, which is absorbed by the substrate, Pchlide. PORA is the predominant protein in the prolamellar body of etioplasts and exists as a ternary complex with Pchlide and NADPH (Lindsten *et al.*, 1988; Ryberg and Sundqvist, 1988). S. Reinbothe *et al.* (2004) showed that PORA binds preferentially Pchlide *b*, a potential precursor of Chl *b*, whereas PORB binds preferentially to Pchlide *a*. Moreover, these investigators reported that in dark-grown barley the developing prolamellar body consists predominantly of a supercomplex of PORA and PORB in an approximately 5:1 ratio with their bound substrates (C. Reinbothe *et al.*, 2003, 2004). Exposure of these seedlings to light results in energy transfer from Pchlide *b* to Pchlide *a*, reduction of the latter to Chlide *a*, and dispersal of the prolamellar body. Dissociation of the PORA: PORB complex possibly allows photoreduction of Pchlide *b* to Chlide *b* (C. Reinbothe *et al.*, 2004). PORA bound to Chlide is then rapidly degraded by proteolysis (C. Reinbothe *et al.*, 1995), along with a dramatic decrease in the amount of *POR* mRNA during the initial stage of development (Armstrong *et al.*, 1995).

More than 90% of the lipids in the prolamellar body are mono- and di-galactosyl diglycerides (Selstam and Sandelius, 1984), which are retained in 'pro-thylakoid' membranes. The rudimentary prothylakoid membranes then expand by addition of proteins and lipids. Some, such as proteins of the core complex of photosynthetic units, are made within the plastid, while others, including the proteins of the light-harvesting antennae and several of the electron transport chain, are imported after synthesis in the cytosol. Synthesis of some of the proteins of the photosynthetic core complexes is regulated at the level of translation elongation, and in etioplasts requires synthesis of Chl *a* for synthesis of the proteins as well as stabilization (Eichacker *et al.*, 1990, 1996). In chloroplasts, elongation also requires the presence of a proton gradient across the membrane (Mühlbauer and Eichacker, 1998), which supports earlier evidence for synthesis of these proteins on ribosomes bound to thylakoid membranes.

Three forms of POR occur in some plants. PORA is abundant in the prolamellar body in etioplasts but is rapidly degraded when plants are exposed to light. PORB is present at a stable level throughout chloroplast development (C Reinbothe *et al.*, 2003, 2004). PORA and PORB are both present in the dark in *Arabidopis*. A third form, PORC, is not detectable in the dark, appears after several hours of illumination and remains at a stable level throughout development (Su *et al.*, 2001; Pattanayak and Tripathy, 2002). The latter steps of Chl synthesis occur on the chloroplast envelope (S Reinbothe and C Reinbothe, 1996; Joyard *et al.*, 1998). Chlide *a* oxygenase, the enzyme that catalyzes oxidation of the 7-methyl group in synthesis of Chl *b*, was recently found on the envelope membranes in partially degreened cells of *Chlamydomonas* (Eggink *et al.*, 2004). A homologous protein was found on the inner membrane of the envelope of barley chloroplasts, which associated with PORA during import of the latter protein (S Reinbothe *et al.*, 2004).

### 2. Interaction of Chlorophyll with Proteins

#### a. Light-Harvesting Complexes

Chl is required not only for photosynthesis but also for assembly of the photosynthetic apparatus. Chls exist mostly, if not entirely, associated with proteins. Complete formation of the thylakoid membrane is observed only in the light when Chl is made, which indicates

that Chl plays an active role in assembly mechanisms. Because the apoproteins of the light-harvesting complexes (LHCPs) are the dominant proteins of thylakoid membranes, they have been useful markers of membrane biogenesis. About one-third of the Chl *a* and essentially all of the Chl *b* reside in peripheral LHCs. LHCPs of the major and minor LHCIIs, associated with PS II, have molecular masses in the range of 25- to 30-kDa. These LHCPs are encoded by a large family of *LHCB* genes in the nuclear genome (The *Arabidopsis* Genome Initiative, 2000), and bind between 8 and 14 Chl molecules per protein molecule. The major LHCII in plants exists as a trimer, as determined by electron cryomicroscopy at 4.2K of two-dimensional crystals (Kühlbrandt *et al.*, 1994), and contains the major LHCPs designated Lhcb1, Lhcb2 and Lhcb3, although not in stoichiometric amounts. Each Lhcb1 protein binds 14 Chl molecules, 8 Chl *a* and 6 Chl *b,* along with 3 xanthophylls, usually 2 lutein and 1 neoxanthin molecules (Croce *et al.*, 1999; Liu *et al.*, 2004). Chl *a*, Chl *b* and the xanthophylls each have defined binding sites with, interestingly, no apparent mixed occupancy. Minor LHCIIs exist as monomers and bind variable amounts of Chls. Lhcb4 (CP24) binds eight Chls (6 Chl *a* and 2 Chl *b*), Lhcb5 (CP26) binds nine Chls (6 Chl *a* and 3 Chl *b*) while Lhcb6 (CP29) binds ten Chls (5 Chl *a* and 5 Chl *b*). The minor LHCs also contain fewer carotenoids, usually with 1 lutein, 0.5 neoxanthin and 0.5 to 1 violaxanthin (Croce *et al.*, 2002; Pascal *et al.*, 2002).

Four apoproteins of LHCI, Lhca1, Lhca2, Lhca3 and Lhca4, are involved in the antenna for PS I. Because of sequence homology, the structure of LHCI monomers is probably quite similar to that of LHCII. Apoproteins of LHCI coordinate 6 to 9 Chl *a*, 3 Chl *b,* 1 lutein, 0.5 violaxanthin and bind sub-stoichiometric amounts of β-carotene instead of neoxanthin (Schmid *et al.*, 2002). LHCI occurs as dimers, generally with heterodimers of Lhca1 with Lhca4, which comprise LHCI-730 that absorbs maximally at 730 nm, and Lhca2 with Lhca3, which provides LHCI-680, with maximal absorption at 680 nm.

### *b. Bonds Between Chlorophyll and Proteins*

$Mg^{2+}$ in Chl usually forms five coordinate covalent bonds. Four of the ligands for such bonds are provided by the pyrrole nitrogens within the Chl molecule. The ligand for the fifth, axial coordination bond is provided by solvent (water) or a functional group on a protein molecule. In the reaction centers and LHCs, the preferred ligands of Chl *a* are the neutral, electron-rich imidazole sidechain of histidine and the charge-compensated ion-pair of glutamate and arginine. These groups are more favorable ligands than those with a large dipole, possibly because of repulsion of the negative, electron-rich end of the dipole by the molecular electron cloud around the central Mg atom. Interestingly, urea, a molecule with a strong dipole [4.56 Debye (D)], competes with Chl *a* for histidine and the glutamate-arginine pair ligands only at relatively high concentrations (Eggink and Hoober, 2000). Chl *b* differs from Chl *a* only in the oxidation of the 7-methyl group on pyrrole ring B to an aldehyde. Although this reaction results in a slight spectral shift, and consequently expands the spectral range for absorbance of light by the chloroplast, the introduction of the additional oxygen atom has a greater function by influencing the coordination chemistry of the central $Mg^{2+}$ atom. The electronegativity of the oxygen atom results in a redistribution of electron density away from the pyrrole nitrogens along the $Q_x$ vector toward the periphery of the molecule. Thus the $Mg^{2+}$ atom in Chl *b* expresses a more positive point charge than that in Chl *a* and becomes a stronger Lewis acid. Consequently, Chl *b* binds more strongly than Chl *a* to molecules with a large dipole such as the dipolar solvent, water (Ballschmitter *et al.*, 1969). The imidazole sidechain of histidine is normally not a ligand for Chl *b*, probably because imidazole does not have a sufficiently large dipole to displace a strongly bound water ligand (Noy *et al.*, 2000). In fact, three of the six molecules of Chl *b* in LHCII retain water as the axial ligand, as found with the recent high-resolution structural determination of the LHCII from spinach (Liu *et al.*, 2004).

Ligands for two other Chl *b* molecules are peptide backbone carbonyl groups (Tyr24 and Val119 in Lhcb1 from spinach). A backbone amide has a dipole (4.2 D, 3.2 D for the carbonyl group) greater than water (2.25 D) (Dudev *et al.*, 1999; Gunner *et al.*, 2000; Georgescu *et al.*, 2002) and thus the carbonyl group is able to displace the water bound to Chl *b*. However, effective competition with water requires an environment with a low dielectric constant such as exists within a protein or membrane. Hydroxyl groups have a smaller dipole than water (Dudev *et al.*, 1999) and are not normally found as ligands in Chl-protein complexes. A possibly important feature of Tyr24 and Val119 is the presence of a proline within 2 or 3 positions of these amino acid residues in the protein sequence, which precludes hydrogen-bonding of the backbone amides and thus frees the carbonyl groups for coordination with Chl *b*. Tyr24 is within a highly conserved sequence, RVK**Y**LGPF, and is nearby to another conserved

sequence, PGDYG, in a proline-rich region of the N-terminal domain of the protein. The interaction of this region of the protein with Chl *b* is possibly the basis for retention of LHCPs in the chloroplast and their near absence in Chl *b*-less mutants. In the absence of Chl *b* synthesis in *Chlamydomonas,* more LHCPs accumulated in the cytosol and in vacuoles (Park and Hoober, 1997). Interestingly, Val119 is next to His120, whose imidazole group is not a ligand to the Chl *b* at this position (Liu *et al.*, 2004).

Ligands that favor interaction with Chl *b* usually contain oxygen and thus exhibit a large dipole, which provides an electrostatic character to the coordination bonds. Water ligands, as well as the 7-formyl oxygen, are hydrogen-bonded to amino acid sidechains.

By engaging electrons on the oxygen atom, hydrogen-bonds from an amino acid sidechain to the 7-formyl group of Chl *b* would enhance the electronegative influence on the central Mg atom and increase its Lewis acid strength. The strengths of the coordination and hydrogen bonds between water, Chl *b* and the protein are expected to instill a high degree of stability of LHCs. The complex network of hydrogen-bonds and the stronger coordination bonds of Chl *b* yield a protein-Chl complex that survives purification, an outcome that is not achieved with only Chl *a* (Hobe *et al.*, 2003). Consequently, in mutant strains of plants that lack the ability to make Chl *b*, very few LHCs accumulate. The stronger interaction between Chl *b* and the proteins appears to be essential not only for complete import of LHCPs into the chloroplast (Eggink *et al.*, 2001, 2004) but also for assembly of stable Chl-protein complexes.

A simple binding assay has shown that the extent of equilibrium binding of Chl *a* to a peptide containing histidine and the glutamate-arginine pair (Eggink and Hoober, 2000) is dramatically different from that of Chl *b*. Fig. 6A and 6B show excitation spectra of Chls *a* and *b* in the presence of various concentrations of the peptide. Binding was monitored by Förster resonance energy transfer from the indole sidechain of tryptophan in the peptide to Chl, with detection of fluorescence emission from the $Q_y$ absorption peak in the red region of the spectrum. Binding of Chl *a* to these ligands was more than an order of magnitude greater than that of Chl *b*. Binding of Chl *c* was also very low and comparable with Chl *b*. This simple experiment demonstrated that modifications to the tetrapyrrole molecule that withdraw electrons along the $Q_x$ axis exert similar characteristics on the coordination chemistry of Chls and that the behavior of Chls *b* and *c* is very different from that of Chls *a* and *d* (Chen *et al.*, 2005)

Balaban *et al.* (2002) and Oba and Tamiaki (2002) examined in detail the positions of the ligands of the Mg atoms in Chl *a* molecules in PSI. Of the 96 Chls, 64 have the imidazole group of histidine as a ligand. The next largest group, 16 Chls, has water as a ligand, which is usually hydrogen-bonded to an amino acid side chain. Because the Mg atom is displaced slightly from the plane of the tetrapyrrole ring system, the ligand is generally on the side from which the Mg protrudes. Furthermore, the stereochemical arrangement of the C17 side chain, with the phytol group, can be either on the same or opposite side as the $C13^2$-methoxycarbonyl group. Most of the ligands are on the 'back side' of the macrocycle, from with the $C13^2$-methoxycarbonyl group protrudes. This orientation places the ligand *anti* to the C17 side chain. In PSI, ligands to 82 Chls are *anti* and 14 are *syn*, which indicates greater stability of the former, calculated to be 4.3 kJ/mol more stable than the latter.

### c. Direct Measurement of Bond Energy

A full understanding of the roles of Chl *b* and Chl *c* in assembly and stabilization of LHCs will require more specific knowledge of the comparative strengths of the interactions between the various Chls and proteins. Recent developments in atomic force micrsocopy or dynamic force spectroscopy (Dudko *et al.*, 2003) may provide insight into these processes. According to Coulomb's law, two opposite charges separated by 3 Å has an energy of 5.9 kJ/mol, in a medium such as water, which has a dielectric constant of 80. This value is supported by direct measurement with atomic force microscopy of the force required to rupture an electron donor-acceptor (charge-transfer) complex, which yielded a value of 70 $\pm$ 15 picoNewtons (pN) or a bond energy of 4 to 5 kJ/mol (Skulason and Frisbie, 2002). However, the interior of a protein molecule or a membrane has a dielectric constant as low as 4 (Georgescu *et al.*, 2002), which makes an electrostatic bond much stronger. In LHCII, two interhelix glutamate-arginine ion pairs in the interior of the protein are thought to stabilize the structure by forming electrostatic links between the helices (Kühlbrandt *et al.*, 1994). Elimination of a glutamate or arginine member of an ion pair by mutation, leaving a noncompensated charge, prevented folding of the protein (Bassi *et al.*, 1999). Also, the electrostatic component of the coordination bond between the more positive Mg atom of Chl *b* and the electronegative end of a ligand dipole should strengthen interaction with the protein.

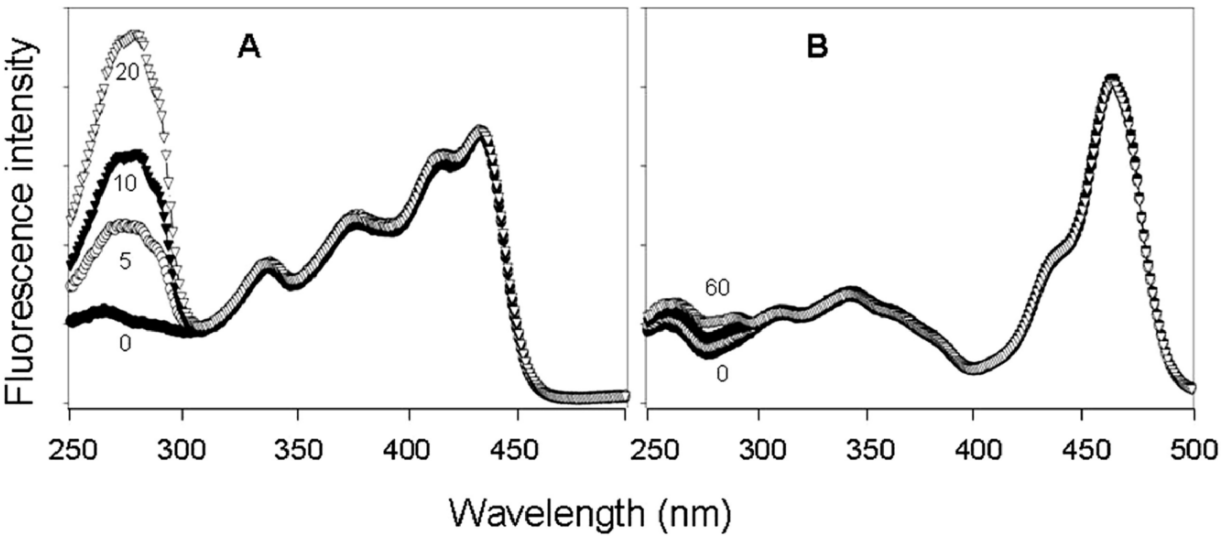


*Fig. 6.* Excitation spectra of Chl *a* and Chl *b* with various concentrations of a peptide maquette containing the conserved Chl-binding motif in LHCPs. (A) The peptide was added to Chl *a* to 0, 5, 10 and 20 μM. Binding of Chl *a* to the peptide was indicated by development of an excitation maximum at 280 nm, the absorption maximum of tryptophan. Excitation spectra were determined with fluorescence emission at 674 nm. (B) The peptide was added to Chl *b* at 0, 10, 30 and 60 μM and excitation spectra were determined with fluorescence emission at 654 nm. Non-specific energy transfer became a factor at the higher concentrations, which indicated that Chl *b* did not bind significantly to the peptide. (Adapted from Chen *et al.*, 2005)

The energy of a hydrogen bond varies widely, depending on the environment, with bond energies of 5 to 40 kJ/mol. Measurements with atomic force microscopy indicate that the force required to rupture a hydrogen-bond is in the range of 100 to 200 picoNewtons (pN) (Grubmüller *et al.*, 1996; van der Vegte and Hadziioannou, 1997). As shown by Merkel *et al.* (1999), the strength of a specific hydrogen bond depends upon the rate at which force is applied and can vary between 100 and 200 pN at relatively rapid loading rates. This aspect has been analyzed more extensively by Dudko *et al.* (2003), who described a rebinding process at low pulling velocities, which are likely to hold within a folded protein structure. The force required to rupture a coordination bond between an imidazole group of histidine and a chelated Ni, about 300 pN (Conti *et al.*, 2000), is slightly above that for hydrogen bonds. In particular, from the discussion in the previous section, the coordination bond between Chl *b* and a water or carbonyl oxygen is expected to be considerably stronger than that determined with Chl *a* and an imidazole ligand and would provide a substantial stabilizing factor in LHCs. As a comparison, the force required to break a covalent bond is an order of magnitude greater, around 3,000 pN (Grandbois *et al.*, 1999) or 350 to 450 kJ/mol. To further understand stability of LHCs provided by Chl *b* requires precise analysis of bond energies, an important area of research.

## *D. Thylakoid Biogenesis*

Data on assembly of photosynthetic complexes are meager. Most efforts have until now been focused on assembly of LHCs. As an in vitro approach, reconstitution of LHCII with mixtures of purified components has been remarkably successful (Bassi *et al.*, 1999; Remelli *et al.*, 1999; Rogl and Kühlbrandt, 1999; Horn and Paulsen, 2004), but definitive assignment of Chl in binding sites was only partially successful, as revealed by comparison with the recently published crystal structure of native LHCII (Liu *et al.*, 2004). Studies on the location of assembly events in vivo require availability of a suitable experimental system. Dispersion of the prolamellar body in the plastid of dark-grown seedlings of several plants, studied in particular with barley, provided a means to study early development in plants. However, the time scale of the developmental process is long and thus definitive mechanistic conclusions are precluded. Experiments with a model organism, the alga *Chlamydomonas reinhardtii,* have been instructive in regard to the pathway for insertion of the major LHCPs into the thylakoid membrane. The chloroplast of cells grown in the light is filled with thylakoid membranes. When "yellow-in-the-dark" (*y*) mutant cells are subsequently grown in the dark, the membranes are diluted among the progeny, leaving the chloroplast nearly depleted of membrane

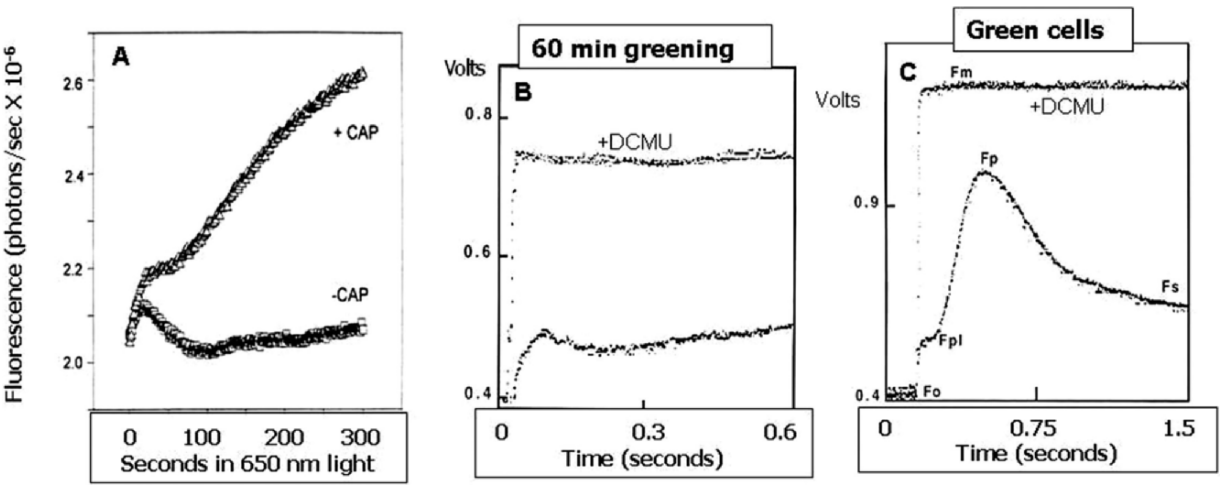


*Fig. 7.* Kinetics of greening of dark-grown *C. reinhardtii y*1 cells and fluorescence induction curves at early and late stages in chloroplast development. (A) Dark-grown cells were preincubated 1.5 h at 38°C and then exposed to 650 nm light. Under these conditions, Chl synthesis occurs linearly upon exposure of cells to light. Assembly of LHCs was monitored by fluorescence at 680 nm, which was excited via energy transfer from Chl *b*. When chloramphenicol (+CAP) was added to one sample to inhibit synthesis of reaction center proteins on chloroplast ribosomes, newly assembled LHCs were fluorescent because the light energy absorbed by the complexes was not photochemically trapped. In the absence of chloramphenicol (−CAP), newly assembled reaction centers quenched the absorbed energy within 30 sec after initiation of illumination. (Adapted from White *et al.*, 1996). (B) Fluorescence induction kinetics typical of the "state 2" condition were displayed by dark-grown cells after 1 h of exposure to white light. The lower curve was essentially unchanged from that obtained at the beginning of greening. Addition of DCMU blocked electron flow out of PSII and consequently some of the energy absorbed by the LHCs was emitted as fluorescence. The large increase in fluorescence when DCMU was added indicated that functional PSII centers were present but did not become sufficiently reduced in the absence of DCMU to cause re-emission of light from LHCs. Essentially no increase in fluorescence was observed when DCMU was added when the measurement was made at the beginning of greening. (C) Fluorescence induction kinetics displayed by cells with a fully-developed chloroplast described a "state 1" condition. The rise to peak fluorescence (Fp) and decline to the steady-state level (Fs), within the maximal fluorescence (Fm) obtained in the presence of DCMU, are typical of results obtained with light-grown algae and leaves of higher plants. (Adapted from White and Hoober, 1994)

(Ohad *et al.*, 1965; Hoober *et al.*, 1991). In these mutants, synthesis of Chl requires light. Exposure of the dark-grown cells to light initiates Chl synthesis and membrane assembly. Assembly of LHCs was monitored by Förster resonance energy transfer from Chl *b* to Chl *a* as these molecules are brought sufficiently close (10 Å or less) as the result of incorporation into LHCs. In these experiments, LHCs accumulated immediately after exposure of cells to light and at a rate that indicated assembly of the complex occurred in seconds (Fig. 7A).

Analysis of the connectiveness of LHCII to reaction centers by photochemical quenching of fluorescence of Chl in the complexes indicated nearly immediate assembly of the complete photosynthetic system (White and Hoober, 1994; White *et al.*, 1996; see Fig. 7A). In these experiments, analysis of the kinetics of greening showed that LHCs were rapidly associated with the photosynthetic apparatus, because light energy absorbed by the complexes was completely trapped. Fluorescence induction kinetics, which reflect the state of PSII, indicated that residual LHCs that existed prior to the light exposure remained unconnected to the electron transfer system. Functional PSI and PSII units were assembled from the beginning of illumination (White and Hoober, 1994) but several hours of greening were required to develop a "state 1" condition, which requires sufficient amounts of thylakoid membranes to allow segregation of the photosystems (Fig. 7B and 7C). During the early period of greening, the chloroplast contained numerous vesicles (Fig. 5) (Hoober *et al.*, 1991), which probably had PSI, PSII and LHCs uniformly distributed, an arrangement that would allow LHCII to transfer energy to the kinetically more rapid PSI, the "state 2" condition. Presumably fusion of vesicles and adherence of nascent thylakoid membranes into grana were required to segregate PSI and PSII so that LHCII was associated predominantly with PSII, as occurs in "state 1" (Depège *et al.*, 2003).

With the technique of immunoelectron microscopy, the first LHCs to appear in the chloroplast were detected in the envelope and associated invaginations, as shown in Fig. 8 (White *et al.*, 1996; Eggink *et al.*, 2001).

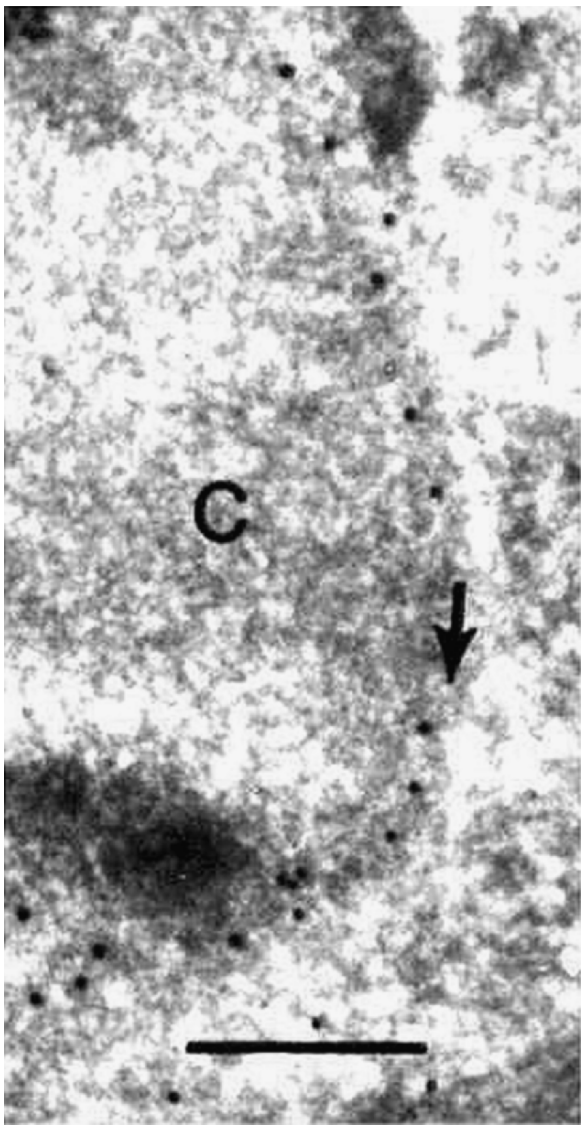


*Fig. 8.* Immunocytochemical localization of LHCP in sections of dark-grown *C. reinhardtii y*1 after exposure to light for 15 min. Sections were treated with antibodies against LHCP and then with 10-nm gold particles conjugated with protein A, which binds to antibodies. The gold particles were found predominantly over the inner membrane of the chloroplast envelope (marked with the arrow), which marked the initial site of integration of the proteins into membrane. Bar, 250 nm. (Adapted from White *et al.*, 1996)

The initial localization of LHCP to the envelope membranes was supported by localization of Chl *a* oxygenase to the envelope (Eggink *et al.*, 2004), which provided the Chl *b* that is essential for LHCII assembly. LHCPs made in excess accumulated outside of the chloroplast in vacuoles (White *et al.*, 1996; Park and Hoober, 1997), evidence that LHCPs that are not incorporated into LHCs are not completely imported into, and thus not retained by, the plastid. Furthermore, when cells were grown in the dark, conditions under which Chl synthesis did not occur, the proteins were not imported into the chloroplast and instead accumulated in the cytosol and vacuoles (Park and Hoober, 1997). These results highlighted the chloroplast envelope as the primary site of assembly of the photosynthetic apparatus during the early stage of chloroplast development. This conclusion is supported by studies on biogenesis of PSII in cyanobacteria, in which proteins involved in processing the precursor of reaction center protein D1 were located in the periplasmic compartment (Zak *et al.*, 2001; Klinkert *et al.*, 2004). These assembly reactions seem to occur at the plasma membrane in cyanobacteria, which is analogous with the inner membrane of the chloroplast envelope.

As demonstrated by Degenhardt and Tobin (1996) in higher plants and more recently in *Chlamydomonas* (Fuhrmann *et al.*, 2004), expression of *LHCP* genes is repressed in the dark and activated by light. The experiments described above, in which the kinetics of initial LHC and thylakoid biogenesis were examined, involved incubation of the algal cells at 38°C prior to exposure to light. The higher temperature apparently reduced the ability of the cells to repress these genes, because *LHCP* mRNA accumulated to the light-induced level in the dark at the higher temperature (Hoober *et al.*, 1982). Thus, this system required light only for Chl synthesis to initiate the developmental process.

The eventual accumulation of LHCPs in thylakoid membranes is determined by molecular interactions that occur initially within the envelope, even before the proteins are completely imported into the plastid. Other than the requirement for Chl, and in particular Chl *b*, little is known about the mechanism of incorporation of these proteins into membranes. A motif was identified in all homologs of LHCPs (Hoober and Eggink, 1999) that binds Chl *a* (Eggink and Hoober, 2000). The sequence ExxHxR in helix-1 of the protein binds two Chl molecules, one to the glutamate(E)-arginine(R) ion-pair and the second to the imidazole sidechain of histidine(H). A similar motif, ExxNxR in helix-3, in which asparagine(N) replaces H, also binds two Chls. As shown in Fig. 6, this motif binds Chl *a* but not Chl *b*. Kohorn (1990) showed that replacement of histidine with alanine or arginine with glutamate in the motif in helix-1 nearly eliminated import into purified chloroplasts. Although these ligands bind Chl *a*, few if any of LHCPs with the wild-type sequence are retained in the chloroplast in mutants that are unable to make Chl *b*,

even though the plants still produce Chl *a*. Therefore, Chl *b* apparently binds to other sites, among which may be the peptide backbone carbonyl ligands found by Liu *et al.* (2004) near the N-terminus of the protein. Notably, the size of Chl *a* oxygenase in *Chlamydomonas,* as estimated by electrophoresis (Eggink *et al.*, 2004), is the same as predicted from the gene sequence (Tanaka *et al.*, 1998). The apparent absence of processing during import into the plastid raises the possibility that binding of Chl to the sequence EQNLQR near the N-terminus may play a role in integration of the oxygenase into the plastid envelope.

Other factors are also likely involved in facilitating assembly of LHCs. For example, mutants that lack the ability to synthesize the major carotenoid in LHCs, the xanthophyll lutein, assemble LHCs much slower than normal (Park *et al.*, 2002). Also, mutants that are deficient in subunits of a stromal complex designated the "chloroplast signal-recognition particle" are deficient in LHCs (Hutin *et al.*, 2002), which suggests that these proteins may be involved in assembly of the complex (Schünemann, 2003). Plants lacking a membrane protein, ALB3, are deficient in LHC assembly (Bellaffiore *et al.*, 2002), although the specific action of this protein is not clear. ALB3 seems to be involved in facilitating insertion of LHCPs into the membrane and is recovered with thylakoid membranes from green cells. However, kinetic measurements of the development of photosynthetic activities indicate that connection between photosystems and LHCs occur immediately upon assembly of the antenna complexes, which leads to the conclusion that assembly occurs as soon as the proteins engage the chloroplast envelope. A brief lag period would be expected were the proteins to enter the chloroplast stroma prior to insertion into membranes, which was not observed (White *et al.*, 1996). A possible explanation for these results is an extensive flow of membrane material from envelope to thylakoid, which would provide a predominant localization of proteins in the latter membrane although their primary site of function is the envelope membrane.

Lipids are synthesized predominantly on envelope membranes (see Chapter 17 in this volume), and the envelope is also the location of the latter steps in Chl and carotenoid synthesis (S Reinbothe and C Reinbothe, 1996; Joyard *et al.*, 1998; Eggink *et al.*, 2004; see also Chapters 15 and 16 in this volume). Thus, the envelope is an important interface between the chloroplast and cytosol and serves as the platform for biogenesis of the extensive thylakoid membrane (Hoober and Eggink, 1999; Hoober and Agyroudi-Akoyunoglou, 2004). In plant cells exposed to light, membrane formation occurs over several hours to several days, depending on the organism, to achieve the mature chloroplast. In some algal systems, membrane formation is induced immediately upon exposure of cells to light, which provides a minute-to-hour time span for experimental studies. The overall process seems to be highly coordinated and other members of a complex do not generally associate when one member of the complex is absent. Often translation of some subunits is controlled by the state of assembly of the complex, a process described as control by epistasy of synthesis (Choquet and Vallon, 2000).

## IV. Overview of Photosynthesis

### *A. Function of the Membrane*

Tetrapyrroles, required cofactors in photosynthesis, are among the most ancient of biological molecules. Availability of cyclic, or rather "macrocyclic", tetrapyrroles that chelate a divalent cation—most commonly a $Fe^{2+}$ or $Mg^{2+}$ ion—allowed development of energy transduction mechanisms, either through electron transport (oxidation-reduction) of the central iron atom in heme or generation of high-energy, excited states of Mg-tetrapyrroles, as with the Chls (see Fig. 2 for structures). Tetrapyrroles strongly absorb visible light. In contrast to hemes, which are not fluorescent because the central iron atom quenches excited states, the Mg-containing Chls are highly fluorescent, which allows transfer of the excited state through many Chl molecules by Förster resonance energy transfer. As deduced from a phylogenetic analysis of genes encoding biosynthetic enzymes, bacteriochlorophyll (BChl) (see Fig. 2) seems to have been the evolutionary precursor of Chl. Comparison of genomic sequences provided evidence that these genes were subsequently introduced into green sulfur bacteria, green nonsulfur bacteria and cyanobacteria by lateral gene transfer.

The essential functional structure for photosynthesis is the thylakoid membrane that physically separates two different compartments and contains an energy transducing apparatus that produces a proton gradient across the membrane and reducing agents such as NADPH. The membrane allows development of an electrochemical gradient, which is an essential intermediate in photosynthesis. Within the membrane reside the reaction centers and a series of electron transfer components. Photochemistry occurs in the reaction centers. The flux of photons, even in full sunlight, is sufficient for only a few photons to be absorbed by a

Chl molecule in a reaction center per second, a rate that is much too slow for productive photosynthesis. Therefore, photosynthetic organisms developed structures that contain a large number of accessory pigment molecules that harvest light energy and funnel the energy into reaction centers. As a consequence, high-energy states are generated at a sufficient rate to efficiently drive synthetic reactions.

Cyanobacteria were the earliest organisms to perform oxygen-producing photosynthesis because they were the first to use Chl rather than BChl as the primary pigment. The higher energies achieved by excited states of Chl made it possible to span the difference in redox potential from oxidation of water to oxygen ($E_m' = +0.816$ V) to reduction of $NADP^+$ to NADPH ($E_m' = -0.342$ V). The radical cation $P680^{\bullet+}$, generated in reaction centers of PSII by absorption of light energy, has a very high oxidizing potential, about 1.3 V (Rappaport *et al.*, 2002; Ishikita *et al.*, 2005), which is sufficient to pull electrons from water. The result is generation of molecular oxygen, which, however, did not appear at a significant level in the atmosphere until about 2,300 MYA (Arnold *et al.*, 2004; Hedges *et al.*, 2004). In contrast, the oxidizing potential of BChl in the special pair of photosynthetic bacteria is about 0.4 V (Ferreira *et al.*, 2004), and thus organisms that contain Bchl cannot evolve oxygen. This difference is also reflected in the higher energy, shorter wavelength (680 nm) of the absorption maximum ($Q_y$ vector) in the red region of the spectrum of Chl *a* as compared with that of BChl *a* (875 nm). Interestingly, the genes for the reaction-center proteins that *bind* (B)Chl have a deeper lineage than those that encode the enzymes that catalyze *synthesis* of (B)Chl. Lack of congruence of the genes that encode the BChl/Chl biosynthetic enzymes and the reaction center proteins that bind these pigments indicates that the photosynthetic apparatus is a composite structure, with components recruited from multiple sources. This concept suggests that the earliest proteins for photosynthetic functions were recruited from those already present. Of particular interest is the proposal that *photosynthetic* reaction center proteins were derived from *respiratory* cytochrome *b* by gene duplication and subsequent divergence of the genes to encode proteins with new functions, from one that bound Fe-porphyrin cofactors (the heme in cytochromes) to one that bound Mg-chlorin (Chl) cofactors (Xiong and Bauer, 2002).

In oxygenic photosynthesis, two reaction centers operate in series. One, photosystem (PS) II, has a quinone as the electron acceptor (type II, from the purple bacterial lineage), while the second, PS I, has an iron-sulfur comlex as the electron acceptor (type I, from the heliobacterial and green sulfur bacterial lineage). The two photosystems were combined for the first time in cyanobacteria by lateral gene transfer and are localized to the thylakoid membranes, which form concentric layers inside, but separate from, the plasma membrane. The structure of PSII was resolved by two groups (Zouni *et al.*, 2001; Ferreira *et al.*, 2004), with only slightly different results. The reaction center exists as a dimer (Nield *et al.*, 2000). Each monomeric complex contains 19 protein subunits, 36 Chl *a* molecules and 7 *all-trans* β-carotenes. Most of the Chls are bound to chloroplast-encoded "core antennae" proteins, CP43 (PsbC, 50 kDa) and CP47 (PsbB, 56 kDa), with 14 and 16 Chl molecules, respectively (Ferreira *et al.*, 2004). The core of the reaction center consists of two similar proteins, D1 (PsbA, 38 kDa) and D2 (PsbD, 39 kDa). Each binds one of the Chls that comprise the special pair, P680. Upon absorption of light energy, the excited state is delocalized over the special pair and two accessory Chl molecules, one on each core protein. An electron is transferred from $Chl_{D1}$ to a pheophytin *a* (a Chl molecule lacking the central Mg atom) on D1, then to a tightly bound plastoquinone on D2, and finally to a loosely bound plastoquinone on D1 (Hankamer *et al.*, 1997). The electron hole on $P680^+$ is then filled by abstraction of an electron from a tyrosine residue, $Tyr_Z$, in D1, which in turn abstracts an electron from the oxygen-evolving complex. This complex, a cubane-like cluster of four Mn ions linked by oxygen atoms, retrieves four electrons from water, one at a time, to generate a molecule of diatomic oxygen (Barber, 2003).

When reduced, the loosely bound plastoquinone in PSII dissociates and diffuses to another large, multisubunit protein complex, the cytochrome $b_6f$ complex. This complex also exists as a dimer, with each monomer containing four large (18- to 32-kDa) subunits (cytochrome $b_6$, the Rieske iron-sulfur protein, cytochrome *f* and subunit IV) and four small polypeptide subunits. Cytochrome $b_6$ contains an atypical heme along with the two heme moieties that are involved in the Q-cycle. A Rieske iron-sulfur ($Fe_2S_2$) protein is the electron transfer link between cytochrome $b_6$ and cytochrome *f*. A Chl *a* molecule, with no apparent amino acid ligand and no known function, sits between two helices of subunit IV. One 9-*cis*-β-carotene molecule resides between helices formed by two of the small, hydrophobic subunits (Stroebel *et al.*, 2003; Kurisu *et al.*, 2003). Because of the Q-cycle, passage of each electron through the complex is accompanied by transfer of two protons ($H^+$) from the stroma to the thylakoid lumen (Sacksteder *et al.*, 2000).

The small, copper-protein, plastocyanin, transfers electrons from cytochrome *f* to a specific docking site on PSI. The crystal structure of PSI revealed 12 protein subunits, 96 Chl *a* molecules, 22 carotenoids, 2 phylloquinone and 3 $Fe_4S_4$ clusters. The core of the complex is a heterodimer of two similar 83-kDa proteins, PsaA and PsaB, that are encoded in the chloroplast genome. Two Chls form the special pair, designated P700 from its absorption maximum, one bound to each core subunit. Electrons move from P700 to a Chl molecule, designated $A_0$, then to a phylloquinine, $A_1$, and finally to the iron-sulfur centers, $F_X$, $F_A$ and $F_B$ (Jordan *et al.*, 2001). Although essentially a symmetrical arrangement of two branches, electrons flow preferentially through the "A" branch (Fromme *et al.*, 2001). The additional 90 Chl molecules that are not part of the reaction center are bound to the N-terminal domains of PsaA and PsaB and provide the "core antenna". The PSI core complex also forms a supercomplex but as a monomer, in contrast to PSII, with its light-harvesting complexes, LHCI (Kargul *et al.*, 2003).

The major light-harvesting function in cyanobacteria is provided by peripheral, exquisitely designed complexes of proteins that contain covalently-bound, linear tetrapyrrole chromophores (phycobilins). These complexes, called "phycobilisomes", are assembled from the subunits phycoerythrin ($\lambda_{max}$ 565 nm), phycocyanin ($\lambda_{max}$ 620 nm) and allophycocyanin ($\lambda_{max}$ 650 nm), named according to their reddish or bluish color, respectively (Grossman *et al.*, 2001). The splayed arrangement of the rod-like structures assembled from these subunits, and the sequential positioning along each rod of the highest energy chromophore (shortest wavelength maximum) at the outer end to the lowest energy chromophore (longest wavelength maximum) at the end adjacent to the reaction center, provides the "downhill" flow of excitonic energy that is necessary for efficient trapping of light energy (Gantt, 1981). This general design for the photosynthetic light-harvesting with phycobilisomes was retained, with slight modifications, in the chloroplast of red algae, which lack Chl *b* and thus the major Chl *a/b*-containing light-harvesting complexes but still contain homologs of LHCPs (Wolfe *et al.*, 1994).

## *B. Differentiation of Carbon Fixation*

Over 90% of land plants contain *only* the reductive pentose-phosphate cycle, i.e., the $C_3$ pathway in which 3-phosphoglycerate is the initial product of the Rubisco-catalyzed reaction between $CO_2$ and ribulose 1,5-bisphosphate. The remainder developed additional pathways for assimilation of carbon (Ku *et al.*, 1996). These alternate pathways are adaptations that provide greater efficiencies in more hostile environments, such as areas with higher temperatures and a more arid climate. In these pathways, $HCO_3^-$ is the initial carbon source instead of $CO_2$. Bicarbonate is formed by dissociation of carbonic acid, which is formed when $CO_2$ in solution is hydrated. Hydration of $CO_2$ is catalyzed very rapidly by the enzyme carbonic anhydrase. Because the solubility of $CO_2$ in water decreases with increasing temperature, the concentration of bicarbonate can therefore reach much higher levels than that of $CO_2$ under these conditions. Consequently, incorporation of carbon into organic molecules is more efficient. The initial reaction in this pathway is catalyzed by phospho*enol*pyruvate carboxylase, which adds the one-carbon unit to phospho*enol*pyruvate to produce the 4-carbon compound, oxaloacetic acid. In contrast to the reaction catalyzed by Rubisco, $O_2$ is not a substrate or inhibitor of this reaction. The product, oxaloacetate, is unstable and is rapidly reduced to malate or converted to the amino acid aspartate by a transamination reaction. The initial product of carbon assimilation is a 4-carbon intermediate, and thus the pathway has become known as the $C_4$ pathway.

The presence of the $C_4$ pathway is usually accompanied by a leaf morphology referred to as "Kranz" (crown) anatomy. The vascular tissue in these plants is surrounded by a layer of "bundle sheath" cells that perform the $C_3$ pathway. Chloroplasts in bundle sheath cells contain thylakoid membranes that are not differentiated into grana or tightly appressed. Surrounding the bundle sheath cells are layers of "mesophyll" cells that initiate the $C_4$ pathway. Thylakoid membranes in the chloroplasts in these cells are differentiated into the typical granal and stromal membranes. Interestingly, some species, such as in the family *Chenopodiaceae*, contain both pathways, with chloroplasts differentiated into the two types that are typical of bundle sheath and mesophyll cells *within the same cell* (Voznesenskaya *et al.*, 1999, 2002).

Phospho*enol*pyruvate carboxylase is located predominantly in mesophyll cells. The initial products of carbon assimilation, malate or aspartate, are transported to bundle sheath cells, where they are oxidized or deaminated and then decarboxylated to generate $CO_2$. The $CO_2$ thus produced is used by Rubisco in chloroplasts of the bundle sheath cells to initiate the typical reductive pentose-phosphate cycle. The other product of the decarboxylation reaction is pyruvate, which in plants that use malate as the "carbon carrier" is returned to the mesophyll cells. In those plants that use

aspartate, pyruvate is transaminated to alanine, which is also returned to the mesophyll cells. In these latter plants, the carrier must transport nitrogen as well as carbon. The advantage of the interplay between these cells is a significantly higher concentration of $CO_2$ in the chloroplast of bundle sheath cells, which results from its production in situ, than could be achieved under normal atmospheric conditions. Moreover, bundle sheath cells have a lower PS II to PS I ratio than mesophyll cells, which reduces the amount of oxygen produced by PS II during the light reactions. Bundle sheath cells thus seem to perform more cyclic photosynthetic electron transport than mesophyll cells. The higher concentration of $CO_2$, combined with a lower oxygen level, increases the efficiency of the "carboxylation" reaction of Rubisco over the "oxygenation" reaction, which allows these plants to thrive under less favorable conditions. However, this adaptation has an energy cost of two additional ATP molecules consumed per $CO_2$ assimilated. This additional energy is required to convert pyruvate to phospho*enol*pyruvate in mesophyll cells, which expends two equivalents of ATP to convert AMP, the product of this reaction catalyzed by pyruvate orthophosphate dikinase, to ADP and then to ATP.

Development of the $C_4$ pathway probably occurred just over 7 MYA, when the carbon isotope ratio in fossil organic material abruptly changed from one that showed strong discrimination against $^{13}C$, a characteristic of $CO_2$ fixation by Rubisco, to a less discriminatory ratio characteristic of bicarbonate incorporation by phospho*enol*pyruvate carboxylase. The change in ratio indicated a rapid expansion of $C_4$ plants across the earth. $C_4$ plants have high rates of photosynthesis and growth, and 11 of the 12 most productive species are $C_4$ species (Furbank, 1998). Although only about 3% of the approximately 250,000 current species of plants contain the $C_4$ pathway, the agricultural importance of several of these species (e.g., maize, sugarcane and sorghum) results in about 30% of the primary productivity of plants accomplished by $C_4$ plants.

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# Chapter 3

# Protein Import Into Chloroplasts: Who, When, and How?

Ute C. Vothknecht* and Jürgen Soll
*Department of Biology I, Botanisches Institut der LMU München, Menzinger Straße 67, D-80638 München, Germany*

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## Summary

Chloroplasts are the characteristic organelles of photosynthetic algae and plants. They originated from a cyanobacterial ancestor that was engulfed by a eukaryotic host cell and subsequently transformed into an organelle. As a result of this heritage, chloroplasts are surrounded by a double membrane, the outer and inner envelope. Plastid proteins that are encoded by the nucleus and translated on cytosolic ribosomes must be transported into the organelle across both of these membranes. This is achieved by two hetero-oligomeric protein complexes within the outer and inner envelope, the Toc and the Tic translocons. Both translocons are composed of a small number of proteins that were inherited from the cyanobacterial ancestor, as well as additional proteins. The genome of

*Author for correspondence, email: vothknecht@lrz.uni-muenchen.de

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*Arabidopsis thaliana* contains multiple paralogs for many of the components of the import apparatus. This finding, which has been corroborated by experimental data, has kindled the idea that there might be specialized import pathways for certain subclasses of chloroplast-imported proteins. Furthermore, alteration of Toc and Tic complex composition could be a means to regulate chloroplast protein import in different tissues or at different developmental stages. This seems likely because the import of precursor proteins into chloroplasts should be regulated in accordance with the physiological and developmental needs of both the organelle and the surrounding cell. Not surprisingly, a functional protein import apparatus is a prerequisite for plastid, and subsequently, plant development. Once inside the chloroplast, proteins must be routed to their final destination within the organelle. The exact manner by which the routing is achieved is so far known only for a small number of proteins. A vesicle transport system of eukaryotic origin that exists in chloroplasts of vascular plants could be the way by which some proteins are transported to the thylakoid membrane.

## I. Introduction

Chloroplasts are plant organelles of endosymbiotic origin. In an allegedly singular event, a photosynthetic bacterium, closely related to contemporary cyanobacteria, was taken up by a heterotrophic host cell (Mereschkowsky, 1905; Margulis, 1970; Palmer, 2000). The endosymbiont was subsequently transformed into an interdependent cell organelle with essential functions for the arising plant cell. An important step in the domestication of the chloroplast was the transfer of the majority of the cyanobacterial genes into the host cell nucleus, through which the host gained control over organelle functions (Martin *et al.*, 2002). Since nuclear-encoded proteins are synthesized on cytosolic ribosomes, development of a system to transfer the plastidal proteins back into the organelle was necessary. The two envelope membranes that surround the chloroplast present a barrier that cannot be traversed by proteins unassisted. Thus, simultaneously with the transfer of genes from the organelle to the nucleus, a proteinaceous import machinery arose in the outer and inner envelope membranes to facilitate the return of proteins encoded by these genes to the plastid (Fig. 1). These import complexes, called Toc (translocon on the outer envelope of chloroplasts) and Tic (translocon on the inner envelope of chloroplast), are essential for organelle development and function. The translocation of proteins into the organelle is a highly regulated process and thereby adds an additional control point to the protein content of the organelle. An intra-organellar sorting machinery ensures that the translocated proteins are directed to their final location within the chloroplast (Fig. 2) and if required, are assembled into multiprotein complexes, which often contain a mixture of subunits with dual genetic origin, i.e., nuclear and organelle encoded.

*Abbreviations:* ER – endoplasmic reticulum; FNR – ferredoxin-NAD(P)$^+$ oxidoreductase; HSP – heat shock protein; IE – inner envelope membrane; Lhcb – light-harvesting chlorophyll-binding protein of photosystem II; OE – outer envelope membrane; OEP – outer envelope protein; pFD – precursor of ferredoxin; pOE – precursors of subunits of the oxygen-evolving complex; pPC – precursor of plastocyanin; PG – phosphatidylglycerol; pSSU – precursor of small subunit of ribulose-bisphosphate carboxylase/oxygenase; SRP – signal recognition particle; SSP – stromal processing peptidase; Tat – twin arginine translocase; Tic – translocon on the inner envelope of chloroplasts; Toc – translocon on the outer envelope of chloroplasts.

### *A. Protein Translocation into Primary Plastids*

About 2,500 nuclear-encoded proteins are synthesised on cytosolic ribosomes and subsequently targeted to and translocated into the chloroplast (Leister, 2003). Most of these proteins are synthesised with an N-terminal, cleavable presequence that functions as an address-tag to guide them to the organelle and across the two envelope membranes (Dobberstein *et al.*, 1977). The presequence ensures the specificity of this process. It allows the protein to be translocated into the destined organelle while at the same time preventing mis-targeting into other cell organelles. This is especially important in plant cells where two endosymbiotic organelles, mitochondria and chloroplasts, reside side by side. The presequence permits the simultaneous translocation across both envelope membranes and it is subsequently cleaved off in the stroma by a single enzyme, the so-called stromal processing peptidase (SPP) (Oblong and Lamppa, 1992). The process of translocation had to be developed early in the course of events that transformed the endosymbiont into a cell organelle. As soon as the first gene was permanently relocated to the host nucleus, the retransfer of the encoded protein into the organelle had to be ensured. It is therefore not surprising that the principal players in the translocation game are evolutionarily conserved within different types of plastid-containing organisms

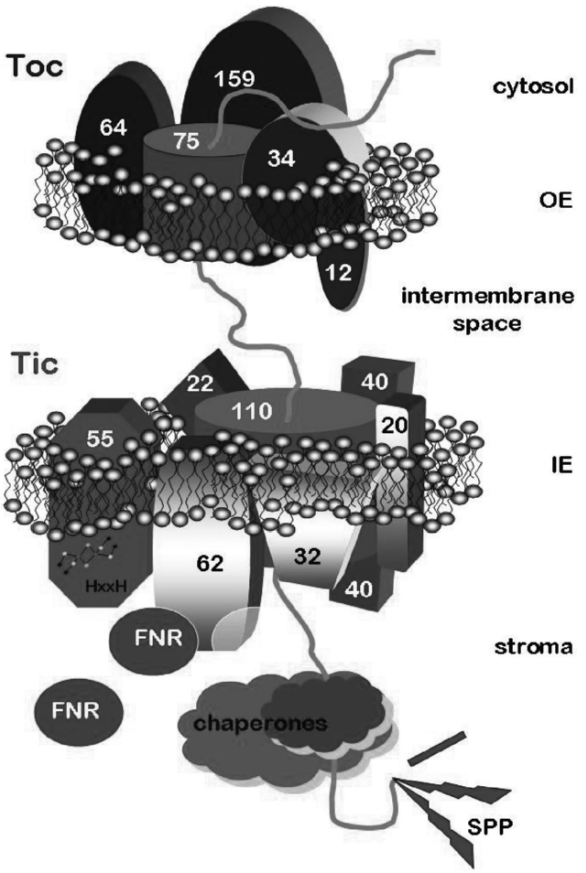


*Fig. 1.* (See also Color Plate 1, p. xxxiv.) Schematic depiction of the chloroplast protein translocon. The majority of nuclear-encoded plastid proteins are synthesized with an N-terminal cleavable presequence. These proteins translocate into the organelle via two proteinaceous heteromeric protein complexes, the Toc and Tic translocons. Components of these complexes are denoted by their molecular weight. After recognition of the precursor proteins by the receptor proteins of the Toc translocon, the translocation occurs simultaneously across the outer and the inner envelope membrane. Chaperones assist the translocation process at the outside as well as from within the organelle. Once the N-terminus of the precursor protein has entered into the stroma the presequence is cleaved off by a soluble enzyme called the stromal processing peptidase (SPP).

ranging from Glaucocystophytes, believed to be the most ancient of plastid-containing eukaryotes, to red and green algae, and up to the highest developed land plants (Schwartzbach *et al.*, 1998; Inagaki *et al.*, 2000; Steiner and Löffelhardt, 2002).

### *B. Protein Translocation in Secondary Plastids*

The original endosymbiosis was most likely a singular event that created a chloroplast surrounded by a

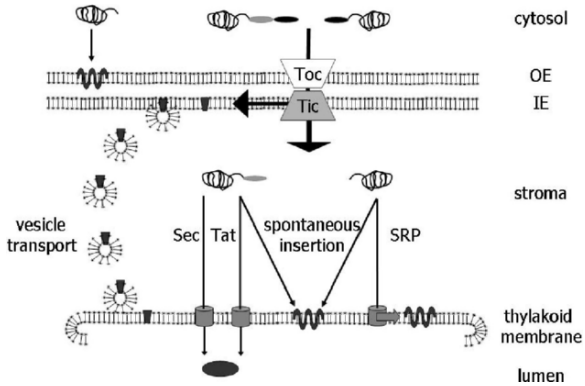


*Fig. 2.* Several different pathways facilitate protein translocation into and across the thylakoid membrane, the Sec-, Tat-, and SRP-pathways, as well as a process dubbed spontaneous insertion. All of these pathways belong to a group of ancient and ubiquitous protein translocation pathways, which are similarly found in bacteria and the cytosol of eukaryotic organisms. Three of these pathways utilize an N-terminal signal sequence in addition to the one required for translocation into the organelle. In chloroplasts of vascular plants, vesicle transport might constitute a further system for protein transport to the thylakoids.

double membrane (Palmer, 2000). Subsequently, multiple events of secondary and even tertiary endosymbiosis between nonphotosynthetic eukaryotes (secondary host) and photosynthetic eukaryotes (the primary host now turning into a secondary endosymbiont) created organisms with so-called complex plastids (Gibbs, 1978). In the course of these events the secondary endosymbiont was again greatly reduced. Mitochondria and most other cell organelles were removed, and its nucleus was downsized to hold only a very few genes or it often vanished completely. In many cases the chloroplast was the only cell organelle that was retained. Since both green as well as red algae acted as secondary endosymbionts, organisms containing complex plastids are found in two different lineages of photosynthetic eukaryotes. In some cases the photosynthetic capacity of the secondary plastid was completely lost but the organelle itself, now called the apicoplast, was retained (McFadden *et al.*, 1996). Included in this latter group are such important human pathogens as *Plasmodium falciparum* and *Toxoplasma gondii*, which cause malaria and toxoplasmosis, respectively.

Because of their origin, the complex (or secondary) plastids are surrounded by three or even four membranes. The two innermost membranes resemble the envelope of the primary plastid, while the outermost membrane is derived from the endomembrane system of the secondary host (Cavalier-Smith, 2003). It is assumed that at the time of the secondary endosymbiosis

most of the genes of the primary plastid had already been transferred to the nucleus of the primary host. Accordingly, the systems for translocation across the envelope membranes had already been developed. After the second endosymbiosis, many genes encoding plastid-targeted proteins were further transferred from the nucleus of the primary host into the nucleus of the secondary host. For these proteins, which are now synthesised in the cytosol of the secondary host cell, it is necessary to cross three or even four membrane in order to translocate into the secondary plastid. In the case of Cryptophytes and Chlorarachniophytes, part of the cytosol of the original primary host, including a translation machinery and a residual nucleus, was retained. This residual nucleus, now called the nucleomorph, still encodes some plastid-targeted proteins, which therefore have to cross only the two innermost membranes of the secondary plastid. To our current knowledge, translocation across these two inner membranes is phylogenetically conserved and follows the same pathway as for primary plastids. On the other hand, a new pathway was developed for crossing the outermost membranes and it involves the endomembrane system of the secondary host (for a comprehensive review, see van Dooren *et al.*, 2001).

## II. On the Road to the Chloroplast

### *A. Membrane Insertion Into the Outer Envelope Membrane*

While most of the proteins destined for the inside of the chloroplast seem to translocate with the help of a cleavable presequence, this is not the case for the majority of the proteins residing in the chloroplast outer envelope itself (Fig. 2). Most outer envelope proteins (OEPs) do not contain a N-terminal presequence and they do not rely on the general import pathway for their insertion into the membrane (for comprehensive reviews, see Soll and Tien, 1998; Schleiff and Klösgen, 2001). Indeed, there is very little knowledge about how OEPs are routed to the organelle. It has to be assumed that the routing information lies within the mature protein. It was consequently shown that targeting information for OEP14 and Com70 resides in the first 30 and 40 amino acids, respectively (HM Li *et al.*, 1991; Wu and Ko, 1993). In contrast, for OEP34 the ten-amino-acid-long hydrophobic core of the C-terminal membrane anchor is important for correct targeting (HM Li and Chen, 1996). GFP fusion experiments argue a similar case for OEP7, where a seven-amino-acid stretch located within the C-terminal transmembrane domain played a critical role in targeting of this protein to the chloroplast (YJ Lee *et al.*, 2001). Whether or not the transport of these proteins to the organelle is assisted by cytosolic factors is not known.

Most studies to date have been aimed at the question of the actual insertion into the outer envelope membrane after the proteins reach the chloroplast. Membrane insertion of proteins is a ubiquitous process within all kind of cells and several of the mechanisms employed are phylogenetically conserved. Yet, no components of known membrane insertion pathways have been identified in the outer envelope of chloroplasts. Therefore, OEPs are either inserted into the membrane without any assistance, by a mechanism dubbed spontaneous insertion, or alternatively, the insertion is assisted by unidentified cytosolic proteins, which, as is the case in mitochondria, reside on the outer envelope (Shore *et al.*, 1995). Detailed studies on the insertion of OEPs have so far been restricted to a very small subset of proteins, i.e. OEP21, OEP14, OEP7 and Toc34 (Salomon *et al.*, 1990; HM Li *et al.*, 1991; May and Soll, 1998; Tsai *et al.*, 1999; Tu and Li, 2000; YJ Lee *et al.*, 2001). In general, the insertion process into the outer membrane shows no strict dependency on nucleotide phosphates, even though some findings indicate a stimulation of Toc34 insertion by ATP and GTP (Seedorf *et al.*, 1995; D Chen and Schnell, 1997; HM Li and Chen, 1997; Tsai *et al.*, 1999). On the other hand, OEP7 as well as Toc34 were inserted into artificial liposomes without the presence of nucleotide phosphates or an ATP-generating system (YJ Lee *et al.*, 2001; Qbadou *et al.*, 2003). Studies on the requirement for proteinaceous components, which might assist the insertion process, yielded even less clear-cut results. It was shown that OEP14, OEP7 and OEP21 could insert into the outer membrane even after treatment of the chloroplasts with the protease thermolysin. This would exclude the participation of soluble cytosolic components as well as proteins that are not fully embedded into the outer envelope (Salomon *et al.*, 1990; HM Li *et al.*, 1991; Fröhlich *et al.*, 2001). Contrary to this, it was shown that insertion of OEP14 is sensitive to a pretreatment of the membrane with trypsin and that Toc34 insertion is at least stimulated by a thermolysin-sensitive component (Tsai *et al.*, 1999). It is clear, on the other hand, that charge distributions within the proteins are important for the insertion process (HM Li and Chen, 1997; May and Soll, 1998; YJ Lee *et al.*, 2001; Schleiff *et al.*, 2001). Also, the lipid composition and distribution of the outer envelope appear to be important for this process. The lipid distribution

within the outer envelope is asymmetric. Compared to the inner leaflet, the outer leaflet of this membrane is enriched in phosphatidylglycerol and depleted in phosphatidylcholine (Dorne *et al.*, 1985). This is significant because charged lipids like phosphatidylglycerol were found to play a role in the association and insertion of proteins into lipid bilayers (van't Hof *et al.*, 1993; van't Hof and de Kruijff, 1995). Furthermore, the outer envelope is the only membrane facing the cytosol that contains the nonbilayer-forming lipid, monogalactosyldiacylglyceride (Bruce, 1998), a lipid believed to be important for protein membrane interaction and insertion.

There is at least one notable exception to the insertion of OEPs and that is Toc75. This translocation pore of the Toc translocon (see below) is synthesized with a bipartite N-terminal presequence and the protein is targeted to the outer envelope via the chloroplast import translocon (Schnell *et al.*, 1994; Tranel *et al.*, 1995). The N-proximal part of its presequence resembles presequences of chloroplast-targeted proteins and it has been shown that this part is cleaved off by the stromal processing peptidase (SPP) (Tranel *et al.*, 1995; Inoue *et al.*, 2001). The C-terminal part of the presequence guides Toc75 into the outer envelope membrane and is processed after or during the insertion by an unknown mechanism (Tranel *et al.*, 1995; Inoue *et al.*, 2001; Inoue and Keegstra, 2003). The exact method by which Toc75 enters the outer envelope has not been elucidated but it is assumed that translocation is halted by means of a stop-transfer signal. It therefore does not enter into the stroma completely. Instead, it might either be inserted from the periplasmic space or it moves into the membrane laterally from the Toc translocon.

### *B. Targeting by Presequence*

Targeting of proteins with the assistance of an N-terminal cleavable presequence is a common feature of many cellular translocation pathways (Dobberstein *et al.*, 1977). Besides chloroplast translocation, this system is employed by the secretion pathway as well as for mitochondrial import. The fact that multiple pathways facilitate a similar recognition process implies two important characteristics of the presequence. The presequence must ensure that proteins are translocated into the correct organelle while at the same time being rejected by all others. Studies so far indicate that *in vivo* the precursor recognition is indeed extremely faithful. No significant mis-targeting of proteins has been observed. Mis-targeting into the wrong organelle has been observed *in vitro* when only a single, albeit wrong, organelle was offered in the assay. If multiple organelles were present in so-called competitive imports, translocation became faithful again and no mis-targeting was observed. Interestingly, it is the mitochondrion that is willing to import chloroplast proteins rather than visa versa. It could be the fact that the chloroplast is the more junior of the two endosymbiotic organelles that makes its import process more restricted.

A question that remains to be answered concerns specific features of the presequence that are responsible for the recognition of plastidal precursors by the Toc-translocon. Regarding their primary structure, presequences of known chloroplast proteins are extremely diverse. They can vary in length from 20 to 150 amino acid residues and appear to have little conservation in their amino acid sequence. Common to all chloroplast precursors, however, is an overall positive charge and an enrichment in the hydroxyl amino acids, serine and threonine. In aqueous solution chloroplast presequences are largely unstructured, with the highest potential to form a random coil. This might change when the precursor proteins arrive at the outer envelope and the presequence encounters the membrane interface. It has been suggested that upon this change in environment the presequence adopts a largely alpha-helical structure in the N-proximate and C-proximate part while the central region remains a random coil. It has been proposed that the interaction with the envelope membrane induces necessary changes within the presequence to allow recognition by the proteinaceous import machinery and has therefore to be considered an integral part of the precursor protein recognition process. While most of these studies have concentrated on the presequence itself, there is some evidence that the mature part of the proteins may influence, although not determine, the import of chloroplast proteins (Rial *et al.*, 2002).

Interestingly, there are several proteins that are deliberately targeted into both mitochondria and chloroplasts by means of an identical presequence. This process is called dual targeting. The first protein known to be dual-targeted was glutathione reductase from pea (Creissen *et al.*, 1995). Since then the process was shown for several more proteins (Peeters and Small, 2001). Most noteworthy in the context of this review, dual targeting includes a processing peptidase, which cleaves the presequence off mitochondrial and chloroplast precursor proteins (Moberg *et al.*, 2003). Dual targeting is achieved by an ambiguous presequence and the way by which these proteins are channelled into the different organelles is very poorly understood. Because these proteins make use of the identical presequence to translocate into mitochondria as well as chloroplasts, it

has to be assumed that their targeting signal is recognized by both import machineries.

### C. Targeting Without Presequence

It was believed for a long time that the cleavable, N-terminal presequence is both necessary and sufficient for the import of all plastid proteins, with the exception of components of the outer envelope. Recent studies have challenged this view on chloroplast import. Ferro *et al.* (2002) identified a chloroplast-located quinone oxidoreductase in the inner envelope of chloroplasts that did not contain a recognizable presequence. They could show that the protein is indeed imported into chloroplasts and that no N-terminal processing of the protein occurs (Miras *et al.*, 2002). Even more, a GFP-fusion construct lacking the first 59 amino acids of the coding region of quinone oxidoreductase was still translocated into chloroplasts. The import of proteins without an N-terminal presequence is further supported by studies on the translocation component Tic32 (Nada and Soll, 2004). The protein does not contain a typical N-terminal presequence and, while the first ten amino acids of the mature protein seem to be essential for translocation into the chloroplasts, they are not cleaved off during this process. The authors could furthermore show that the protein does not rely on the Toc complex and that the ATP requirement for its translocation is significantly lower than for proteins that use the "general import pathway" (Nada and Soll, 2004). The implication of these studies is that a system exists besides the known Toc- and Tic-translocation that can import proteins into chloroplasts without the requirement of an N-terminal presequence. Further studies are required to show whether there are many more proteins targeted to the chloroplast in this manner. For now the fact remains that the vast majority of all known chloroplast proteins use the "classical" Toc-Tic pathway and the future will show whether such alternative import pathways are the tip of an iceberg that will lead to a new understanding of chloroplast protein translocation or whether they remain the proverbial exception that proves the rule.

## III. Protein Translocation via Toc and Tic

### A. Cytosolic Components

There are a number of cytosolic components that assist the protein translocation process. Chaperones of the Hsp70 (heat shock protein of 70 kDa) class interact with all kinds of chloroplast precursor proteins the moment they emerge from the ribosome. Their function is to prevent the premature folding of the precursor proteins, because the import machinery admits only unfolded proteins. At the same time, they prevent aggregation of hydrophobic proteins in the cytosol. The requirement of chaperones for the import reaction was first shown *in vitro* for the precursor of the light-harvesting chlorophyll-binding protein (Waegemann *et al.*, 1990). Small and nonhydrophobic proteins like pSSU (precursor of small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) and pFD (precursor of ferredoxin) can be imported *in vitro* without the assistance of chaperones (Pilon *et al.*, 1990, 1992). While the requirement for chaperones can be overcome *in vitro*, it is assumed that *in vivo* all precursor proteins interact with cytosolic chaperones at one point or the other (Beckmann *et al.*, 1990). This concept is supported by an analysis of chloroplast transit sequences, which revealed that over 70% of all known precursor proteins have a potential binding site for DnaK, the *Escherichia coli* Hsp70 homolog (Ivey III *et al.*, 2000). *In vitro* studies that followed up this analysis showed that chloroplast precursor proteins do indeed interact with Hsp70 proteins from plants and that they do so directly and nonspecifically (Ellis and van der Vies, 1991; Rial *et al.*, 2000).

A second cytosolic component of the import process is a so far unidentified serine/threonine kinase (Waegemann and Soll, 1996). Phosphorylation of precursor proteins within the transit sequence is a specific feature of chloroplast import, although it is not required for targeting fidelity. Phosphorylated precursor proteins will bind to the Toc translocon but they have to be dephosphorylated prior to translocation.

The analysis of precursor phosphorylation led to the discovery of another cytosolic targeting component. When chloroplast transit sequences were analysed for a consensus phosphorylation motif, it turned out that a potentially common motif found in transit sequences displayed similarity to the phosphopeptide binding motif of 14-3-3 proteins. A subsequent study revealed the binding of several precursor proteins to a so-called guidance complex (May and Soll, 2000). The guidance complex is composed of 14-3-3 proteins, Hsp70, and possibly other proteins. Phosphorylation of the transit sequence seems to be a precondition for binding to the guidance complex. Interaction with the guidance complex on the other hand, increases the import rate of the precursor protein several-fold (May and Soll, 2000). Probably not all chloroplast-targeted precursor proteins interact with the guidance complex. *In vitro,* most proteins are imported without the assistance of

this system and its requirement is stimulatory rather than mandatory.

### *B. The Toc Translocon*

The term Toc-translocon in its broadest sense denotes the complete set of proteins involved in the translocation of precursor proteins across the outer membrane of the chloroplast envelope. Mostly it is reserved for the set of proteins that form the actual membrane-embedded translocation unit (Fig. 1). Most of the Toc components were identified initially by biochemical studies. A majority of these studies were performed on isolated chloroplasts of *Pisum sativum* and, if not specifically mentioned, the text refers to the pea proteins. Due to the availability of the completed genome, the research on protein translocation has partially shifted to *Arabidopsis thaliana* as the model organism and those proteins will be identified by an "at" prefix.

To our current knowledge, the Toc translocon comprises a core of three proteins, called Toc159, Toc75 and Toc34 according to the molecular masses of the proteins (Schnell *et al.*, 1997). These core proteins build a molecular machine that is responsible for the recognition of the precursor protein as well as its translocation across the outer membrane. Together they form a complex with an apparent mass of about 500 kDa (Schleiff *et al.*, 2003) that contains a single Toc159, four copies of Toc75 and four to five copies of Toc34. The core complex appears to form a solid ring with a width of about 130 Å built around four translocation channels. Two more components, Toc64 (Sohrt and Soll, 2000) and Toc12 (Becker *et al.*, 2004a), are associated with the Toc translocon but do not appear to be part of the core unit (Fig. 1).

Toc75 is the most prominent protein in outer membrane preparations from pea chloroplasts. The protein is deeply embedded into the membrane and therefore inaccessible to proteolytic digestion. Structural analysis of the amino acid sequence indicates that Toc75 can form a beta-barrel anion channel formed by 16 transmembrane β-sheets (Sveshnikova *et al.*, 2000). Heterologously expressed Toc75 that was reconstituted into liposomes formed a voltage-dependent, cation-selective channel with an opening of about 26 Å (Hinnah *et al.*, 1997, 2002). Channel properties were affected by the presence of precursor proteins or peptides that mimic the presequence but not by mature chloroplast proteins lacking a presequence. Together these findings support the role of Toc75 as the import channel of the Toc translocon (Fig. 1). It was furthermore shown that Toc75 has a binding site for precursor proteins and acts independently of the Toc receptors Toc34 and Toc159 (Kouranov and Schnell, 1997).

The two remaining core components, Toc159 and Toc34, have been characterized as precursor protein receptors. They can each be phosphorylated and this feature has been implicated in the regulation of precursor recognition (Sveshnikova *et al.*, 2000b; Fulgosi and Soll, 2002). The phosphorylation process is catalysed by two independent kinases, which themselves reside within the outer envelope (Fulgosi and Soll, 2002). Furthermore, Toc159 and Toc34 were shown to bind GTP via a classical GTP-binding site, which is part of a larger GTP-binding domain (Hirsch *et al.*, 1994; Kessler *et al.*, 1994, Seedorf *et al.*, 1995). Both proteins share significant sequence homology within this GTP-domain that extends well beyond the actual GTP-binding motif.

Toc34 seems to be the primary receptor of the Toc translocon. Interaction with precursor proteins was shown by cross-linking experiments and this interaction appears to be tightly regulated. While phosphorylation of the precursor protein is not a prerequisite for translocation, phosphorylated precursor proteins exhibit enhanced binding to Toc34 (Sveshnikova *et al.*, 2000b; Schleiff *et al.*, 2002). Recognition of precursor proteins is dependent on the binding of GTP to the GTP-binding site close to the N-terminus of Toc34 (Kouranov and Schnell, 1997; Gutensohn *et al.*, 2000; Sveshnikova *et al.*, 2000b; Jelic *et al.*, 2002). Binding of precursor proteins stimulates the endogenous GTPase activity of Toc34 as high as 50-fold, indicating that the precursor protein itself functions as a GTPase-activating factor. Two Toc34 isoforms exist in *Arabidopsis*, namely atToc33 and atToc34, of which atToc33 appears to be the true homolog of Toc34 from pea (Table 1). Regulation of Toc34 seems to be a complex process, because phosphorylation of Toc34 and atToc33 inhibited GTP binding and thereby precursor recognition (Sveshnikova *et al.*, 2000b, Jelic *et al.*, 2003). The proteins cannot be phosphorylated in their GTP-bound form. Hence, phosphorylation and GTP-binding create a molecular switch that can interchange the protein between an active and inactive state. The second *Arabidopsis* isoform, atToc34, cannot be phosphorylated, indicating that it is only subject to regulation by GTP binding and hydrolysis. Both receptor isoforms show distinct but not exclusive substrate selectivity as deduced from *in vitro* binding studies (Jelic *et al.*, 2003) and *in vivo* experiments (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). The soluble domain of Toc34 was the first component of the chloroplast

*Table 1.* Protein homologs to components of the Toc and Tic translocon of *Pisum sativum* from the genomes of *Arabidopsis thaliana* and *Synechocystis*. The protein marked with an asterick was shown to reside in mitochondria by Chew *et al.* (2004).

| Pisum sativum | Arabidopsis thaliana | Atg-number | Synechocystis |
|---|---|---|---|
| Toc159 | atToc159 | At4g02510 | homologies only to the |
| | atToc132 | At2g16640 | GTP-binding domain |
| | atToc120 | At3g16620 | |
| | atToc90 | At5g20300 | |
| Toc75 | atToc75-III | At3g46740 | synToc75 |
| | atToc75-I | At1g35860 | |
| | atToc75-IV | At4g09080 | |
| | atToc75-V | At5g19620 | |
| Toc34 | atToc33 | At1g02280 | homologies only to the |
| | atToc34 | At5g05000 | GTP-binding domain |
| Toc64 | atToc64-III | At3g17960 | homologies only to the |
| | atToc64-V (mtOM64*) | At5g09420 | amidase domain |
| | atToc64-I | At1g08980 | |
| Tic110 | atTic110 | At1g06940 | none |
| Tic62 | atTic62 | At3g18890 | ycf39 |
| Tic55 | atTic55 | At2g24820 | homologies to other Rieske-iron-sulfur center containing proteins |
| Tic32 | atTic32 | At4g23430 | homologies to other |
| | | At4g23420 | short chain dehydrogenases |
| | | At4g11410 | |
| Tic22 | atTic22-I | At3g23710 | none |
| | atTic22-II | At4g33350 | |
| Tic20 | atTic20-IatTic20-II | At1g04940 | low homology to ycf60 |
| | | At4g03320 | |
| Tic40 | atTic40 | At5g16620 | None |

translocation machinery to be crystallized (Sun *et al.*, 2002). The crystal structure obtained from heterologously expressed protein showed the majority of Toc34 in an oligomeric state. Therefore, it was deduced that homodimerization could play a role in the translocation process *in vivo*. Yet, because of the similarity of the soluble domains of Toc34 and Toc159, Sun *et al.* (2002) could not exclude the possibility that *in vivo* a heterodimer of these two proteins exists.

Toc159 is the second GTP-binding protein of the Toc translocon. It was identified early as a possible Toc component because of its presence in purified translocon preparations (Waegemann and Soll, 1991) and its capacity to interact with precursor proteins with whom it builds a prominent cross-linking product (Perry and Keegstra, 1994; Ma *et al.*, 1996; Bauer *et al.*, 2000). Toc159 consists of three domains, an acidic N-proximal A-domain, a central GTP-binding or G-domain with similarity to a corresponding domain in Toc34, and a C-proximal membrane anchor or M-domain. The A and G-domains are exposed to the cytosol and therefore protease sensitive during envelope preparation. This feature is responsible for Toc159 being described originally as Toc86, which contains only the M and G-domains. A further removal of the G-domain leads to a 52 kDa membrane-spanning fragment of Toc159. Toc159 was shown by cross-linking experiments to directly interact with precursor proteins (Perry and Keegstra, 1994) and antibodies raised against Toc159 can inhibit the protein import reaction *in vitro* (Hirsch *et al.*, 1994). These experiments indicate that the action of Toc159 precedes the actual translocation process and that the protein might function as a precursor receptor. Recent studies suggest that Toc159 makes contact with the precursor protein after it has interacted with Toc34 (Schleiff *et al.*, 2003), but an inverse order of preprotein interaction has also been suggested (Wallas *et al.*, 2003).

A soluble population of Toc159 was observed in the cytosol of mechanically disrupted pea leaves (Bauer *et al.*, 2002). In addition, expression of the fusion protein Toc159-GFP under the strong CaMV-35S promotor resulted in a dual location of the fusion protein in the cytosol and the chloroplast envelope (Bauer

*et al.*, 2002). It was therefore proposed that Toc159 functions as a shuttle receptor, which recognizes and binds preproteins in the cytosol. It subsequently travels with its cargo to the chloroplast surface where Toc159 inserts into the membrane and thereby delivers the preprotein to another Toc subunit, most likely Toc34 (Weibel *et al.*, 2003). Several pieces of evidence argue, however, against this idea. First, Toc159 behaves as an integral membrane protein, which is resistant to extraction with 0.1 M $Na_2CO_3$ or 6 M urea. Second, the "soluble" form of Toc159 is bound to galactolipids, which reside only in chloroplast membranes. It therefore appears more likely that upon disruption of the cell some Toc159 is present in membrane vesicle fragments of low density, which might contaminate the cytosol during chloroplast isolation (Becker *et al.*, 2004b). Third, expression of the M-domain of Toc159 fused to GFP resulted exclusively in chloroplast but not in cytosolic localisation (Bauer *et al.*, 2002; K.H. Lee *et al.*, 2003). It was demonstrated earlier for mitochondria that overexpression of a protein can lead to mis-localization and a failure of the overexpressed protein to translocate to its correct cellular compartment (Sickmann *et al.*, 2003), which might be the case for the full-length Toc159-GFP construct.

*In vitro* reconstitution experiments using either a purified Toc-complex, or single subunits, indicate a different sequence of events during Toc translocation. Toc components reconstituted into proteoliposomes bind and import precursor proteins in a manner dependent only on the presequence and hydrolysis of GTP, while molecular chaperones are not required (Schleiff *et al.*, 2003). The combination of Toc34 and Toc75 leads to GTP-dependent precursor protein binding but not to translocation into liposomes. On the other hand, the combination of Toc75 and Toc159 leads to GTP-dependent binding and membrane translocation indicating that Toc159 functions not only as a receptor but also as a GTP-driven motor. One can envision that GTP hydrolysis fuels a conformational change in Toc159 that pushes the precursor protein through the Toc75 channel. Alternatively, the hydrolysis of GTP could change the affinity of Toc159 to precursor proteins, whose binding could subsequently induce the conformational changes in Toc159. The function of Toc159 as a motor protein aside, these results also imply that Toc159 and Toc75 represent the minimal functional translocation unit of the Toc complex, while Toc34 forms the initial receptor for incoming precursor proteins. The Toc translocon is not static but is rather a dynamic structure, whose configuration is strongly influenced by GTP-binding, which seems to favour the association of Toc34 with the Toc75/Toc159 core unit while GDP induces the dissociation of Toc34 (Svesnikova *et al.*, 2000b; Schleiff *et al.*, 2002).

### C. The Intermembrane Space

Of all the components involved in the translocation process, least is known about those that reside within the intermembrane space. In contrast to mitochondria, the outer envelope of chloroplasts cannot be removed without concomitant disruption of the inner envelope. It is therefore very difficult to isolate proteins specific for this compartment. Even proteins like Tic22, which are attached to the intermembrane surface of the inner envelope, are generally accounted for as Tic subunits (Fig. 1). So far, there is very little experimental evidence for soluble components of the translocation machinery within the intermembrane space.

### D. The Tic Translocon

To date at least seven different proteins are identified as components of the Tic translocon, namely Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20 (Kessler and Blobel, 1996; Lübeck *et al.*, 1996; Caliebe *et al.*, 1997; Kouranov *et al.*, 1998; Stahl *et al.*, 1999; Küchler *et al.*, 2002; Hörmann *et al.*, 2004). Nevertheless, the exact composition of the Tic translocon is not clear.

Tic110 is the largest component of the Tic translocon and also one of the most abundant proteins of the inner envelope membrane. Tic110 was shown to interact with precursor proteins as well as with several of the other components of the Tic translocon, including Tic40, Tic22, and Tic32 (Kessler and Blobel, 1996; Lübeck *et al.*, 1996; E. Nielsen *et al.*, 1997; Kouranov *et al.*, 1998; Stahl *et al.*, 1999; Hörmann *et al.*, 2004). Because the interaction partners of Tic110 also include Toc75, it was suggested that its function lies at the interface between the two translocons and that Tic110 is involved in the formation of contact sites between the two envelope membranes during the translocation process (Lübeck *et al.*, 1996). On the other hand, Tic110 not only spans the entire inner envelope membrane but also extends a significant domain into the stroma, where it is believed to recruit stromal chaperones to which it could be cross linked (Kessler and Blobel, 1996; E. Nielsen *et al.*, 1997). Lately, a completely different function for Tic110 was suggested by Heins and coworkers (2002), who showed that heterologously expressed Tic110 forms a cation-selective high-conductance channel when reconstituted into

liposomes. The channel formed by Tic110 contains an inner pore opening of about 1.7 nm and interacts with precursor proteins. pSSU but not the mature SSU was able to regulate the opening of the channel. These findings are in accordance with studies on isolated inner membrane vesicles, which revealed the presence of a channel that mirrors the *in vitro* features of Tic110 (Heins *et al.*, 2002). It was therefore concluded that Tic110 forms the import channel of the Tic translocon. Further studies will have to show which of these functions, or perhaps all of them, are fulfilled by Tic110.

Tic62 was first identified through co-purification with Tic110 by Blue-Native-PAGE (Küchler *et al.*, 2002). The protein is embedded into the inner envelope membrane by two N-terminal transmembrane helices but extends part of its C-terminal domain into the stromal compartment (Fig. 1). This C-terminal domain comprises a novel, repetitive module that was shown to interact with ferredoxin-NAD(P)$^+$ oxidoreductase (FNR). The module is repeated three times in Tic62 from pea and four times in atToc62. This difference does not appear to be essential for protein function, because one of the modules is enough to bind FNR (Küchler *et al.*, 2002). More important is the implied role that this domain has for the function of Tic62 in the translocation process. So far it is not known whether FNR is bound permanently to Tic62 or whether a binding-release mechanism exists. FNR catalyses the final electron transfer of oxygenic photosynthesis and is an acknowledged transducer of the redox state of the chloroplast. Therefore, binding of FNR to Tic62 is one of several indications that Tic translocation is regulated by the redox state of the organelle. Such a redox regulation of chloroplast import could be a way of fine-tuning the amount of certain nuclear-encoded chloroplast proteins whose nuclear expression is also regulated by a redox mechanism (Escoubas *et al.*, 1995; Oswald *et al.*, 2001; Pfannschmidt *et al.*, 2001).

Tic55 can be co-purified with both Tic110 and Tic62 by Blue-Native-PAGE as well as on sucrose gradients. It is believed to have a regulatory function for the translocation process (Caliebe *et al.*, 1997). Tic55 has two distinct characteristics, which implicate this function; the protein contains a Rieske-type iron-sulfur center and a mononuclear iron site (Fig. 1). Import of precursor proteins into chloroplasts was inhibited by the Rieske-type protein-modifying agent diethylpyrocarbonate especially at the inner membrane, indicating that this domain plays an important role in the regulation of Tic translocation *in vivo* (Caliebe *et al.*, 1997). Together with the redox sensing features of Tic62, it appears that the perception of the redox state of the chloroplast and a subsequent specific channelling of the import process might be the dominating regulation mechanism underlying Tic translocation.

Tic32 is the most recently identified member of the Tic translocon family and further supports the idea of a redox regulation of chloroplast import (Hörmann *et al.*, 2004). Tic32 is embedded in the inner envelope membrane and can be cross-linked to Tic110. Most noteworthy are the presence of a functional NAD(P)$^+$-binding site and the homology of the protein to a class of short-chain dehydrogenases. The exact topology and function of Tic32 in the translocation process remains to be elucidated. Interestingly, import of Tic32 into the chloroplast is independent of a cleavable presequence and the Toc-Tic machinery (Nada and Soll, 2004).

Tic20 is a small integral membrane protein of the inner envelope. The protein has been identified as part of the Tic translocon by its association with Tic110 and Tic22. It can interact with precursor proteins during the translocation process but its function in the process is not clear. It is supposedly anchored into the membrane by three predicted transmembrane alpha helices, and two short soluble domains extrude into the stroma and the intermembrane space (Kouranov *et al.*, 1998). Tic20 is distantly related to Tim17, a component of the mitochondrial import machinery (Reumann and Keegstra, 1999). Because Tic20 is deeply embedded into the inner membrane, it has been proposed as an alternative to Tic110 as part of the translocon pore (Kouranov and Schnell, 1997; Kouranov *et al.*, 1998). However, as of now, there is no experimental evidence for such a function of Tic20.

Tic22 is another small component of the Tic complex, where it is found associated to Tic20. The protein is attached to the surface of the inner envelope from the intermembrane space side (Fig. 1). Cross-linking experiments show that Tic22 interacts with both Tic20 and precursor proteins. Interaction with the latter can occur before the precursor protein engages the rest of the Tic translocon. Potentially, Tic22 could therefore be involved in passing on the precursor protein from the Toc to the Tic translocon. In addition, Tic22 was found to interact with Tic32 during the translocation of Tic32 into the chloroplast and therefore might play a role in the import of inner membrane proteins without N-terminal presequences (Nada and Soll, 2004).

Tic40 was originally not considered as a component of the Tic translocon. The protein was identified as a component of the outer envelope membrane of *Brassica napus* or alternatively was suggested to reside in

both the inner and outer membranes. It was therefore called bnToc36 and Com44/Cim44, respectively (Wu *et al.*, 1994; Ko *et al.*, 1995). It was later shown that the protein is an exclusive component of the inner envelope (Stahl *et al.*, 1999; Chou *et al.*, 2003), and co-immunoprecipitation with Tic110 identified it as a component of the Tic translocon (Stahl *et al.*, 1999). Clues about the function of Tic40 were derived from the presence of a potential binding site for Hsp70 proteins in the N-terminal part of the protein. This suggests a function as a chaperone-binding protein (Stahl *et al.*, 1999). The potential Hsp70 binding site is part of a larger hydrophobic domain, which was shown to extend into the chloroplast stroma (Chou *et al.*, 2003). It is therefore believed that Tic40 is part of the stromal chaperone complex that is an integral part of protein translocation across the chloroplast envelope.

### *E. Stromal Components*

Even before precursor proteins complete their translocation into the chloroplast, stromal components join the process. Several chaperones have been shown to interact with precursor proteins from the moment the presequence has reached the inside of the organelle (Fig. 1). Subsequently, the presequence is cleaved off by the so-called stromal processing peptidase (SPP) (Oblong and Lamppa, 1992). All current investigations indicate that there is only a single SPP that recognizes, binds, and cleaves all precursor proteins. A potential candidate for this SPP was recently identified in *Arabidopsis* (Moberg *et al.*, 2003). While searching for mitochondrial processing peptidases, Moberg and coworkers showed that the gene locus At3g19170 encodes a protein with similarity to zinc-metallopeptidases, which was able to process both mitochondrial as well as chloroplast precursor proteins. The protein is furthermore dually targeted into both mitochondria and chloroplast, indicating that a single protein is responsible for the important processing step in both organelles (Moberg *et al.*, 2003). SPP removes the complete presequence in one endoproteolytic cleavage step (Richter and Lamppa, 1998, 1999) and simultaneously releases the mature protein. The presequence is cleaved by SPP into smaller fragments before these are released from the protein. All the information required for the function of SPP seem to reside in the C-proximal domain of the presequence, while the N-proximal domain has no influence (Richter and Lamppa, 2002). The resulting fragments of the presequences are subsequently degraded into amino acids by a second, ATP-dependent metallopeptidase, the transit peptide subfragment-degrading enzyme (Richter and Lamppa, 1999). In accordance with its function in presequence degradation, the presently unidentified protein can recognize both the N- and C-proximal domain of the presequence.

## IV. Variations on Toc and Tic Translocation

Detailed analysis of the *Arabidopsis* genome has revealed homologs to all the Toc and Tic components previously identified in pea or other plants (Table 1). Additionally, it was found that in many cases the *Arabidopsis* genome encodes more than one, and often slightly varying, homologs (Jarvis *et al.*, 1998; Bauer *et al.*, 2000; Eckart *et al.*, 2002; Jackson-Constan and Keegstra, 2001; Vojta *et al.*, 2004). In some cases the presence of multiple isoforms might present a unique situation within *Arabidopsis,* but they are common in other plants as well. Thus a specific function of these paralogs in the import process has to be more generally considered.

Toc75 is the most prominent protein of the outer membrane and for a long time no isoform of this protein was known. A recent analysis of pea outer membranes discovered the presence of a second homolog to Toc75 (Eckart *et al.*, 2002). Both proteins are expressed in green leaves, with the "original" Toc75, now called Toc75-III, being the considerably more abundant of the two. The discovery of a second Toc75 led to the subsequent finding that the *Arabidopsis* genome encodes a whole family of at least four different Toc75-like proteins, called atToc75-I, -III, -IV and -V in correspondence to the chromosome on which they are encoded. The homology between the different Toc75 proteins is mainly restricted to the C-terminal part, which is predominantly involved in channel formation. So far it is not known whether the different Toc75 homologs facilitate distinct functions in chloroplast import. It seems clear that atToc75-III is the major translocation pore and is the protein found associated with the Toc core unit (Schleiff *et al.*, 2003). atToc75-V is found in the outer envelope membrane of chloroplasts but a function for this protein in protein import remains to be shown. Nothing is known about the expression, localization or function of the other two atToc75 homologs or whether they can be found in other plants.

Four different Toc159 homologs were identified in *Arabidopsis* (Bauer *et al.*, 2000; Jackson-Constan and Keegstra, 2001), namely atToc159, atToc132, atToc120, and atToc90. None of the smaller isoforms

has been identified so far in any other organism. In principle, all four *Arabidopsis* isoforms share the same three-domain structure as described for Toc159 but with decreasing size, as the A-domain of these isoforms becomes progressively smaller and is completely absent in atToc90. atToc159 is the most abundant of the four proteins and because of its size is thought to represent the true homolog of Toc159 from pea. Of the four isoforms, at least the largest three seem to represent proteins with nonredundant functions in protein translocation (Bauer *et al.*, 2000; Ivanova *et al.*, 2004).

Within pea chloroplasts only a single Toc34 protein has been identified, while two homologs are found in the outer envelope of *Arabidopsis*, atToc34 and atToc33 (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). Of these two proteins, atToc33 seems to be the functional ortholog of Toc34. The gene is primarily expressed in rapidly expanding as well as meristematic tissues, while atToc34 is expressed at a basic, housekeeping level in all cells. Studies on knockout mutants suggest that the function of the two proteins is partially redundant. A biochemical analysis revealed that the proteins possess somewhat different properties. They have different affinities for different precursor proteins and only Toc33 appears to be regulated by phosphorylation (Jelic *et al.*, 2003).

Toc64 has at least three homologs in *Arabidopsis*, called atToc64-III, atToc64-V and atToc64-I, of which atToc64-III is the functional ortholog to Toc64 from pea (Sohrt and Soll, 2000). atToc64-V was shown recently to reside in mitochondria (Chew *et al.*, 2004) and nothing is known so far about the expression and localisation of atToc64-I. It is also not known whether multiple isoforms of Toc64 occur in other plants.

Isoforms of Tic components seem to be much less abundant in the *Arabidopsis* genome (Table 1). Tic110, Tic62, Tic55 and Tic40 all seem to be encoded by a single, unique gene. In contrast, Tic32, Tic20 and Tic22 are present with at least two homologs of each, but in neither case is it known whether these isoforms play distinct roles in the protein import process.

## V. Protein Translocation and Chloroplast Biogenesis

Because roughly 95% of the plastidal proteins are nuclear-encoded, it is not surprising that mutations in genes coding for components of the translocon machinery have a vast influence on the development of the organelle. Studies on mutants in *Arabidopsis*, and to a lesser extent in other plants and algae, have shown that the effect of such mutations differs in correlation to the importance of the component and the redundancy of isoforms. Therefore, much insight into the specific function of different translocon components can be gained from the analysis of such mutants.

### *A. Mutations in Toc Components*

As mentioned above, the *Arabidopsis* genome encodes two homologs of Toc34, called atToc33 and atToc34 (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). As shown for the *Arabidopsis* mutant *ppi1*, a loss of atToc33, the likely ortholog to Toc34 in pea, is not lethal. The plants can grow on soil and produce viable seeds. Nevertheless, leaves of younger plants are uniformly yellow-green and chlorophyll levels remain reduced even in older plants, while, outwardly, older leaves recover their appearance and become indistinguishable to wild-type leaves. A detailed analysis of the *ppi1* mutant showed that in particular the import of proteins related to photosynthetic function is affected (Kubis *et al.*, 2003), a feature that is supported by biochemical studies (Gutensohn *et al.*, 2000). In accordance with the observed phenotype, only chloroplasts isolated from younger leaves display reduced import capacity *in vitro*. This would give atToc33 a specific function in the translocation process at an early stage of plant development. The fact that older leaves recover nearly completely indicates that the function of atToc33 is not exclusive. Most likely atToc34, which exists in mesophyll cells at a much lower level, can compensate for the lack of atToc33 to a certain extent and with time. This is supported by complementation experiments, which show that over-expression of either atToc33 or atToc34 can rescue the *ppi1* mutant phenotype (Jarvis *et al.*, 1998). In contrast to the *ppi1* (atToc33) mutant, two independent mutant lines for atToc34 did not differ significantly from wild-type plants (Constan *et al.*, 2004). At all stages of development, the plants appeared normal and they contained properly developed chloroplasts. Furthermore, protein import into leaf chloroplasts was not affected in either mutant. On the other hand, both mutants showed a slightly reduced root growth, which remained the only visible phenotype. Double mutations in atToc33 and atToc34 turned out to be embryo lethal (Constan *et al.*, 2004).

From the four homologs of Toc159, only atToc159, atToc132 and atToc120 have been studied by means of *Arabidopsis* mutants (Bauer *et al.*, 2002; Ivanova *et al.*, 2004). The atToc159 mutant, called *ppi2*, is seedling

lethal on soil. When grown heterotrophically, the plants display an albino phenotype and consequently chloroplast development is heavily affected. Chloroplasts in mesophyll cells of young leaves are smaller, i.e., resembling proplastids, and display a complete lack of thylakoid membranes. In contrast to the *ppi1* mutant, chloroplasts seem to be impaired in the import of all kinds of plastidal proteins, suggesting a general function of atToc159 in the translocation process. Interestingly, the disruption of *ppi2* does not affect plastid biogenesis in guard cells and in roots (Yu and Li, 2001). As a possible explanation, in these cells the presence of either one of the other atToc homologs, atToc132, atToc120 or atToc90, may compensate for the lack of atToc159 or even fully replace its function. Interestingly, complementation of the *ppi2* mutant with a protein comprising only the M-domain of atToc159 leads to a recovery of Toc function and chloroplast differentiation, though at a reduced level. Expression of M and G domains together leads to a full recovery of the mutant. These experiments imply that the M-domain is essential and that the M and G domains together are sufficient for the function of atToc159, raising the question about the role of the A-domain in the import process *in vivo*. On the other hand, the three atToc159 homologs, atToc132, atToc120 and atToc90 cannot complement the *ppi2* mutant, indicating that these isoforms are not redundant but instead seem to have a function distinct from atToc159.

Results from mutant analyses have been substantiated by genetic data. Using a transgene-based positive screen, Sun *et al.* (2001) identified a transcription factor, CIA2 that is involved in regulating the expression of *atTOC33* and *atTOC75-III*. Both genes are specifically up-regulated in leaf tissue and disruption of the *CIA2* gene resulted in a reduction of transcription of both genes. Other genes, like *atTOC34* or *atTOC159,* are not affected by the loss of CIA2, indicating that they are regulated in a different manner. Whether CIA2 is directly responsible for the regulation of these genes or acts via another unidentified factor is unknown. Nevertheless, these data provide additional support for differential expression and function of distinct translocon components.

### B. Mutations in Tic Components

Mutants in gene loci of Tic components have been much less studied. A reduction of atTic20 by antisense expression created mutant plants with pale leaves, a reduced accumulation of chloroplast proteins, and an abnormal plastid ultrastructure (X Chen *et al.*, 2002). The disruption of *atTIC20* seemed to affect most chloroplast proteins independently of their involvement in photosynthetic function. In addition, the fact that the severity of the phenotype correlated directly with the reduction in the amount of atTic20 indicated that this protein is essential for translocation through Tic. Notable exceptions are the components of the Toc and Tic translocons themselves, which are not affected by the *atTIC20* antisense expression (X Chen *et al.*, 2002).

Mutant analysis of atTic40 in *Arabidopsis* revealed that the protein is not essential for plant viability (Chou *et al.*, 2003). Yet, mutant seedlings were pale-green in color, developed more slowly then wild-type plants, and remained very small throughout their entire life cycle. Chloroplasts of Δ*TIC40* plants displayed an underdeveloped thylakoid membrane system with a nearly complete lack of grana, together with a reduction in chloroplast proteins. *In vitro* import experiments confirmed a reduced rate of chloroplast import for the mutant. Again, other components of the import machinery itself did not appear to be affected by the lack of atTic40 protein (Chou *et al.*, 2003).

### C. Mutation of SPP

Only mature chloroplast proteins without their presequences are able to fold into a functional form (Della-Cioppa *et al.*, 1986). Therefore, it is not surprising that interference with the SPP has an immense impact on organelle function, which consequently triggers effects on organelle biogenesis. A severe reduction in the amount of SPP in the pea mutant *AS14* resulted in chlorotic plants. Growth of both shoots and roots was retarded and the number of chloroplasts per cell dropped significantly (Wan *et al.*, 1998). This phenotype was accompanied by a reduction in protein import across the envelope membranes. Subsequent studies in *Arabidopsis* supported the findings with the pea mutant. Plants with a reduced level of SPP showed a phenotype similar to the pea mutant, and precursor proteins fused to GFP accumulated in the cytosol instead of being imported into the organelle. A complete loss of SPP rendered the mutant seedling lethal, which supported the essential function of SPP (Zhong *et al.*, 2003). These studies imply that the processing of the precursor protein is an integral part of the translocation process and that its specific function cannot be replaced by other proteases within the organelle. How the lack of SPP feeds back to the import machinery remains to be elucidated.

## VI. The Evolutionary Origin of Translocon Components

The necessity for chloroplast protein import arose only after the endosymbiotic creation of the organelle. The prokaryotic ancestor of the chloroplast had no need for such import machinery. This raises the question whether any of the translocon components are of prokaryotic origin or whether these proteins evolved fresh in concert with the gene transfer from the newly arising organelle to the nucleus of the host cell. This question is especially interesting in light of the remarkable differences between the plastidal and the mitochondrial translocons. The endosymbiotic event that created the mitochondrion is believed to precede the creation of the chloroplast and it is generally assumed that gene transfer into the nucleus and consequently protein import into mitochondria had already occurred at the time of the endosymbiotic event that created the chloroplast. Therefore, it seems odd that the organism developed a completely different set of machinery for the new organelle and did not just alter the already existing mitochondrial system to fit its needs. The development of the chloroplast import translocon from existing cyanobacterial translocases would provide a possible alternative.

So far, the phylogenetic origin of many of the plastidal translocon components are not clear. Only for Toc75 have true homologs been identified in the genomes of contemporary cyanobacteria and bacteria (Heins and Soll, 1998; Reumann and Keegstra, 1999). Several cyanobacterial proteins show sequence similarity to Tic55, Toc34, Tic62 and Tic20, but in neither case is it clear whether these proteins represent true homologs of the translocon components. For other components such as Toc159, Toc64, Toc34, Tic32 and Tic110 no bacterial ancestor could be recognized at all. It is likely that the Toc and Tic translocons were built around a few existing cyanobacterial proteins, whose properties were adapted to the new function. Other components were further built around these proteins either completely from scratch or by adaptation of proteins with useful domains.

How would one envision that this transformation of cyanobacterial proteins to a chloroplast translocon took place? In the case of *Synechocystis*, the Toc75 homolog, called synToc75, resides in the outer membrane and the heterologously expressed protein forms a high conductance channel with affinity for polyamines and peptides (Bölter *et al.*, 1998). Even more, all known Toc75 homologs, bacterial as well as eukaryotic, belong to a large protein family related to bacterial transport proteins such as Omp85, which are involved in the export and integration of proteins across and into the outer membrane of gram-negative bacteria (Bölter *et al.*, 1998; Heins and Soll, 1998; Reumann and Keegstra, 1999; Genevrois *et al.*, 2003; Voulhoux *et al.*, 2003). Intriguingly, it appears from very recent findings that mitochondria possess proteins in their outer membrane that are related to this family and are involved in the insertion of outer membrane proteins (Paschen *et al.*, 2003; Gentle *et al.*, 2004). These findings imply that Toc75 is part of an ancient bacterial channel family and that this pore protein was recruited after the endosymbiotic formation of the chloroplast to build up the channel of the newly arising import machinery. In the case of the inner envelope membrane, the origin of the translocation channel is less clear, because Tic110 has no homolog in bacteria and the alternative pore component Tic22 has only very low homology to bacterial proteins. It is therefore more difficult to imagine how the Tic translocon arose. It would be feasible to imagine that, in the beginning, the chloroplast used one of the cyanobacterial plasma membrane secretion pores in a reversed direction. Gram-negative cyanobacteria contain several systems for translocating proteins across or into the plasma membrane, i.e., Tat, Sec, and SRP pathways, all of which include a translocation pore. In cyanobacteria, these pathways seem to be located exclusively in the inner membrane where they facilitate the transport of inner membrane as well as thylakoid proteins. How the latter get from the inner membrane to the thylakoids is not known. With respect to the arising chloroplast, it is noteworthy that the pathways for thylakoid insertion are located in the thylakoid membrane and no longer in the inner envelope membrane. The need for this change in the localization of thylakoid insertion is not clear. Nevertheless, it would necessitate the development of a new Tic translocon pore to avoid the mistargeting of thylakoid proteins to the inner envelope membrane. While this idea so far remains mere speculation, the question of the localization of biogenesis of the thylakoid membrane and the insertion and translocation of thylakoid proteins in cyanobacteria versus chloroplasts has become a new focus of present research.

## VII. Intraplastidal Transport

Chloroplasts consist of six different subcompartments; namely the outer envelope membrane, the intermembrane space, the inner envelope membrane, the stroma,

the thylakoid membrane, and the thylakoid lumen. Consequently, proteins imported into the chloroplast have to be sorted to their final intracellular destination within the organelle. As mentioned above, most of the outer envelope proteins do not enter the chloroplast but are inserted from the cytosolic side of the outer membrane. The only exception known to date is Toc75 (Schnell *et al.*, 1994; Tranel *et al.*, 1995). It is known that the protein reaches deep enough into the stroma for SPP to cleave off the N-terminal portion of the presequence but the subsequent steps of insertion of Toc75 into the outer membrane have not yet been discovered. Nothing is known about the routing of proteins residing in the intermembrane space, and the means by which inner membrane proteins reach their final destination is not very well understood. For the routing of inner membrane proteins, two different pathways could be envisioned. First, they could translocate through the Toc-complex but only insert into the Tic-complex far enough for the presequence to be removed. The proteins could then be inserted laterally into the membrane as discussed for insertion into the ER membrane or the mitochondrial inner membrane (Herrmann and Neupert, 2003). Alternatively, the inner envelope proteins could be translocated completely into the stroma and subsequently reinserted into the envelope membrane from the inner side. Short of suggesting a double function of the Tic complex for the latter insertion, this would require a separate translocation system. Interestingly, chloroplasts do contain a protein called Artemis, a functional homolog of ALB3, a translocase pore of the Oxa1-YidC family, which is located in the inner envelope membrane (Fulgosi *et al.*, 2002). *Arabidopsis* mutant plants of this protein do not display a specific effect on inner envelope protein insertion but the exact function of the protein remains to be shown.

Last but not least, the organelle must deal with all the proteins that are destined for the thylakoid membrane and lumen. A variety of translocation pathways into and across the thylakoid membrane have been discovered in chloroplasts and only a basic overview can be given within this chapter (for comprehensive review, see Robinson and Mant, 2005). Nuclear encoded proteins can be translocated into or across the thylakoid membrane by at least four different pathways (Fig. 2). To date there are two pathways described for the insertion into the thylakoid membrane: the signal recognition particle-dependent (SRP) and the spontaneous insertion pathway. Two further pathways are known for the translocation into the thylakoid lumen: the twin-arginine translocase (Tat) and the secretory (Sec) pathways. Each of these pathways appears to serve a different set of proteins.

### *A. Signal Recognition Particle (SRP) Pathway*

The SRP pathway was first shown for the Lhcb1 (light-harvesting chlorophyll-binding protein 1) protein (Cline, 1986) and, other than that, it has only been shown to function for Lhcb4.1 and Lhcb5 (Woolhead *et al.*, 2001). It is therefore believed that this pathway might be restricted to the nuclear-encoded family of light-harvesting chlorophyll-binding proteins. All these proteins are synthezised with a singular presequence, cleaved off after envelope translocation, indicating that no second thylakoid-specific sequence tag is employed (Fig. 2). Insertion via the SRP pathway requires two stromal components, the so-called signal recognition particle and FtsY (X Li *et al.*, 1995; Kogata *et al.*, 1999). The signal recognition particle consists of two soluble proteins, a 54-kDa (cpSRP54) and a 43-kDa (cpSRP43) subunit (Schünemann *et al.*, 1998). While it is relatively certain that both cpSRP54 and cpSRP43 are involved in binding Lhcb proteins, the exact role of FtsY in the process remains to be elucidated. Insertion of Lhcb proteins into the membrane is achieved with the assistance of ALB3, a protein that belongs to the Oxa1-YidC family (Moore *et al.*, 2000). Whether this pathway comprises further components or indeed requires additional interaction with the Sec machinery remains to be seen. It should be pointed out that an alternative hypothesis suggests that the insertion of Lhcb takes place at the inner envelope of chloroplast in conjunction with the addition of chlorophyll as an essential prerequisite (Hoober and Eggink, 2001). This alternative to the thylakoid insertion of Lhcb is further detailed in Chapter 2 of this book.

### *B. Spontaneous Insertion*

The majority of thylakoid proteins seem to insert via the spontaneous insertion pathway (Fig. 2). These proteins range from simple hairpin proteins like PsbW or SecE to more complex proteins like PsaK and PsbY (Mant *et al.*, 2001; Steiner *et al.*, 2002). Only a few of these proteins, i.e., CFoII, PsbW, PsbX and PsbY, contain a bipartite presequence that guides translocation into the chloroplast and subsequently into the thylakoid membrane. All other proteins using this pathway are lacking such a feature. Furthermore, spontaneous insertion does not appear to require stromal components, nucleoside triphosphates, SecA, SRP or ALB3, and it

is not affected by protease treatment of the thylakoid membrane.

### C. Twin-Arginine Translocase (Tat) Pathway

A bipartite presequences is a common feature of nuclear-encoded proteins that are targeted into the thylakoid lumen (Fig. 2). These proteins can take two different routes across the thylakoid membrane. Proteins like PsbN, OE23 or OE16 use the Tat pathway, which relies on a conserved twin-arginine motive inside the presequence as well as showing a strict dependence on the $\Delta$pH across the thylakoid membrane (V.S. Nielsen *et al.*, 1994; Clark and Theg, 1997). This motif within the C-terminal part of the presequence is an important determinant to distinguish Tat from Sec-translocation. One of the most important features of this pathway is its ability to translocate proteins in a fully folded state and even with specific cofactors required for the folding already attached (Clark and Theg, 1997; Hynds *et al.*, 1998). Proteins known to be translocated by this pathway can be between 4,000 and 40,000 Da in size. Three proteins have been identified biochemically as well as genetically as the primary components of this translocation pathway, i.e., Hcf106, Tha4, and cpTatC (Settles *et al.*, 1997; Mori *et al.*, 2001). They are orthologs of the *Escherichia coli* Tat components TatB, TatA/E and TatC, respectively. Hcf106 and Tha4 are somewhat homologous, single membrane-spanning proteins with a C-terminal domain exposed to the stroma. The exact mechanism of Tat translocation is not yet fully determined but it has been suggested that a complex containing cpTatC as well as Hcf106 acts as a receptor for the precursor proteins and that a subsequent Tha4 recruitment allows translocation of the protein (Cline and Mori, 2001). It is not known which component or components form the actual translocation channel.

### D. Secretory (Sec) Pathway

The second pathway involved in thylakoid translocation is the so-called Sec pathway (Fig. 2), which was shown to translocate proteins such as plastocyanin and OE33 into the thylakoid lumen. Like the Tat pathway, it relies on a bipartite presequence but does not seem to be able to translocate folded proteins (Hynds *et al.*, 1998). Components of Sec translocation include the two membrane-bound proteins SecY and SecE as well as the soluble stroma protein SecA (Valentin, 1993; Laidler *et al.*, 1995; Schünemann *et al.*, 1999). The Sec pathway has not been studied well in chloroplasts but from analogy to the bacterial Sec-dependent export, it is assumed that SecA interacts with precursor proteins in the stroma. Upon ATP-hydrolysis, SecA inserts itself into the membrane carrying the precursor with it. SecY, SecE, and maybe other not yet identified proteins form the translocation channel by which the proteins are routed through the membrane into the lumen.

### E. Vesicle Transport in Chloroplasts

Even though each of these pathways was shown to serve several different proteins, for the vast majority of nuclear-encoded proteins the means by which they insert into or translocate across the thylakoid membrane is not known. Most of them are likely to take one or the other of the already described pathways, but it cannot be excluded that additional pathways exist that we do not know of at the moment. Furthermore, as mentioned above, it was suggested that the insertion of Lhcb1 takes place at the inner envelope of chloroplast in conjunction with the addition of chlorophyll as an essential prerequisite (Hoober and Eggink, 2001). Such an assembly of protein complexes at the inner envelope would require transport of the whole protein-pigment-lipid unit to the thylakoid membrane.

In regard to the many unresolved questions concerning protein targeting to thylakoid membranes, it is noteworthy that a vesicle transport system exists in chloroplasts of vascular plants (Fig. 3). Early electron microscopic studies led to the conclusion that under certain conditions vesicle-like structures can be found in the stroma space between the inner envelope and the thylakoid membrane (von Wettstein, 1959; Mühlethaler and Frey-Wyssling, 1959). A first indication that these vesicles resemble vesicular traffic systems came from studies by Morré *et al.* (1991). Later,

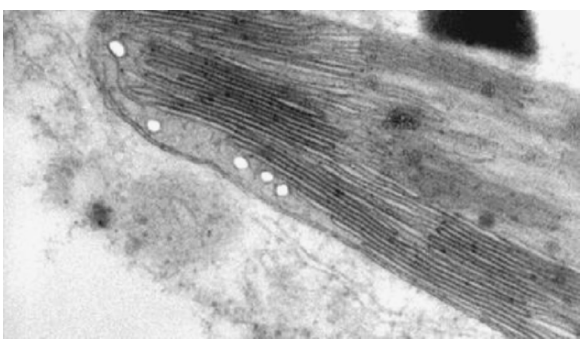

*Fig. 3.* Ultrastructure of a leaf chloroplast from *Selaginella* after treatment with microcystin LR. Vesicles have accumulated in the stroma between the envelope membrane and the thylakoids. Micrograph courtesy of Sabine Westphal.

it was shown by Westphal and coworkers that these vesicle structures are indeed part of a vesicular transport system that appears common to all vascular plants (Westphal *et al.*, 2001a, 2003). They could furthermore show that vesicle formation and fusion in chloroplasts was affected by inhibitors of typical components of cytosolic vesicle transport systems (Fig. 3). This led to the suggestion that this transport system is at least partially of eukaryotic origin and that it was transferred into the organelle well after the endosymbiotic formation of the chloroplast. Indications that chloroplast vesicle transport is a late evolutionary addition also came from studies on a number of different chloroplast-containing organisms. These studies confirmed that, with the possible exception of *Chlamydomonas reinhardtii*, no vesicle transport has been observed in any branch of photosynthetic eukaryotes other than vascular plants (White and Hoober, 1994; Westphal *et al.*, 2003). Nevertheless, it cannot be excluded that the absence of microscopic evidence for vesicle transport in some of the organisms analysed results from technical issues.

Originally, it was suggested that chloroplast vesicles could transport lipids from the inner envelope to the thylakoid membrane, because the synthesis of membrane lipids occurs at the inner membrane and it is generally believed that no connection exists between this membrane and the thylakoids. Yet, recent studies have given rise to the suggestion that vesicle transport might also function in the transfer of thylakoid membrane proteins. The protein Vipp1 is found in photosynthetic eukaryotes as well as cyanobacteria and mutants lacking this protein are devoid of thylakoid membranes (Kroll *et al.*, 2001; Westphal *et al.*, 2001b). In vascular plants the lack of Vipp1 protein also results in the abolishment of chloroplast vesicle transport, supporting the idea that a connection between this transport system and thylakoid biogenesis exists (Kroll *et al.*, 2001). Vipp1 originated from a bacterial ancestor protein, PspA from which it can be distinguished by an additional C-terminal domain (Westphal *et al.*, 2001b). It was shown recently that PspA and Vipp1 both form a homo-multimeric, high-molecular weight complex, which forms a large ring-shaped structure (Hankamer *et al.*, 2004; Aseeva *et al.*, 2004). In chloroplasts the complex is found attached to the inner side of the inner envelope membrane. Studies of the *E. coli* PspA protein furthermore suggest that PspA can enhance protein translocation via the Sec and Tat pathways (Kleerebezem and Tommassen, 1993; DeLisa *et al.*, 2004). Because the Vipp1 homolog from *Synechocystis* could replace PspA in these experiments, DeLisa and coworkers concluded that both proteins might still have a similar function and the structural conservation between Vipp1 and PspA would support this idea. The exact function of Vipp1 in the Tat and Sec translocation pathways and how it relates to vesicle transport in chloroplasts have yet to be elucidated.

## VIII. Protein Translocation into Complex Plastids

Complex plastids are derived from secondary and even tertiary endosymbiotic events, which involved green as well as red algae (Gibbs, 1978). Due to this rather complex phylogenetic origin, the routing system into this organelle is more multifaceted than in primary organelles (for comprehensive review, see van Dooren *et al.*, 2001). For the majority of genes that have been relocated into the nucleus of the secondary host, the plastid-targeted proteins have to translocate across three or even four membranes to enter the organelle. This is achieved by means of a bipartite presequence. The first, N-proximal part of the presequence resembles the signal sequence that targets proteins into the endoplasmic reticulum. Indeed, the outermost membrane of all complex plastids is derived from the host cells phagocytic membrane (Cavalier-Smith, 2003). Therefore, it is feasible that the already developed sorting machinery through the endomembrane system facilitates reimport of proteins across this membrane. We can distinguish between two potentially different ways by which this reimport is achieved. In the case of the chlorarachnophytes and euglenoids, which have three membranes surrounding the plastid, the proteins are synthesized in the cytosol and enter the ER cotranslationally like other secreted proteins (Sulli and Schwartzback, 1995; Waller *et al.*, 2000). The N-proximal part of the bipartite presequence is cleaved off and the plastid-destined proteins are further routed from the ER to the organelle by means of vesicle transport. After fusion of the vesicle with the outermost plastid membrane, the proteins are released into the periplastidal compartment. In the case of haptophytes, cryptomonads and heterokont algae, the outermost membrane of the complex plastids is actually a continuation of the ER membrane and the plastid is therefore nominally within the ER of the cell. The nuclear-encoded proteins are therefore cotranslationally imported directly into the periplastidal space. In principle, these two processes present a similar way to cross the outermost plastid membrane. In both cases the proteins reach the periplastidal compartment with their N-proximal presequence removed and still in possession of the

C-proximal presequence that is required for further routing. In complex plastids surrounded by four membranes, the next obstacle on the way into the organelle is a membrane thought to be derived from the plasma membrane of the secondary endosymbiont (the previous primary host). To date there is no information available as to how this membrane might be traversed. It cannot be excluded that the membrane has become passable unassisted even for large proteins but this seems unlikely considering that this would have made the whole membrane superfluous. Scientists have postulated different ways to solve this problem but all theories so far are awaiting experimental confirmation. The space surrounding the two innermost membranes of the plastid represents the cytosol of the primary host cell and at this point the nuclear-encoded proteins might be joined by another group of proteins. These are proteins encoded by genes still residing on the remaining nucleus of the primary host. Only recently has the nucleomorph DNA of the cryptomonad *Guillardia theta* been completely sequenced and found to encode proteins destined for the plastid (Douglas *et al.*, 2001). For both the nuclear-encoded as well as the nucleomorph-encoded proteins, the translocation process that guides them across the two innermost membranes is phylogenetically conserved. It resembles the translocation mechanism of primary plastids. For this purpose the nuclear-encoded proteins posses the C-terminal part of their bipartite presequence, while the nucleomorph-encoded proteins posses a classical chloroplast presequence. Several of such proteins have been successfully imported into plant chloroplasts *in vitro*, thereby giving evidence for the conservation of the plastidal import process (Lang *et al.*, 1998; Wastl and Maier, 2000).

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# Chapter 4

# Origin and Evolution of Plastids: Genomic View on the Unification and Diversity of Plastids

Naoki Sato*
*Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba 3-8-1, Meguro, Tokyo 153-8902, Japan*

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*Author for correspondence, email: naokisat@bio.c.u-tokyo.ac.jp

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## Summary

The notion "plastid" unifies the diversity of various plastids in diverse photosynthetic eukaryotes and certain non-photosynthetic parasites. Plastid diversity can be seen in the photosynthetic or metabolic capacities, the photosynthetic accessory pigments, the architecture of plastid membranes, and the size and content of plastid genomes. The central unifying principle is that all plastids are bound by two envelope membranes and possess plastid DNA, which was inherited from an ancestral cyanobacterial endosymbiont. Although phylogenetic analysis of the relationship between cyanobacteria and plastids does not identify the cyanobacterial species nearest to the plastid origin or the branching order of various plastids lineages, the radiation of both extant cyanobacteria and plastids is estimated to have occurred on a similar geological timescale. In addition to two major secondary endosymbiogenesis each involving a red and a green algal endosymbionts, tertiary endosymbiotic events have been proposed to explain the origin of diverse dinoflagellates. A new concept of plants suggests that all hosts of secondary or tertiary plastid endosymbiogenesis had once possessed primary plastids and subsequently lost them, and thereby they had been prepared to accept new plastids. In spite of these recent developments in plastid phylogeny that demonstrate continuity of plastid genomes, discontinuous evolution of plastid genomic machinery is another aspect of plastid evolution. Plastids gained various regulatory mechanisms from their host organisms during the evolution of land plants such that the genomic machinery that runs the plastid genome of the flowering plants no longer looks like red algal counterparts, nor even the cyanobacterial genomic machinery.

## I. Introduction: Unification and Diversity

The plastids archetype unifies various allied organelles within plant and algal cells. The concept is based on the relatedness of various plastids, which are either photosynthetic or non-photosynthetic, in different tissues of a single plant as well as similarity of various plastids in different photosynthetic eukaryotes (Kirk and Tilney-Bassett, 1967; Chapter 1). The close relationship of such different plastids is based on the fact that all plastids contain plastid DNA and are bound by a double-membrane envelope. This definition inevitably emphasizes the unifying aspect of plastids, by disregarding differences associated with plastids of different tissues or organisms. Classical observation of the morphology of plastids in various types of organs and tissues of plants and algae, first by optical microscopy and then by electron microscopy, led to various hypotheses on the origin and evolution of plastids. Biochemical analysis of chloroplasts obtained after fractionation revealed resemblance of plant and algal chloroplasts to cyanobacterial cells. The origin and evolution of plastids remained speculative until the early 1980's when DNA sequencing identified the nature of plastid DNA. It was only a decade ago that the identity of all plastids, such as amyloplasts, chromoplasts, proplastids etc., was unequivocally established by DNA analysis, although the DNA might be modified depending on the tissue in which plastids are located (Ohta *et al.*, 1991).

Elucidating the phylogeny of plastids is an effort to find links between these diversified plastids and to reconstruct a unified history of all plastids. The link may be continuous in many cases, but may be

*Abbreviations:* DGTS – diacylglyceryl trimethylhomoserine; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; HGT – horizontal gene transfer; LHC – light-harvesting complex; LPOR – light-dependent protochlorophyllide reductase; MGDG – monogalactosyl diacylglycerol; NEP – nuclear-encoded RNA polymerase; PEP – plastid-encoded RNA polymerase; PG – phosphatidylglycerol; RRM – RNA-recognition motif; SQDG – sulfoquinovosyl diacylglycerol.

*Table 1*. List of completely sequenced plastid genomes and related bacterial genomes. Sequencing of *Nostoc punctiforme* is not finished, but the data are available from the web site of Joint Genome Institute (JGI). Data of other genomes are available from GenBank. This table was compiled in late 2003.

| Species | Accession | bp | Proteins | Group | G + C % |
|---|---|---|---|---|---|
| Plastids | | | | | |
| *Adiantum capillus-veneris* | AY178864 | 150,568 | 87 | Pteridophytes | 42.0 |
| *Anthoceros formosae* | AB086179 | 161,162 | 90 | Hornworts | 32.9 |
| *Astasia longa* | ALO294725 | 73,345 | 46 | Euglenophytes | 22.4 |
| *Arabidopsis thaliana* | AP000423 | 154,478 | 87 | Dicots | 36.3 |
| *Atropa belladonna* | NC_004561 | 156,687 | 87 | Dicots | 37.6 |
| *Chaetosphaeridium globosum* | NC_004115 | 131,183 | 98 | Charophytes | 29.6 |
| *Chlamydomonas reinhardtii* | BK000554 | 203,395 | 68 | Chlorophytes | 34.6 |
| *Chlorella vulgaris* C-27 | AB001684 | 150,613 | 174 | Chlorophytes | 31.6 |
| *Cyanidioschyzon merolae* | AB002583 | 149,987 | 207 | Rhodophytes | 37.6 |
| *Cyanidium caldarium* RK1 | AF022186 | 164,921 | 197 | Rhodophytes | 32.7 |
| *Cyanophora paradoxa* | CPU30821 | 135,599 | 149 | Glaucophytes | 30.5 |
| *Epifagus virginiana* | M81884 | 70,028 | 24 | Dicots | 36.0 |
| *Euglena gracilis* | X70810 | 143,171 | 67 | Euglenophytes | 26.1 |
| *Guillardia theta* | NC_000926 | 121,524 | 147 | Cryptophytes | 32.9 |
| *Marchantia polymorpha* | X04465 | 121,024 | 89 | Liverworts | 28.8 |
| *Mesostigma viride* | AF166114 | 118,360 | 105 | Chlorophytes | 30.2 |
| *Nephroselmis olivacea* | NC_000927 | 200,799 | 155 | Chlorophytes | 42.2 |
| *Odontella sinensis* | NC_001713 | 119,704 | 140 | Diatoms | 31.8 |
| *Oenothera elata* subsp.*hookeri* | AJ271079 | 159,443 | 120 | Dicots | 39.2 |
| *Oryza sativa,* cv *japonica* | NC_001320 | 134,525 | 108 | Monocots | 39.0 |
| *Physcomitrella patens* subsp. *patens* | AP005672 | 122,890 | 85 | Mosses | 28.5 |
| *Pinus thunbergii* | D17510 | 119,707 | 160 | Gymnosperms | 38.5 |
| *Plasmodium falciparum* | X95275-6 | 29,422 | 30 | Apicomplexa | 13.1 |
| *Porphyra purpurea* | U38804 | 191,028 | 209 | Rhodophytes | 33.0 |
| *Psilotum nudum* | AP004638 | 138,829 | 102 | Pteridophytes | 36.0 |
| *Spinacia oleracea* | AJ400848 | 150,725 | 98 | Dicots | 36.8 |
| *Nicotiana tabacum* | NC_001879 | 155,939 | 101 | Dicots | 37.9 |
| *Toxoplasma gondii* | U87145 | 34,996 | 26 | Apicomplexa | 21.4 |
| *Triticum aestivum* | NC_002762 | 134,545 | 84 | Monocots | 38.3 |
| *Zea mays* | X86563 | 140,384 | 111 | Monocots | 38.4 |
| Cyanobacteria | | | | | |
| *Anabaena sp.* PCC 7120 | BA000019 | 6,413,773 | 5,364 | Cyanobacteria | 41.3 |
| *Gloeobacter violaceus* PCC 7421 | BA000045 | 4,659,019 | 4,430 | Cyanobacteria | 62.0 |
| *Nostoc punctiforme* PCC 73102 | JGI | (9.0 Mb) | 7,281 | Cyanobacteria | 41.4 |
| *Prochlorococcus marinus* MED4 | NC_005072 | 1,657,990 | 1,712 | Cyanobacteria | 30.8 |
| *Prochlorococcus marinus* MIT9313 | NC_005071 | 2,410,873 | 2,265 | Cyanobacteria | 50.7 |
| *Prochlorococcus marinus* SS120 | AE017126 | 1,751,080 | 1,882 | Cyanobacteria | 36.4 |
| *Synechococcussp.* WH8102 | NC_005070 | 2,434,428 | 2,517 | Cyanobacteria | 59.4 |
| *Synechocystissp.* PCC 6803 | AB001339 | 3,573,470 | 3,264 | Cyanobacteria | 47.7 |
| *Thermosynechococcus elongatus* BP-1 | BA000039 | 2,593,857 | 2,475 | Cyanobacteria | 53.9 |
| Photosynthetic bacteria | | | | | |
| *Chlorobium tepidum* TLS | AE006470 | 2,154,946 | 2,252 | Green-sulfur | 56.5 |
| *Rhodopseudomonas palustris* | BX571963 | 5,459,213 | 4,836 | Proteo alpha | 65.0 |
| Bacteria | | | | | |
| *Bacillus subtilis* 168 | AL009126 | 4,214,814 | 4,100 | Low GC Gram + | 43.5 |
| *Escherichia coli* K-12 MG1655 | U00096 | 4,639,221 | 4,289 | Proteo gamma | 50.8 |

discontinuous in others. The discontinuity appears in various aspects of plastid evolution, such as the origin of plastids, gene transfer to nucleus, replacement of genomic machinery, and so on. Such discontinuities are the origin of the flourishing diversity of plastids. We will focus on some important aspects of plastid evolution, with opposing principles, such as unification/diversification and continuity/discontinuity. Comparison of hitherto unavailable genome sequences is now revolutionizing the notion of plastids and even the notion of plants. Exploitation of genomic data is another focus of this chapter. Table 1 lists completely sequenced plastid genomes and some related prokaryotic genomes, which are discussed in this chapter. For

additional recent reviews on the evolution of plastids, the reader is referred to McFadden (2001), Sato (2001, 2003), Archibald and Keeling (2002), Wilson (2002), Cavalier-Smith (2003), and Palmer (2003).

## II. Endosymbiotic Origin of Plastids: The Major Unifying Principle

### A. Endosymbiosis as a Basic Assumption

It was not very difficult for the researchers in the 19th century to imagine that chloroplasts have something to do with algae. See the illustrations of Schimper (1885) as reproduced in Kirk and Tilney-Bassett (1967). However, it was not until detailed composition, structure and physiology of chloroplasts and algae were elucidated that a realistic view on the origin of chloroplasts could be envisaged. The fundamental distinction between prokaryotes and eukaryotes as revealed by electron microscopic observation and biochemical analysis in the 1960's provided the basis of the endosymbiotic origin of organelles. Early evidence included structural characteristics such as membrane systems (thylakoid membrane as the place of photosynthetic electron transport), but common physiological properties such as oxygenic photosynthesis were taken as evidence as well (Whatley, 1983). Existing examples of tight symbiosis of cyanobacterial cells and various hosts such as liverworts and ferns also provided evidence for the transition from symbiosis to endosymbiosis. Biochemical characterization of major pigments, major components of the electron transport chain, as well as the transcription and translation machinery also provided solid evidence for the endosymbiotic theory.

In the Margulis' theory on endosymbiosis, the origin of plastids was part of a larger hypothesis that attempted to explain the origin of several cellular components by endosymbiosis of some free-living organisms (Margulis, 1970). The cyanobacterial origin of plastids and $\alpha$-proteobacterial origin of mitochondria are currently accepted as proven (Gray and Doolittle, 1982). The essential point is the sequence conservation between the plastid DNA and cyanobacterial DNA as described in detail below. However, we still need more study of the putative symbiotic host, because the ancestral host cell may have arisen by multilateral bacterial fusion (Gupta, 1998). We should also consider the possibility that photosynthetic genes may have already existed in the primitive eukaryote that later harbored plastids (pre-endosymbiotic gene transfer). We cannot clearly distinguish if nuclear photosynthetic genes arose by gene transfer from some cyanobacteria or if the photosynthetic function of plants originated entirely from the endosymbiont. In addition, such arguments always assume "minimal evolution", namely, the most probable way of evolution should explain the current status of living organisms by minimum number of events (see questions and answers of Cavalier-Smith, 2003). However, this is not proven *a priori*, and we can even argue how many times endosymbiosis occurred (see Section III C).

### B. Molecular Evidence for the Endosymbiotic Origin of Plastid Genomes

#### 1. rRNA

Phylogenetic analyses based on ribosomal RNA (rRNA) sequences are one of the standard methods of inferring relationships among diverse organisms, since rRNA exists in all organisms and organelles as a single molecular species even in organisms in which rDNA exists in multiple copies. This was the basis for construction of the universal tree of life and distinction of the three kingdoms by Dr. Carl Woese (1987). The SSU (small subunit) rRNA tree of cyanobacteria and plastids (Douglas and Turner, 1991) suggested that all plastid rRNAs of various algae and plants form a single cluster, with branches within the cyanobacterial radiation. This was supported by a detailed analysis with complete 16S rRNA sequences (Nelissen *et al.*, 1995). In contrast with preceding studies using partial 16S rRNA sequences, the phylogenetic analysis using such complete 16S rRNA sequences suggested that the branching of plastids occurred early in the cyanobacterial diversification, leaving *Gloeobacter* and *Pseudanabaena* as only the genera that diverged before the divergence of plastids and cyanobacteria (see Fig. 2b). Essentially similar results were reported using the rRNA gene sequences of 53 cyanobacteria and 10 plastids (Turner *et al.*, 1999). The phylogeny of cyanobacteria was further studied using 16S rRNA sequences, and an important outcome of such studies was that various strains having the same genus name such as *Synechococcus* are polyphyletic (Honda *et al.*, 1999). A more recent analysis of the phylogeny of cyanobacteria including various marine species of *Prochlorococcus* and *Synechococcus* also suggested deep origin of plastid clade within the cyanobacterial divergence, although the *Gleoebacter* sequence was not included in this particular analysis (Hess *et al.*, 2001).

Another line of evidence for the similarity of cyanobacteria and plastids using RNA sequences is the

presence of Group I introns within the $tRNA^{Leu}$ gene (Besendahl *et al.*, 2000). Many, though not all, of the tRNA genes in cyanobacteria and plastids contain a Group I intron, which is highly conserved. All plastid sequences of this intron diverged according to the major phylogeny of algae and plants, but the plastid clade diverged from the root of the cyanobacterial diversity. Therefore, the origin of plastid divergence is not clearly mapped in the cyanobacterial diversity by the intron sequences.

## 2. Protein Sequences

Protein-coding genes in the plastid genomes have, in general, homologues in cyanobacterial genomes. This is true for most proteins involved in photosynthesis, such as subunits of photosystems, electron transport complexes, and the ATP synthase. Many ribosomal proteins and subunits of prokaryotic RNA polymerase are also encoded by the plastid genome and are highly similar to their counterparts in cyanobacteria. However, it is also true that many photosynthesis-related proteins and ribosomal proteins found in plastids are encoded by the nuclear genome. In such cases, the similarity of nuclear-encoded plastid proteins with the cyanobacterial counterparts is still markedly high. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is one of the first proteins that was used for the phylogenetic analysis of plastids and cyanobacteria. The large subunit of Rubisco is invariably encoded by the plastid genome (*rbcL*) in photosynthetic eukaryotes, and more than 440 sequences are currently available in the SwissProt database. Figure 1a shows a simplified phylogenetic tree of *rbcL* using selected species. The phylogenetic tree of *rbcL* is complicated by the fact that there are different types of Rubisco. The red algal *rbcL* genes (Form ID) are similar to the *rbcL* of $\alpha$-proteobacteria (Form IC), and considered to be a product of horizontal (or lateral) gene transfer (HGT) (Delwiche and Palmer, 1996; Tabita, 1999). The same type (Form ID) of *rbcL* genes is found in chromophytes, which resulted from secondary endosymbiogenesis by a red alga (see Section III C 1). In cyanobacteria, marine cyanobacteria including *Prochlorococcus* and *Synechococcus* also acquired *rbcL* from another bacterium, possibly a $\gamma$-proteobacterium (Form IA) (Tabita, 1999; Hess *et al.*, 2001). The plastids of green algae, land plants and *Cyanophora* form a single clade, which is sister to the main cluster (Form IB) of cyanobacteria (Fig. 1a). The relationship among various green algae and plants estimated by *rbcL* is incongruent with known phylogenetic relationship, such as the strange position of pteridophytes, and the non-root position of *Mesostigma viride*, which diverged from the root of all green algae and plants (Lemieux *et al.*, 2000).

The *atpB* gene is a good marker of plastid phylogeny. The *atpB* gene is also widely used in phylogenetic analysis within a family or at higher levels. Both *rbcL* and

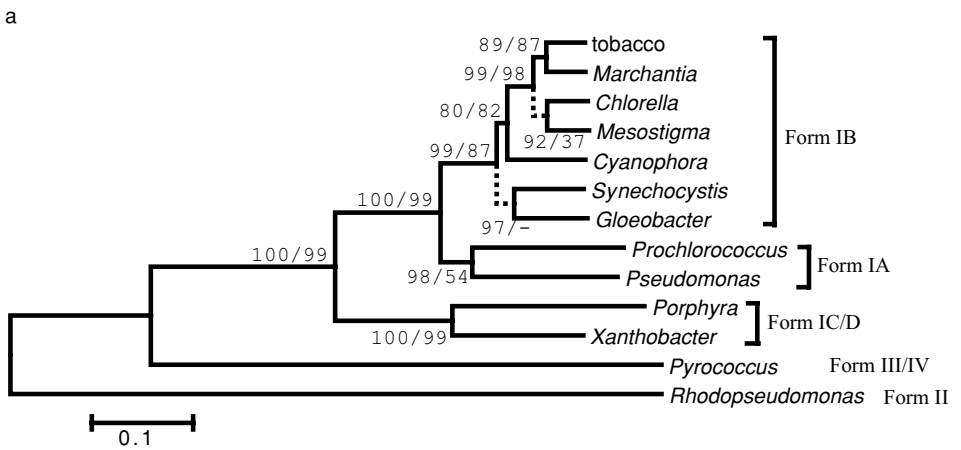



*Fig. 1.* (a) A simplified phylogenetic tree based on Rubisco protein sequences. RbcL sequences of representative species are used to illustrate various different forms of Rubisco. The tree was constructed by the neighbor-joining (NJ) and maximum parsimony (MP) methods using the MEGA2 software (Kumar *et al.*, 2001). Each set of numbers on the branch indicates bootstrap confidence levels with the two methods (NJ/MP). The dotted lines indicate branches that are not supported at high confidence level. Scale bar represents substitutions per site. Source of RbcL sequences (SwissProt accession number): *Pseudomonas hydrogenothermophila* ($\gamma$-proteobacterium), Q51856; *Xanthobacter flavus* ($\alpha$-proteobacterium), P23011; *Pyrococcus abyssi* (Euryarchaeota), Q9UZD7. Other sequences were taken from gene models based on complete genome sequences (Table 1).

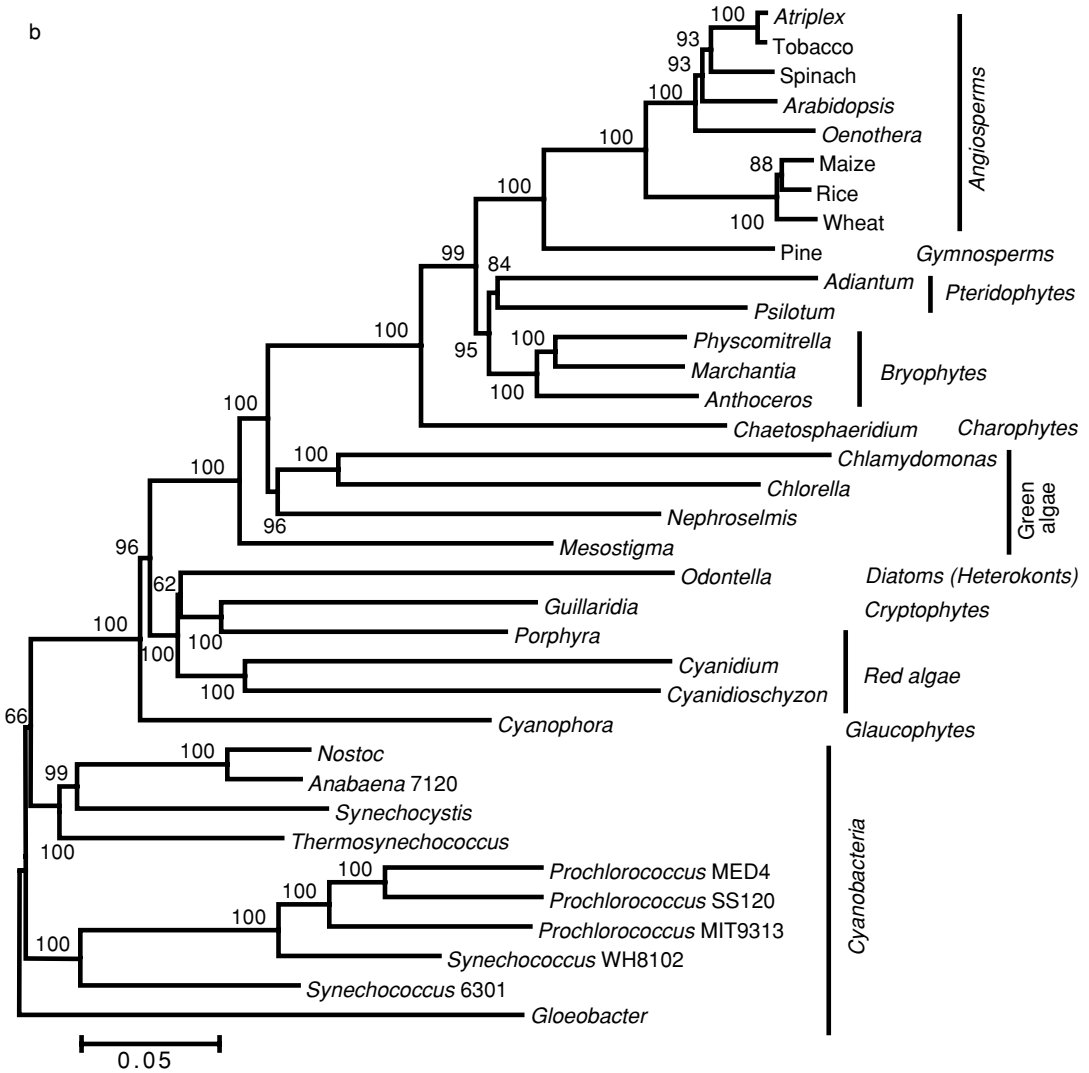



*Fig. 1.* (b) An NJ tree of plastids and cyanobacteria based on 27 protein sequences. Each number on the branch indicates the bootstrap confidence level. The 27 proteins consist of two sub-sets of 11 and 16 proteins, respectively. The 11 proteins (housekeeping proteins that are also conserved in bacteria) were: Rps7, Rps12, Rpl2, Rpl16, Rps2, Rpl14, Rps3, Rps4, Rps19, RpoB, and RpoC1. The 16 proteins (mostly photosynthesis-related proteins) were: AtpA, AtpB, AtpH, Ycf4, PetA, PetB, PetD, PsaA, PsaB, PsaC, PsbA, PsbB, PsbC, PsbD, RpoC2, and Rps8. AtpI is not encoded by *Cyanophora* plastid genome, and was not included. The sequences of *Euglena*, *Astasia*, *Plasmodium*, and *Toxoplasma* are highly divergent, and were not included in this tree. Data of *Synechococcus* sp. PCC 6301 were kindly provided by Dr. M. Sugita (Nagoya University).

*atpB* give similar results and the combined use of these two genes gives a better resolution in phylogenetic reconstruction (Savolainen *et al.*, 2000). This reflects the slow and relatively uniform evolution of the *atpB* gene. Rate of evolution is an important factor that significantly influences any reconstructed phylogenetic relationship. For example, the evolutionary rate of the *rpoB* gene is high among plastids, but low among cyanobacteria, whereas the evolutionary rate of the *psbA* gene is high among cyanobacteria, but low among plastids (N. Sato, unpublished data).

Another approach to studying plastid phylogeny involves use of concatenated protein sequences encoded by both plastid and cyanobacterial genomes. There are different opinions about the use of such concatenated sequences. Yoon *et al.* (2002b) argued that only those proteins that show low rate of evolution should be used, whereas Martin *et al.* (2002) used 117 protein

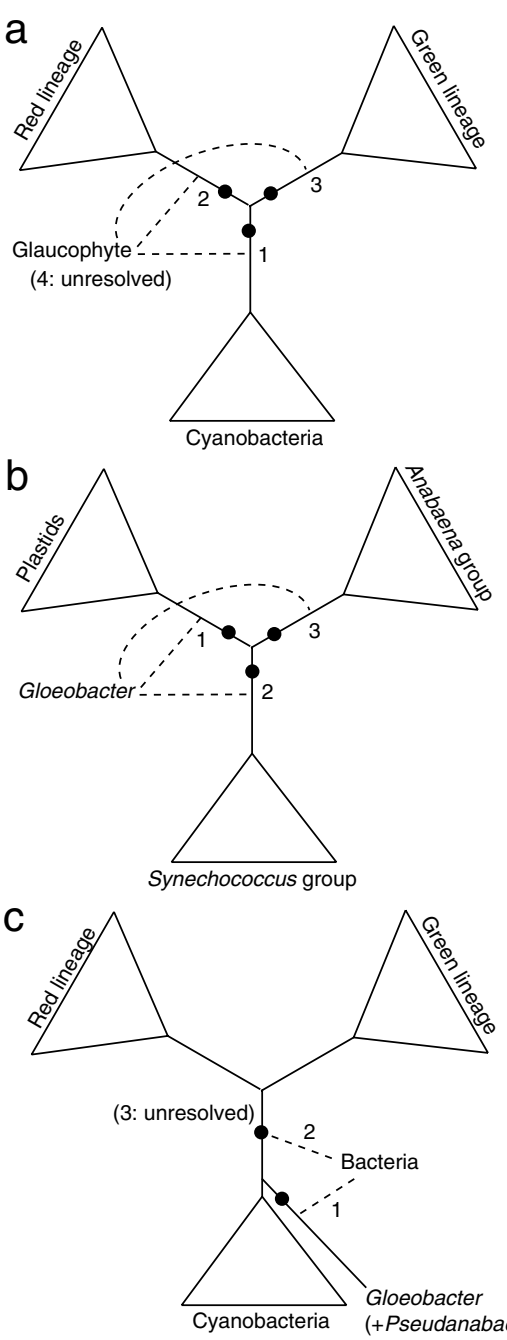
sequences that are conserved in 20 plastid genomes. We tested the effect of concatenation in our hands, and confirmed that various ribosomal proteins give various topologies of phylogenetic relationship, but concatenation of such proteins gives a more reasonable phylogenetic relationship. However, we should still be cautious about which sequences to use in such analysis. Various sets of proteins have been used for the construction of trees, such as those involved in photosynthesis, ATP synthesis, translation and transcription (Martin *et al.*, 1998, 2002; Yoon *et al.*, 2002b). Figure 1b is an example of such a phylogenetic tree, which is made with the newest data set available at the time of writing (see Table 1 for organisms). Similar trees included only a single species of cyanobacterium as an out-group to plastids, but Fig. 1b includes all available cyanobacterial genomes. Although this is an unrooted tree, the radiations of cyanobacteria and plastids do not overlap each other, namely, the plastid progenitor is outside the extant cyanobacterial diversity or very close to the root of it. Within the cyanobacteria, two major clusters are recognized as in the case of *rbcL* tree (Fig. 1a). The plastids consist of three lineages, green, red and glaucophyte. Within the green lineage, land plants diverged from green algae, but the precise branching is somewhat distorted because pteridophytes and bryophytes form a subcluster.

Ribosomal proteins are also useful in inferring phylogeny of plastids and bacteria. Both concatenated amino acid sequence and synteny of ribosomal protein gene cluster suggested monophyly of all plastids, and the closest bacterial group was the cyanobacteria (Ohta *et al.*, 1997). This approach was extended with larger number of species, and indicated essentially identical phylogenetic relationship (Stoebe and Kowallik, 1999).

Here we will focus on some important branching patterns (Fig. 2). Figure 2a illustrates three possible branching patterns of Glaucophyta (*Cyanophora paradoxa*) with respect to red lineage, green lineage and cyanobacteria. All three possible branchings are supported by various methods of analysis. Use of rRNA or proteins, number of proteins included, as well as method of phylogenetic analysis all affect estimation of the lineage most related to Glaucophyta. It is therefore highly speculative to suppose that glaucophyte plastids diverged first from the plastid lineages.

Figure 2b shows the relationship of *Gloeobacter* (a cyanobacterium without thylakoid membranes) to plastids and the two major groups of cyanobacteria. Both the rRNA data as described above and some protein data are consistent with the position of *Gloeobacter* at the root of all other cyanobacteria and all plastids. However, some data also support inclusion of *Gloeobacter* within *Synechococcus* group. This is related to the crucial problem on whether the plastid progenitor is within the diversity of extant cyanobacteria. To root the tree, bacteria are included using proteins that are shared by bacteria, cyanobacteria and plastids (Fig. 2c). An eleven-protein set includes ribosomal proteins and RNA polymerase subunits, which are shared by all plastids including those in apicomplexans and euglenophytes. The *atpA* and *atpB* genes are also useful for the rooting with bacteria. A slightly different set of plastids was used in the analysis with *atpA* and *atpB*. Curiously, three different data sets are compatible with *Gloeobacter* being the first cyanobacterium that diverged from other cyanobacteria and plastids. There is some ambiguity about the position of *Gloeobacter* in the analysis without rooting (Fig. 2b), but available data using the rooting with bacteria supports the initial diversification of *Gloeobacter*. All these data suggest a deep origin of plastids within extant cyanobacteria. *Gloeobacter* might be related to the cyanobacteria that have diverged first, but the large genome size and the unique cellular structure (photosynthesis is performed in the cytoplasmic membrane rather than in internal membranes) of this cyanobacterium allow doubt about the similarity of plastid progenitor with present-day *Gloeobacter*.

Proteins involved in the chlorophyll/bacteriochlorophyll synthesis are used to infer phylogenetic relationships among photosynthetic bacteria, cyanobacteria and plastids (Xiong *et al.*, 2000). The results showed that the purple bacteria are the first photosynthetic organisms, which is in clear contrast with the results with 16S rRNA or Hsp60/Hsp70 sequences that showed green non-sulfur bacteria are the earliest photosynthetic bacteria. However, the relationship between plastids and cyanobacteria as deduced by the analysis of chlorophyll synthesis enzymes was markedly different from the above-mentioned results, namely, the plastid cluster is a sub-cluster that diverged after cyanobacteria (only one species *Synechocystis* sp. PCC 6803). In the *chlB* tree, plastids diverged even later, *i.e.*, after *Plectonema boryanum*. In addition, the branch leading to the plastid cluster was significantly long. The putative *chlB* gene in red algae might not be a true orthologue (see Section III A 1). It is probable that the photosynthetic genes evolved differently from other proteins or 16S rRNA genes. The anomalies in the evolution of photosynthesis-related genes were noted in a later study using whole-genome datasets (Raymond *et al.*, 2002). Since the core components of photosynthesis are considered to have been subject




| | | |
|---|---|---|
| 1 | 27 proteins | ME 96%, NJ 59%, ML, QP 72% |
| | SSU rRNA | [a] NJ (JC) 99% |
| | 117 DNA | [d] ML |
| | 27 DNA | ML |
| 2 | 16 proteins | MP |
| | SSU rRNA | [a] MP 46%, NJ (trans) 100% |
| | 27 DNA | Bayes 100% |
| | 16 DNA | Bayes 100% |
| 3 | 16 proteins | ME 65%, NJ 75% |
| | 27 DNA | ME/NJ 65% |
| | 11 DNA20sp | QP 79% |
| | 11 DNA | Bayes 100% |
| | atpAB | ME 64% |
| | RP synteny | [b] |
| | Cladistic | [e] 84% |
| 4 | 27 proteins | MP |
| | 16 proteins | QP |
| | 16 DNA | QP |
| | 11 proteins | QP |
| | 11 DNA | NJ,/ME (1 or 3), ML, QP |
| | atpAB | MP |

| | | |
|---|---|---|
| 1 | 27 proteins | NJ 85% MP 73% |
| | atpAB | ME 69%, QP 70% |
| | SSU rRNA | [a] NJ 81-100%, MP 40% |
| | SSU rRNA | [c] ML 86-95% |
| 2 | 27 proteins | ME 66%, ML, QP 87% |
| | 16 proteins | ME 98%, MP 66% QP 91% |
| | 27 DNA | NJ 60%, ME 55% |
| | 16 DNA | QP 92% |
| | SSU rRNA | Bayes 90% |
| | 11 DNA | Bayes 100% |
| 3 | Gene content (gclust-MP) | |
| | 11 DNA | QP 60% |
| | 27 DNA | ML |
| | 27 DNA | Bayes 100% |
| | 16 DNA | Bayes 100% |
| | 11 DNA | Bayes 100% |

| | | |
|---|---|---|
| 1 | atpAB | ME 100%, MP 99% |
| | SSU rRNA | [a] NJ 52-60%, MP 77% |
| | 11 proteins | MP 86% |
| | 11 DNA20sp | QP83% |
| | 11 DNA | fastDNAm |
| | 11 DNA | Bayes 100% |
| | 11 DNA20sp | Bayes 100% |
| 2 | 11 DNA20sp | ME100%, NJ 99% |
| 3 | 11 proteins | QP, ME |
| | 11 DNA | QP |

References

[a] Nelissen *et al.*, 1995 [b] Ohta *et al.*, 1997b
[c] Turner *et al.*, 1999 [d] Martin *et al.*, 2002
[e] Nozaki *et al.*, 2003b

*Fig. 2.* Topology of important problematic branchings. (a) Branching of Glaucophyte (*Cyanophora paradoxa*) with respect to cyanobacteria, and red and green lineages of plastids. Three possible patterns are shown as 1, 2 and 3. Results of phylogenetic analysis that support each of the three possibilities are shown. Each percentage indicates bootstrap confidence level of the branch marked by a dot that links the trichotomous center and each hypothetical branch point. The 27 protein data (and sub-data: 11 or 16 proteins, or AtpAB. See legend to Fig. 1) were analyzed by the following methods and softwares. Minimum evolution (ME) and NJ methods, MEGA2 software version 2.1; maximum likelihood method (ML), protml software (MOLPHY package version 2.3 beta3. Adachi and Hasegawa, 1996), quartet puzzling maximum likelihood method (QP), tree-puzzle software version 5 (reference for original version: Strimmer and von Haeseler, 1996); maximum parsimony (MP), PAUP software version 4 beta 10 (Swofford, 2002); Bayes, MrBayes software version 3.1 (Ronquist and Huelsenbeck, 2003). Other data were obtained from references shown. "DNA" indicates protein-coding DNA sequences. "DNA20sp" indicates protein-coding DNA sequences of 20 species (in other analyses, 35 species as shown in Fig. 1 were used). (b) Branching of *Gloeobacter* with respect to two major groups of cyanobacteria and plastids. (c) Branching of bacteria (four species shown in Table 1) with respect to cyanobacteria and plastids.

to HGT, it is difficult to infer correct phylogeny with only photosynthesis-related genes.

### 3. Genomic Comparison

The nucleotide sequence of plastid DNA has been determined in various plants and algae (Table 1) and such organellar genome data has been useful in analyzing phylogenetic relationships between and among cyanobacteria, plastids and plants. In particular, the Gclust project is an attempt to compare all homologous proteins in photosynthetic prokaryotes and eukaryotes, either by sequence-based phylogenetic analysis or by the presence/absence of gene clusters (Sato, 2002, 2003). It is a combined project consisting of software development (gclust software, current version 3.52. See http://nsato4.c.u-tokyo.ac.jp/old/Gclust/), compilation of genomic data (Table 1 and many others), and mass analysis of phylogeny. Phylogenetic trees shown in the present article are prepared based on the Gclust databases.

Whole genome comparison of cyanobacteria, bacteria, and eukaryotes (17 species) by the Gclust database identified more than 38,117 homologue groups (each group being a set of homologous sequences from different organisms), and a phylogenetic relationship was inferred from the presence/absence of such groups in each species (Sato, 2003). Based on such clustering, the number of homologue groups that include the two photosynthetic eukaryotes (*Arabidopsis* and *Cyanidioschyzon*) and the eight cyanobacteria but no non-photosynthetic organisms was estimated to be 44. This number moderately increased to 69 or 107, if the number of cyanobacteria to be included is allowed to decrease to five or even only one among the eight species. In other words, many of the genes that are shared by photosynthetic organisms are shared, not by just some species of cyanobacteria, but by most of the cyanobacteria. This suggests that many genes in photosynthetic eukaryotes originated from a common ancestor of all eight cyanobacteria, but not from an ancestor of a certain group of cyanobacteria (see Section III.C.3).

## C. Related Topics

### 1. Multiple Origins of Chlorophyll a/b—Type Photosystems

Since the discovery of prochlorophytes (Lewin, 1976), chlorophyll *a/b*-containing prokaryotes were thought to be the origin of green algae and land plants. However, prochlorophytes were subsequently found closely related to cyanobacteria. Different genera of prochlorophytes such as *Prochloron*, *Prochlorothrix*, and *Prochlorococcus* are polyphyletic and are related to different groups of cyanobacteria (Honda *et al.*, 1999; Hess *et al.*, 2001). Some species of *Prochlorococcus* contain both chlorophylls *a/b* and phycobiliproteins as light-harvesting pigments (Hess *et al.*, 1996). The synthesis of chlorophyll *b* from chlorophyll *a* is catalyzed by a single oxygenase (Tanaka *et al.*, 1998), and therefore, could have been transferred to an organism that originally did not have chlorophyll *b* such as *Synechocystis* (Xu *et al.*, 2001). The fundamental difference in the chlorophyll *b*-binding light-harvesting proteins in prochlorophytes and algae/plants in the green lineage also suggests that the occurrence of chlorophyll *b* in prochlorophytes and the green lineage are not evolutionarily related (see also III A 1).

### 2. The Progenitor of Plastids

As shown above, the search for the progenitor of plastids still produces controversial results. Analysis using the 16S rRNA sequence suggests that the plastids diverged near the cyanobacterial root. However, protein-based analysis (sequence, synteny, clustering) suggests that the plastid diversity resides outside the diversity of extant cyanobacteria (Fig. 2). A reasonable guess is that the radiation of plastids and cyanobacteria overlap only slightly and that plastids diverged from cyanobacteria after *Gloeobacter*. In other words, the diversification of most or all of present-day cyanobacteria began just before the endosymbiotic creation of plastids, which is estimated to be about 1.58 Ga (billion years ago: see Fig. 3), which is the date of plant-animal-fungal divergence (Wang *et al.*, 1999; Yoon *et al.*, 2002b). This looks strange because we know that ancient microfossils of putative trichomic cyanobacteria have been assigned a date of about 3.5 Ga (Schopf, 1993). The origin of cyanobacteria (divergence of cyanobacteria from other bacteria) is currently estimated to be 2.6 Ga, which immediately preceded the rise in atmospheric oxygen concentration (2.4-2.2 Ga) (Hedges *et al.*, 2001). The deep origin of plastids among extant cyanobacteria suggests that, after the creation of photosynthetic eukaryotes, most cyanobacteria of the day were not competitive and went extinct. Photosynthetic eukaryotes are quite different from cyanobacteria with respect to facultative heterotrophy, motility with flagella and sexual reproduction. We can imagine that cyanobacteria survived only in extreme environments such as hot springs, Antarctica, and the ocean,

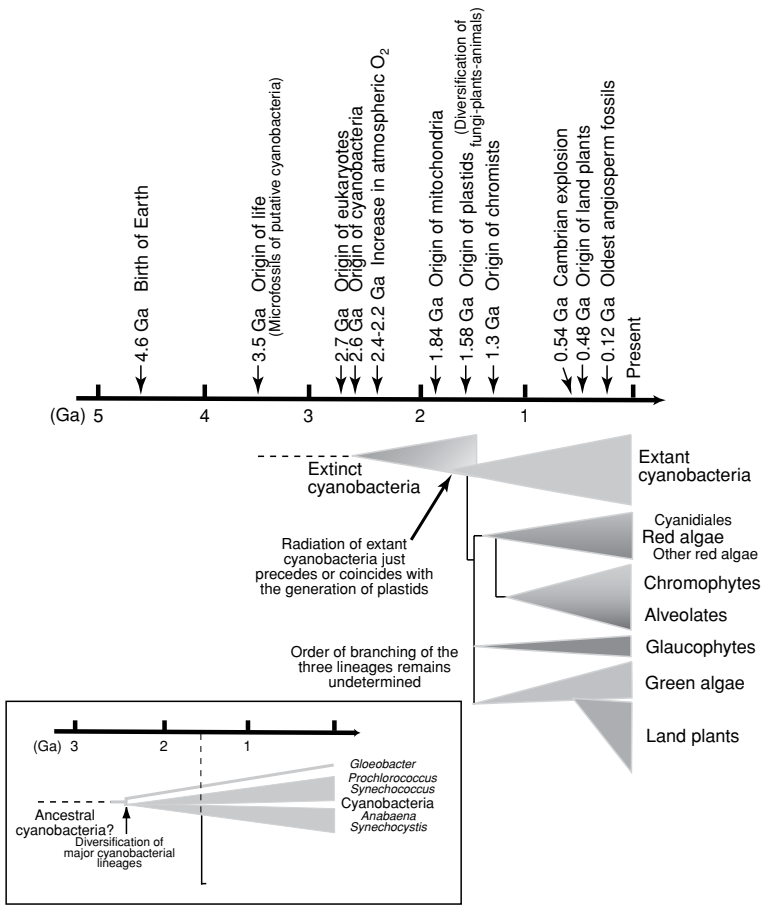



*Fig. 3.* (See also Color Plate 1, p. xxxiv.) Evolution of cyanobacteria and plastids in the geological time scale. Origin of cyanobacteria dates from 2.6 Ga (billion years ago) according to Hedges *et al.* (2001). One view is that the radiation of extant cyanobacteria just precedes or coincides with the generation of plastids (see text). Shown in the inset is another possibility that plastids diverged from one of the two major cyanobacterial lineages. The origin of plastids is estimated to be 1.58 Ga (Wang *et al.*, 1999). Origin of chromists (and possibly alveolates) was 1.3 Ga according to Yoon *et al.* (2002b). The origin of land plants was 0.48 Ga (Kenrick and Crane, 1997).

where photosynthetic eukaryotes are unable to grow. With their ability to form heterocysts and tolerate desiccation, cyanobacteria in the order Nostocales may have been the pioneers of land surface, but this was much later in the history and before the origin of land plants (0.48 Ga: Kenrick and Crane, 1997). In other places, only the species of cyanobacteria that performed highly efficient photosynthesis could survive. This hypothesis can explain the relatively young origin of extant cyanobacteria. (See Note Added in Proof.)

## *3. Plant Proteins of Non-Cyanobacterial Origin*

Not all plant proteins that have homologues in cyanobacteria originated from a cyanobacterial endosymbiont. There is a large group of RNA-binding proteins having one or more RNA-recognition motif(s) (RRM) in plants and animals. Poly(A)-binding proteins and U1A spliceosomal protein have four and two RRM domains, respectively. Another type of RNA-binding protein is glycine-rich RNA-binding protein (GRP), which consists of a single RRM and a glycine-rich C-terminal domain. In plastids, RNA-binding proteins having two RRM domains are present and some are involved in RNA processing (Nakamura *et al.*, 2001). Cyanobacteria also contain RNA-binding proteins (called Rbp) having a single RRM and a short glycine-rich C-terminal domain, similar to plant GRPs. The plant RNA-binding proteins were thought to be homologues of cyanobacterial Rbps, but a detailed phylogenetic analysis indicated that both originated separately. The structural and physiological similarity of plant GRPs and cyanobacterial Rbps are a

result of convergent evolution (Maruyama *et al.*, 1999). The plastid RNA-binding proteins diverged from plant GRPs (Nomata *et al.*, 2004).

Another example is phytochrome, a red/far-red reversible photoreceptor in plants. Genome sequencing revealed that phytochrome homologues called bacteriophytochromes are present in cyanobacteria and various bacteria. Plant phytochromes, however, are not directly related to cyanobacterial bacteriophytochromes (Montgomery and Lagarias, 2002). Bacteriophytochromes and plant phytochromes share the photoreceptor domain but the C-terminal domains are different. The histidine kinase domain in the bacteriophytochromes transfers signal to downstream receiver molecules, while the signal produced by the plant phytochromes is still unclear. The phylogenetic analysis of the photoreceptor domains revealed that the domain is present in various bacteria, and the plant type domain is considered to originate from common ancestors of bacteria and eukaryotes.

Fatty acid desaturases are present in various plants and cyanobacteria as well as some bacteria. Various types of desaturases exist depending on the position in the acyl chain to which the double bond is introduced. The $\Delta 15$ desaturases that produce $\alpha$-linolenic acid in plants and cyanobacteria originated from $\Delta 12$ desaturases that produce linoleic acid in cyanobacteria. However, the ER- and plastid-located $\Delta 12$ desaturases are paraphyletic. Different types of $\Delta 12$ desaturases are also present in cyanobacteria. No simple relationship exists between the cyanobacterial enzymes and plant enzymes. Diversification of this enzyme occurred in the common ancestor of cyanobacteria, bacteria, plants and fungi. Desaturases of different lineages remained in different phyla, and this is a reason why the phylogeny of desaturases is apparently incongruent with known phylogenetic relationship of organisms (Sato, 2003b; Sato, in preparation).

## III. Origin and Evolution of Plastid Diversity

### *A. Diversity of Plastids in Different Aspects*

#### *1. Pigments and Related Topics*

Originally, diversity of various lineages was primarily characterized by pigment composition. Chlorophyll *a* is essential in photosynthesis and present in all algae and plants. Chlorophyll *b* is present in the green lineage, while chlorophyll *c* is present in chromophytes and dinoflagellates. Carotenoids are more variable. Phycobiliproteins are present in red algae and glaucophytes. However, pigments alone are not good markers for the phylogeny of plants and algae. Cellular structures such as membrane architecture (see below) are also important in the phylogeny of plastids.

Chlorophyll biosynthesis provides an interesting perspective of plastid evolution. Two different types of protochlorophyllide reductases that synthesize chlorophyllide *a* are known, namely, a light-independent enzyme and a light-dependent enzyme (Fujita, 1996). The light-independent, oxygen-sensitive reductase (DPOR) encoded by *chlB*, *chlN* and *chlL* genes is similar to nitrogenase complex and is conserved in cyanobacteria, algae, and land plants up to gymnosperms. A homologous system is also present in photosynthetic bacteria. In many algae and land plants (except angiosperms) a complete set of the three genes is encoded by the plastid genome. Extensive phylogenetic studies have been done on the *chlB* (Boivin *et al.,* 1996) and *chlL* (Kusumi *et al.*, 2000) genes. Among the completely sequenced plastid genomes, the two species of *Pinus* possess *chlB*, *chlN* and *chlL* genes. The *chlB* gene is widely distributed in various gymnosperms and pteridophytes except *Psilotum* and *Gnetum* (Boivin *et al.*, 1996). In bryophytes, *Anthoceros* (hornwort) has a complete set of the three genes, while *Marchantia* (liverwort) and *Physcomitrella* (moss) have only *chlB* and *chlL* within the plastid genome. In these species, *chlN* gene is supposedly encoded by the nuclear genome, but no EST sequence homologous to *chlN* has been reported in the large EST database of *Physcomitrella*. In the red algae, *Porphyra* (a macrophytic marine red alga) has the three genes, but unicellular algae such as *Cyanodioschyzon* and *Cyanidium* have only a *chlN* homologue within the plastid genome. These homologues are markedly different from other plastid-encoded *chlN* genes, and are considered to be the *moeB* gene involved in molybdopterin biosynthesis. The nuclear genome of *C. merolae* does not contain *chlB*, *chlL* or *chlN* gene (Matsuzaki *et al.,* 2004). In addition, none of the plastid genomes of the secondary endosymbionts encodes these genes. The light-independent protochlorophyllide reductase was, therefore, lost in different lineages or taxa: unicellular red algae (Cyanidiales), *Psilotum*, and angiosperms, as well as after secondary endosymbiosis.

In contrast, the light-dependent protochlorophyllide reductase (LPOR) requires NADPH as a cofactor and is present in all photosynthetic eukaryotes and cyanobacteria. This enzyme is not sensitive to oxygen, and is

thought to have evolved after the rise in atmospheric oxygen pressure, presumably from a short-chain alcohol dehydrogenase. This is the only enzyme that synthesizes chlorophyllide *a* in angiosperms. In addition, in various species of plants and algae that do not have light-independent enzyme (see above), LPOR is the sole enzyme that reduces protochlorophyllide. The replacement of DPOR by LPOR probably represents an evolutionary adaptation to high level of oxygen in the atmosphere.

The phylogeny of light-harvesting complexes (LHCs) has been analyzed in detail. Cyanobacterial antenna consists of PCB proteins, which are distant homologues of CP43 encoded by *psbC*. Photosynthetic eukaryotes have a different type of antenna protein belonging to the LHC superfamily (Durnford *et al.*, 1999). The eukaryotic LHCs consist of two major classes, namely, chlorophyll *a/b*-binding proteins that are conserved in the green lineage, and chlorophyll *a/c*-binding proteins that are conserved in the red and chromophyte lineage. In the green lineage, two types of LHCs diverged, namely, LHCI and LHCII. The origin of LHCs is thought to be a pre-LHC similar to early light-inducible proteins (ELIPs) having three helices, which was created by duplication and fusion of cyanobacterial high light-inducible proteins (HLIPs), having a single membrane-spanning domain (Dolganov *et al.*, 1995).

## 2. Membrane Systems

Plastids are typically enclosed by two layers of envelope membrane, which are major barriers to metabolites and proteins. The envelope membranes are also major site of biosynthesis of various lipophilic compounds, such as lipids, pigments and terpenoids, within the plant cell. The plastid envelope may be compared with the inner and outer membranes of cyanobacteria. The isolated envelope membranes look yellowish-orange due to pigmentation with carotenoids, just as the inner and outer membranes of cyanobacteria. However, the outer envelope membrane of plastids is more similar to the endoplasmic membrane, as indicated by the presence of phosphatidylethanolamine and phosphatidylcholine (see Douce and Joyard, 1990). The envelope membranes of chloroplasts and proplastids are similar in composition and function. However, we do not have enough information about the composition and function of envelope membranes of various plastids in diverse plants and algae.

The membrane surrounding the plastids is an important structural marker in plastid phylogeny. In many algae that are thought to be products of secondary endosymbiosis, plastids are surrounded by one or two additional membranes, called periplastid ER and periplastid membrane (or chloroplast ER, collectively). In these algae, at least one membrane (periplastid membrane) completely encloses the plastid, while the outer one (periplastid ER) is continuous with the outer layer of the nuclear membranes in some algae. When two membranes are present, the outer one resembles rough ER. The diversity in membrane systems surrounding the plastids is a clue to the understanding of plastid phylogeny (see below).

A vesicle transport system, similar to that in cytoplasm, was found in the plastids (von Wettstein, 1959; Chapter 3). By this system, vesicles formed from the envelope membrane move through the stroma and contribute to the formation of thylakoid membranes. Detailed survey among various algae and plants indicated that this vesicle transport system is, however, detected in only land plants (Westphal *et al.*, 2003) and, therefore, considered to be a result of recent evolution.

## 3. Membrane Lipids

Plastid membranes contain glycerolipids that are characteristic of photosynthetic membranes (see Chapter 17). They include monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), phosphatidylglycerol (PG), and sulfoquinovosyl diacylglycerol (SQDG). All of these glycerolipids are invariably present as major constituents of thylakoid membranes as well as envelope membranes in all photosynthetic eukaryotes analyzed. The biosynthesis of MGDG, however, is different in cyanobacteria and in plastids. In plastids, diacylglycerol is galactosylated with UDPgalactose to give MGDG, whereas, in cyanobacteria, monoglucosyl diacylglycerol is synthesized first from UDPglucose and diacylglycerol, and then, it is converted to MGDG, possibly by epimerization (Sato and Murata, 1982). This is also true in *Prochloron*, a cyanobacterium in which chlorophyll *b* is present (Murata and Sato, 1983). SQDG has been known to be specific to plastids and cyanobacteria, as well as to some photosynthetic bacteria, but *Gloeobacter violaceus* does not contain this sulfolipid (Selstam and Campbell, 1996). In addition, mutants lacking this lipid are known in various organisms (Sato *et al.*, 1995). In contrast, PG is known to be essential in photosynthesis (Sakurai *et al.*, 2003). A betaine lipid diacylglyceryl trimethylhomoserine (DGTS) is also found as a plastid constituent in many algae, bryophytes and ferns (Sato, 1992). DGTS is clearly a

component of plastids in *Chlamydomonas* and some algae, but it is not clear whether it is also found in lower plants.

Fatty acid composition was one of the important molecular markers used in phylogeny (Erwin, 1973). Acyl chain length, and the number and position of double bonds are unique to each taxon. A tremendous accumulation of fatty acid data existed already in the 1970's for each representative taxon. The unusual acid *trans*-Δ3-hexadecenoic acid is present at the *sn*-2 position of PG of plastids in all photosynthetic eukaryotes analyzed. Since this acid is not found in cyanobacteria, its origin is unknown. The glycerolipids in plastids contain $C_{16}$ and $C_{18}$ acids in general. Both may contain as many as three (or four, depending on organisms) double bonds, such as 16:1, 16:2, 16:3, 18:1, 18:2 and 18:3. The position and number of double bonds are different in different taxa and different lipid classes. In the galactolipids of plants, double bonds are inserted at Δ7, Δ10, and Δ13 in $C_{16}$ acids in this order, while they are inserted at Δ9, Δ12, and Δ15 in $C_{18}$ acids. In cyanobacteria, however, double bonds are inserted at Δ9 and Δ12 positions in both $C_{16}$ and $C_{18}$ acids. In red and chromophyte algae, $C_{18}$ acids are less abundant and $C_{20}$ and/or $C_{22}$ acids predominate, such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid (DHA). The $C_{20}$ acids are also common in lower green plants such as bryophytes and pteridophytes. The polyunsaturated fatty acids are therefore common in photosynthetic membranes, but their importance to photosynthesis is not clear. An *Arabidopsis* mutant almost devoid of 18:3 can grow photosynthetically, although the pollen development suffers from the lack of jasmonic acid, a product of 18:3 (McConn and Browse, 1996). The unicellular thermophilic species of red algae, such as *Cyanidioschyzon merolae*, also lack 18:3 (Moretti and Nazzaro, 1980). Many unicellular species of cyanobacteria such as *Synechococcus* lack all polyunsaturated acids (Kenyon, 1972). Therefore, the polyunsaturated acids are clearly not essential in photosynthesis, but may be important for the maintenance of membrane physical properties such as fluidity at low temperature (Routaboul *et al.*, 2000). We recently found that, in *C. merolae*, the origin of the three fatty acid desaturases, which are the only desaturases known in this alga, is not from endosymbiont but from eukaryotic host (Sato, 2003; Sato, in preparation). In contrast, the desaturases functioning in plastids of green plants originate from cyanobacterial endosymbiont. The red and green lineages are quite different in the composition and synthesis of fatty acids in plastids.

An interesting comparison of lipid biosynthesis in different taxa suggests there exists a "prokaryotic" and a "eukaryotic" pathway of plastid lipid synthesis (Roughan and Slack, 1982). Plastids are the major site of fatty acid synthesis in plants. Fatty acids are exported to ER, where glycerolipids such as phosphatidylcholine are synthesized. Then some part of the diacylglycerol moiety of phosphatidylcholine is reimported to plastids for the synthesis of MGDG. This is called the eukaryotic pathway, because the metabolism occurs mainly in the eukaryotic compartment of plant cell. In contrast, a significant fraction of plastid lipids is synthesized within the plastid envelope, and this is called prokaryotic (or plastid) pathway. The flow of the two pathways is about 1:1 in *Arabidopsis*, a 16:3 plant, which contains 16:3 acid in MGDG, while essentially all MGDG is synthesized via the eukaryotic pathway in pea, an 18:3 plant, which lacks 16:3 (Browse *et al.*, 1986). In green algae such as *Chlorella*, only the prokaryotic pathway is functional, while in *Chlamydomonas* and some marine green algae, in which phosphatidylcholine is totally replaced by DGTS, a role of DGTS synthesized in ER in the synthesis of MGDG was also suggested (for a review, see Harwood and Jones, 1989). Almost no data are available for the synthesis of lipids in lower green plants, but the prokaryotic pathway must be important in these organisms, because the 16:3 content is high. In *Cryptomonas* (a cryptophyte), no metabolic flow from phosphatidylcholine to MGDG was found, suggesting the lack of eukaryotic pathway (Sato, 1991). Although data are largely missing in red and chromophyte algae, the metabolism of lipids and fatty acids might be very different from those in green plants, and therefore, the role of plastids in lipid metabolism may also be different.

## *B. Evolution of Plastid and Nuclear Genomes*

### *1. Evolutionary Loss and Minimal Set of Plastid Genes*

Comparison of various plastid genomes reveals loss of genes during evolution. The number of protein genes encoded by the plastid genome according to the GenBank entry (including duplication and unidentified genes) ranges from 207 in a unicellular red alga *C. merolae* to 105 in a prasinophyte *Mesostigma viride* (Table 1). However, in non-photosynthetic plastids, the number of genes is further limited: 46 in a non-photosynthetic euglenophyte *Astasia longa*, 24 in a parasitic plant *Epifagus virginiana*, 30 in *Plasmodium*

*falciparum*, and 26 in *Toxoplasma gondii*. The difference in gene content between photosynthetic and non-photosynthetic plastids is primarily due to the genes involved in photosynthesis, such as photosystems, electron transport, ATP synthesis, and carbon fixation. In contrast, the genes found in the plastids of lower algae include genes involved in various biosynthetic pathways found within the plastid, such as the biosynthesis of lipids (acyl carrier protein, acetyl CoA carboxylase, condensing enzyme, etc.) and chlorophyll (*chlB, chlN, chlL, chlI* etc.), among others. The plastid genomes of red algae and glaucophytes also encode structural genes for phycobiliproteins. Some regulatory proteins and DNA-binding proteins are also encoded by the plastid genomes of red algae and chromophytes (III D 1).

In contrast, the smallest cyanobacterial genome known to date (*Prochlorococcus marinus* MED4) encodes 1,712 protein genes. It is clear that a drastic reduction in genome size occurred soon after the primary endosymbiosis. It is interesting to note that many parasitic bacteria have reduced genome sizes. For example, a human parasite *Mycoplasma genitalium* has only 480 protein genes within the 580 Mbp genome (L43967), and an endocellular symbiont of aphids *Buchnera* sp. APS has 564 protein genes within the 640 Mbp genome (BA000003) (Andersson, 2000). These bacteria lack many enzymes necessary for biosynthesis, and therefore, absolutely dependent on the host organism. However, they still retain their own genetic systems, such as replication, transcription and translation. Plastids are dependent on the eukaryotic host cell not only for metabolites but also for proteins. This is the fundamental difference between the parasitic bacteria and the organelles. In plastids, genes that are redundant with nuclear counterparts can be eliminated. In addition, many plastid proteins are now encoded by the nuclear genome and imported to plastids (III.C.1.b). The data on the loss of various genes have been used to estimate phylogenetic relationship among plastids (Martin *et al.*, 2002; Nozaki *et al.*, 2003b). However, many parallel losses of *infA* gene have also been known in various different lineages within angiosperms (Millen *et al.*, 2001).

## 2. Gene Transfer from Plastids to Nucleus

Two decades ago, the presence of a plastid DNA sequence in the mitochondrial genome was reported and named "promiscuous DNA" (Ellis, 1982; Stern and Lonsdale, 1982). Since then, many cases of transfer of a part of plastid genome to either the mitochondrial or nuclear genome have been reported (Thorsness and Weber, 1996; Cummings *et al.*, 2003). However, evidence is accumulating that gene transfer is more often and more extensive than we initially supposed it to be. The transfer of whole mitochondrial genome to the chromosome II of *Arabidopsis thaliana* was revealed as a result of genome sequencing project (Arabidopsis Genome Initiative, 2000). A similarly extensive transfer of plastid DNA to the nuclear chromosome was reported in rice (Yuan *et al.*, 2002; Shahmuradov *et al.*, 2003). Many insertions of plastid DNA containing intact protein coding sequences that cover 83% of plastid DNA were detected in the nuclear genome. In addition, some of the nuclear copies of plastid genes are expressed as evidenced by the presence of ESTs. In *A. thaliana*, a less extensive but still significant number of DNA fragments originating from the plastid genome were identified in the nuclear genome. These findings indicate that gene transfer from organelles to nucleus is not an exceptional event taking place very rarely in the history of plants, but is a rather frequent event that occurred many hundreds or thousands of times within the history of each plant species. The transfer might involve an RNA intermediate as originally suggested (Schuster and Brennicke, 1987), but a more extensive transfer involving a whole genome is also common. In the latter case, the synteny of genes is largely conserved in the remaining plastid DNA fragment within the chromosome (Shahmuradov *et al.*, 2003).

Recently, various approaches to detect the transfer of genes from plastids to nucleus have been tested. Huang *et al.* (2003) used a drug resistance gene inserted in plastid DNA as a marker to detect gene transfer to nucleus and found that the rate of gene transfer was as high as 1/16,000 in cultured cells. This rate was surprisingly high. Subsequently, a lower but still very high rate was reported in tobacco plants (Stegemann *et al.*, 2003). The high rate of gene transfer from plastids to nucleus is not very strange, because we already know that the DNA introduced into the plant cell, either by particle bombardment or by polyethylene glycol-mediated procedure, is effectively incorporated into the nucleus. It is also quite reasonable that chloroplasts may break within the cell at a certain frequency. Thorsness and Weber (1996) suggested that the leak of genetic material from organelles occurs at a rate equivalent to spontaneous mutation of nuclear genes, and is under genetic and environmental control. However, we still do not know how such transferred DNA is fixed within the nuclear genome and inherited for generations. It is still not clear yet how the transferred plastid gene acquired a promoter and a plastid-targeting signal.

All these recent findings convinced us that gene transfer from plastids (and mitochondria) to nucleus is rather a frequent phenomenon, and is part of massive gene flow within the eukaryotic cell.

## C. Continuity of the Plastid Lineages

### 1. Single Origin of Green and Red Lineages

#### a. Overview of the Three Lineages in Plastid Evolution

There are three major lineages of plastids: green, red and glaucophyte. *Cyanophora paradoxa* is a glaucophyte that contains a muroplast (formerly called cyanelle or cyanoplast, see Chapter 1) having peptidoglycan layer around the periphery, and this was taken as evidence that the muroplast is a remnant of endosymbiotic cyanobacterial cell (Aitken and Stanier, 1979). Sequencing of the muroplast genome clearly indicated that the muroplast is just a special type of plastid, and the muroplast DNA is very similar to the plastid DNA of various algae and plants (Stirewalt *et al.*, 1995). The green lineage includes plastids in green algae and land plants. In addition, the plastids of euglenophytes and chlorarachniophytes are thought to originate from secondary endosymbiogenesis by a hypothetical green algal cell (Hallick *et al.*, 1993; Marin *et al.*, 2003). The red lineage includes plastids in red algae and the chromophytes, various chlorophyll *c*-containing algae such as heterokonts (brown algae, diatoms, etc), cryptophytes, and haptophytes. The plastids of chromophytes are thought to originate from secondary endosymbiogenesis by a hypothetical red algal cell. The plastids of dinoflagellates might be polyphyletic, since analysis with PsbO protein sequences suggested that multiple tertiary endosymbiogenesis yielded divergent dinoflagellates (Ishida and Green, 2002). Among them, the single origin of peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis by a haptophyte has also been suggested (Yoon *et al.*, 2002a).

#### b. Single Origin of Red and Green Lineages Based on Protein Translocation Apparatus

The importance of protein translocation machinery (translocon) in determining the identity of an organelle is evident, because structure and function of an organelle are determined by the proteins imported into the organelle as well as the products of organellar genomes. Recent advances in the structural and functional elucidation of the translocon of plastids (for reviews see Jarvis and Soll, 2001; Chapter 3) suggest that some translocon components are of cyanobacterial origin. The outer membrane components such as Toc75, a translocation channel (Eckart *et al.*, 2002), and the inner membrane components such as Tic20 and Tic22 have homologues in cyanobacteria, and therefore, are believed to be products of endosymbiosis. Tic110 is unique to photosynthetic eukaryotes, and is an essential component of the transport machinery in the inner membrane. Cavalier-Smith (1982, see also 2003) argued that the presence of a common translocon would be strong evidence for the single origin of red, green and glaucophyte lineages. Later on, *in vitro* import experiments using heterologous combinations of transit peptide and plastid suggested that the translocon is essentially similar in the three major lineages (Cavalier-Smith, 2003). This is true for the targeting of red algal plastid proteins to plant chloroplasts (N. Sato, unpublished data in the author's laboratory among others) and the targeting of muroplast proteins to plant chloroplasts (Steiner and Löffelhardt, 2002). However, direct information about the translocon components in red lineages was missing until recently. The complete genome sequence of *C. merolae* indicates that many of the translocon components such as Tic20, Tic22, Tic110, Toc34 and Toc75 are present in the red alga (Matsuzaki *et al.*, 2004). Toc34, a GTP-binding protein, which is necessary for the recognition of transit peptide, has a similar domain within Toc159, but Toc159 is not present in *C. merolae*. The absence of Toc159 and Tic40 in the red alga suggests that some auxiliary components of translocon might be different. Despite such differences, the presence of essential common components of translocon is a strong evidence for the single origin of red and green lineages.

We also have evidence that the protein import into plastids is not as simple as it is currently understood (see Chapter 3). Some plastid proteins lack a "typical" transit peptide, but are indeed imported into chloroplasts both *in vitro* and *in vivo*. A well-documented example is the chloroplast envelope quinone oxidoreductase homologue (Miras *et al.*, 2002), which is targeted to the inner envelope membrane without N-terminal processing. Another is the PEND (plastid envelope DNA-binding) protein (Sato *et al.*, 1998), which has a very short (15 amino acid) presequence, but the targeting to the inner envelope membrane requires a longer N-terminal domain (see Sato *et al.*, 2003). The N-terminal region including the presequence is

not predicted to be chloroplast transit peptide by any prediction software, in other words, it is not similar to known transit peptides. These fragmentary examples might suggest that another translocation system is present. We still do not know if this is related to the recently identified Toc75 homologue (Eckart *et al.*, 2002). We also need information on the structure of translocon(s), especially that of glaucophytes.

### *c. Secondary Endosymbiogenesis and the Three Lineages*

Various lines of evidence support occurrence of secondary endosymbiogenesis (see Cavalier-Smith, 2003). First, the phylogenetic analysis of the SSU rRNA sequences of plastid and nuclear genomes showed that the plastid-encoded 16S rRNAs of red and chromophyte algae are monophyletic (Nelissen *et al.*, 1995). The nuclear-encoded 18S rRNA sequences, however, are divergent. This suggests that a red algal cell was captured within an unrelated host eukaryote cell and that only the plastids remained after subsequent degradation of the endosymbiont. Similarly, sequence comparison of 16S rRNA suggested relatedness of euglenophytes, chlorarachniophtytes and green algae. Second, there are extra membrane systems surrounding the plastids that are thought to originate from secondary endosymbiogenesis. Originally, the number of "chloroplast ER" was recognized as an important structural character in the classification of algae (see e.g. Table III in Dodge, 1973). There are two layers of endoplasmic reticulum that surround plastids in heterokonts. In the case of euglenophytes and dinoflagellates, only a single ER membrane surrounds the plastid. These chloroplast ER membranes are thought to represent remnants of surrounding membranes associated with endocytosis: one originates from the plasma membrane of the host, while another originates from the plasma membrane of the endosymbiont. Third, the nucleomorph, which is found in some algae such as cryptophytes and chlorarachniophytes is supposed to be the reduced nucleus of endosymbiont. The localization of the nucleomorph within a space between the plastid and chloroplast ER is consistent with this idea. Subsequently, this notion was verified by complete sequencing of the 551-kbp nucleomorph genome in *Guillardia theta* (cryptophyte) (Douglas *et al.*, 2001). The three chromosomes of the nucleomorph are linear with a telomeric region on both ends, encode histone and other eukaryotic proteins, and some genes contained eukaryotic introns. In addition, 18S rRNA as well as various tRNAs are also found to be encoded by the nucleomorph genome. Phylogenetic analysis of the nucleomorph 18S rRNA suggested its close similarity with the 18S rRNA of red algae (Douglas *et al.*, 1991).

It is clear that there were different events of secondary endosymbiogenesis, because the secondary endosymbiogenesis in the red and green lineages must have occurred independently. The origin of various chromophytes such as cryptophytes and heterokonts is still controversial. Accumulating sequence data of the nuclear genome allowed phylogenetic analysis of the origin of the host cells of these organisms. Recent molecular clock analysis suggested a single, ancient (1.26 Ga) origin of chromophytes (Chromista) (Yoon *et al.*, 2002b), but more data are needed to be conclusive.

### *d. Plastids in Non-Photosynthetic Organisms*

Various non-photosynthetic organisms also have plastids. The most easily recognizable non-photosynthetic plastids are those of parasitic plants such as *Epifagus* (Wolfe *et al.*, 1992). *Astasia* is a non-photosynthetic member of euglenophytes (Marin *et al.*, 2003). There are also non-photosynthetic members of dinoflagellates (Fast *et al.*, 2001; Yoon *et al.*, 2002a). In these cases, the phylogenetic relationship of photosynthetic and non-photosynthetic species is established by morphological and cytological characters, and therefore, the identity of plastids in non-photosynthetic organisms is irrefutable. However, we now know other non-photosynthetic organisms that have plastids, such as the malaria parasite *Plasmodium falciparum*, and related human parasites. These organisms have an organelle similar to plastids with respect to ultrastructural and cytochemical properties (see Chapter 24). The identity of these organelles was established by sequencing of the organellar DNA (Wilson *et al.*, 1996), which contains genes for rRNAs and tRNAs, as well as genes for prokaryotic RNA polymerase and ribosomal proteins. All these genes are similar to known plastid genes, and hence the organelles in *Plasmodium* and *Toxoplasma* were identified as plastids. In addition, various nuclear-encoded proteins that are targeted to plastids, such as those involved in fatty acid and isoprenoid biosynthesis, were discovered (Wilson, 2002). Such cellular function is equivalent to the functions of plastids in plants and algae. These biosynthetic enzymes as well as plastid RNA polymerase are targets of drug design (Fichera and Roos, 1997).

Phylogenetic analysis of plastid and nuclear genes suggested that the malaria parasites and related

organisms cluster with red algae and chromophytes. Although sequence-based phylogeny gave variable results, the affinity with red lineage is estimated by the fact that the *str* operon is translocated in the ribosomal gene cluster of plastid genome as in the plastids of red lineage (Ohta *et al.*, 1997). Recent phylogenetic analysis using nuclear genes established that alveolates including malaria parasites are similar to chromophytes, and the large group chromalveolates was proposed (for explanation, see Cavalier-Smith, 2003). Alveolates are considered to have diverged from chromophytes after acquiring red algal plastids by secondary endosymbiosis. Supporting evidence was provided by the nuclear-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase, or GAPDH (Fast *et al.*, 2001). The plastid-targeted GAPDH enzymes diverged from the cytosolic ones, and both isozymes are present in apicomplexans, dinoflagellates, heterokonts and cryptomonads. The plastid-targeted and cytoplasmic GAPDHs diverged before the diversification of these organism groups. In red algae, green algae and plants, the plastid-targeted GAPDH of cyanobacterial origin are present. Such data favors single origin of "chromalveolates". However, evidence for green origin of apicoplasts is also presented based on unusual "split *cox2* gene", which is encoded by the nuclear genome and targeted to mitochondria (Funes *et al.*, 2002). The two data sets can be reconciled by assuming multiple endosymbiosis, once of a green alga and once of a red alga (see comments in Palmer, 2003). In this respect, the "extended Plantae hypothesis" arguing that all organisms that gained plastids by secondary endosymbiosis originally had plastids and lost them (III C 2, Nozaki *et al.*, 2003a) may also explain the apparent discrepancy. We should also examine the GAPDH data in more detail, because *Arabidopsis* encodes two copies each of additional cytoplasmic and putative plastid-targeted GAPDHs of eukaryotic origin (see supplementary figure, Matsuzaki *et al.*, 2004).

### *e. Additional Evidence for the Single Origin of Red and Green Lineages*

A line of evidence that is not based on sequence data comes from analysis of complex origin of Calvin-Benson cycle enzymes in photosynthetic eukaryotes. In 1997, Martin and Schnarrenberger published a review article showing that the Calvin-Benson cycle enzymes have different origins: Rubisco, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, transketolase, ribulose-5-phosphate 3-epimerase, and phosphoribulokinase originate from cyanobacterial endosymbiont, whereas triosephosphate isomerase, fructose-1, 6-*bis*phosphate aldolase, fructose-1, 6-*bis*phosphatase, sedoheptulose-1, 7-*bis*phosphatase, and ribose-5-phosphate isomerase originate from eukaryotic host or mitochondria. We recently re-examined this issue with complete genome data of *A. thaliana* (green plant) as well as of *Cyanidioschyzon merolae* (red alga). The dual origin of Calvin-Benson cycle enzymes is totally conserved in both organisms (Matsuzaki *et al.*, 2004), except for red algal Rubisco, which has been known to originate from $\alpha$-proteobacteria by HGT (as described in II.B.2). This is a clear indication that such chimeric origin of Calvin-Benson cycle enzymes had been established early after the primary endosymbiosis, and the complexity was maintained all through the plant and algal evolution after the separation of red and green lineages. In other words, this supports the single origin of red and green lineages.

### *f. Minicircles in Dinoflagellates*

Dinoflagellates are highly divergent photosynthetic organisms with non-photosynthetic relatives sporozoa, which also have a plastid. Both are members of alveolates (see Cavalier-Smith, 2003). Their plastids are thought to originate from secondary (from a red alga) or tertiary (from a haptophyte) endosymbiogenesis according to the phylogenetic analysis with PsbO (Ishida and Green, 2002). The plastid genome of the dinoflagellate *Heterocapsa triquetra* was found to split into minicircles each harboring a single gene (Zhang *et al.*, 1999). More recent analysis indicated that unigene minicircles are common in various photosynthetic dinoflagellates (Zhang *et al.*, 2002), and the minicircular plastid DNA was probably established early in the evolution of dinoflagellates. However, the mechanism of such transition in plastid DNA organization remains speculative.

### *g. Arguments Against Single Origin of Plastids*

Although many data support a single origin of plastids, convergent evolution was also suspected (Stiller *et al.*, 2003). These authors analyzed genome content of plastids and mitochondria, and showed that similar sets of ribosomal protein genes and tRNA genes are conserved in the mitochondrial genome of *Reclinomonas* and various plastid genomes, notably the plastid genome of *Marchantia* (liverwort). They argued that these arose by parallel loss of similar sets of genes. However, this

analysis involves gene content, but not sequence similarity. This argument does not decrease the value of sequence-based phylogeny. Nevertheless, we will see some examples of convergent evolution of mitochondria and plastids later in this article (III.E), which is a new aspect of plastid evolution.

### *2. New Notion of Plants: Are the Eukaryotic Hosts in the Red and Green Lineages Monophyletic?*

The arguments described above strongly suggest that plastids in the red and green lineages are of a single origin. However, does this mean that the organisms in the two lineages are monophyletic within the tree of life? Accumulating genomic data of various plants and algae of red and green lineages allow us to construct phylogenetic trees based on various nuclear genes. Until recently, the data on the red algae were incomplete (Baldauf *et al.*, 2000), or the nucleomorph data were used in place of red algal data (Moreira *et al.*, 2000). However, using the complete genome data of *C. merolae*, Nozaki *et al.* (2003a) presented a phylogenetic relationship of all eukaryotes based on concatenated sequences of α- and β-tubulins, actin, and elongation factor 1a. The results indicate that the plants and algae in the green and red lineages are not monophyletic in the sense that many other phyla also clustered with these lineages. In their analysis, all eukaryotes are divided into three major clades, Group A (Metazoa and Fungi: Opisthokonta), Group B (green plants and algae, red algae, Glaucophyta, Heterokontophyta, Apicomplexa, Kinetoplastida, Euglenophyta, etc: Plantae), and Group C (Amoebozoa). The authors consider various non-photosynthetic phyla within Group B might have experienced primary cyanobacterial endosymbiosis, but later lost plastids (Kinetoplastida, Heterolobosea, and Ciliophora). Then, secondary endosymbiosis resulted in photosynthetic organisms again in Heterokontophyta and Euglenophyta. Apicomplexa has lost the ability of photosynthesis but still retained the secondary plastids. This new notion of Plantae (in Latin, or "plants" in English) clearly explains the single origin of plastids as well as the multiple secondary endosymbiogenesis of plastids. An essentially new idea is that the secondary endosymbiosis was only possible in host organisms that had already lost primary plastids. This is reasonable, because many genes encoding plastid proteins had been present before the secondary endosymbiosis, which is clearly easier than the primary endosymbiosis.

### *3. Novel Conserved Photosynthesis-Related Proteins*

Comparison of various completely sequenced genomes is useful in finding genes that are common in a defined clade of organisms, but can also shed light on the genes that are transferred from one clade to another by HGT. In most cases, HGT is sporadic, but in the case of endosymbiogenesis, a large number of genes are transferred from endosymbiont to the new organism. Based on this idea, attempts have been made to find genes that are transferred from cyanobacterial endosymbiont to photosynthetic eukaryotes. Originally, the *ycf* genes were defined as conserved genes in plastid and cyanobacterial genomes. But some *ycf* genes are encoded by nuclear genomes, depending on organism. Therefore, the notion of *ycf* gene should be extended to include genes that are conserved in the nuclear and plastid genomes of photosynthetic eukaryotes and cyanobacterial genomes. Abdallah *et al.* (2000) compared the genomes of *A. thaliana* (nucleus and plastid), *Saccharomyces cerevisiae* and *Synechocystis* sp. PCC 6803 and estimated that between 650 and 900 chloroplast proteins and between 500 and 650 non-chloroplast proteins of *A. thaliana* originated from cyanobacterial endosymbiont. In a more extended analysis including *A. thaliana* and 20 reference genomes, Martin *et al.* (2002) selected *A. thaliana* proteins that are more similar to cyanobacterial orthologues than to orthologues in other non-photosynthetic organisms. This fraction was about 18% or 14%, depending on bootstrap significance level, of the total proteins in *A. thaliana*, and they estimated about 4,500 or 3,500 proteins, respectively, in *A. thaliana* might be of cyanobacterial origin. Among the 3,628 proteins analyzed, more than a half are not targeted to plastids, and the functional categories of these proteins encompass various categories. Therefore, there is no doubt that a large number of plant proteins originate from cyanobacteria. However, it is not clear whether the genes for these proteins were acquired by the plants by sporadic HGT, or by HGT before the endosymbiosis, or by endosymbiogenesis. It is also not clear either how many cyanobacteria share these plant proteins of putative cyanobacterial origin. In most analyses, *Synechocystis* has been used as a reference cyanobacterium, but this is clearly not the representative of all cyanobacteria. Another important point is that the number of genes estimated by these studies is the number of *A. thaliana* genes, and the actual number of cyanobacterial genes that are transferred to all photosynthetic eukaryotes remains unknown.

Another comprehensive approach is to compare all the protein sets in selected organisms. The Gclust project (Sato, 2002, 2003a) compares total protein sets of eight cyanobacteria, three photosynthetic bacteria, two non-photosynthetic bacteria, two non-photosynthetic eukaryotes, and two photosynthetic eukaryotes. Homologue groups were estimated by recurrent clustering with increasing BLASTP E-values as thresholds. The use of homologue groups removes the risk of counting various homologues (paralogues) that might originate from a single gene. A drawback of this approach is the difficulty (at least in the current version of the software) of handling multidomain proteins, which are supposed to originate from domain fusion or domain shuffling. Based on such clustering, 69 homologue groups are found shared by all cyanobacteria and photosynthetic eukaryotes analyzed, but not by the other taxa included in the study. Twenty-five of them include genes that are known to function in photosynthesis or biogenesis of plastids. The remaining 44 homologue groups include genes that have no clear annotation and therefore represent putative plastid proteins of endosymbiont origin. Interestingly, most of these genes in photosynthetic eukaryotes (*A. thaliana* and *C. merolae*) have a putative transit peptide for plastid targeting. A functional genomic project to identify the function of these genes is in progress in the author's laboratory, and will identify many novel photosynthesis-related genes in plants.

## *D. Discontinuity and Complex Origin of Plastid Genomic Machinery*

### *1. Eukaryotic and Prokaryotic Components of Plastid Nucleoids*

The nucleoid, which consists of various proteins and RNA as well as multiple copies of plastid DNA, represents the functional unit of the plastid genome. Isolated nucleoids have been prepared by solubilizing the membranes and then sedimentation by high-speed centrifugation. The purity of the nucleoids prepared by such procedure remains low, and many hydrophobic proteins are associated with the preparation. Therefore, proteomic study has been difficult. Recently, the major nucleoid protein of plastids of pea, tobacco and soybean was identified as an enzyme sulfite reductase (SiR) (Sato *et al.*, 2001). The plant type SiR catalyzes the reduction of sulfite to sulfide using ferredoxin as an electron donor, and this reaction is essential in the assimilation of sulfate. SiR is also a major component of plastid nucleoids of a moss *Physcomitrella patens* (Sato *et al.*, 2003, 2004). A purified recombinant SiR binds to plastid DNA without sequence specificity, and forms microscopically identifiable particles that look similar to nucleoids. It reversibly tightens the compaction of plastid nucleoids and reduces the transcription activity of nucleoids (Sekine *et al.*, 2002). A small amount of SiR is also found attached to the plastid nucleoids of a red alga *C. merolae* and in the nucleoids of a cyanobacterium *Anabaena variabilis* M3 (Miyajima *et al.*, 2004), but in these nucleoids, HU is the major DNA-binding protein (see below). Hence, extensive binding of SiR to plastid DNA was achieved during the evolution of green plants or land plants with the loss of HU. We still need data of green algal nucleoids.

In the nucleoids of red algae and cyanobacteria, a prokaryotic DNA-binding protein called HU is present and tightly binds to DNA. The HU protein has been extensively studied in *Escherichia coli*, and is a major DNA-binding protein in exponentially growing cells. In the *E. coli* nucleoids, 12 proteins were identified, such as HU/IHF (two copies each), Fis, and Dps. Dps is known to accumulate in the stationary phase, when HU is no longer present at high levels. Dps is found in cyanobacteria, but Dps and Fis are not found encoded in the nuclear or plastid genomes of any algae and plants. HU is also encoded by various prokaryotic genomes including cyanobacteria. A homologue of HU, called HU-like protein or HlpA, is encoded by the plastid genomes of *Guillardia theta* (cryptophyte: formerly, *Cryptomonas)* (Wu and Liu, 1997; Grasser *et al.*, 1997) and *C. merolae* (Kobayashi *et al.*, 2002). We call all these homologues "HU" for simplicity in this article, but this does not necessarily mean that the physiological function of HU is identical in different organisms. HU is believed to be present in the plastids of the red lineage. Indeed, EST clones or genomic sequences encoding HU have been reported in apicomplexans *Toxoplasma* (W06256) and *Plasmodium* (mal IX 154g10), and a diatom *Thalassiosira pseudonana* (Joint Genome Institute web site, many overlapping contigs such as PQI43382). HU protein is neither encoded in the plastid genomes of green lineage, nor in the nuclear genomes of *A. thaliana*, rice, moss, and *C. merolae*. Curiously, an HU homologue is encoded in the nuclear genome of a green alga *Chlamydomonas reinhardtii*. This HU is distant from other algal HU, and is likely to be targeted to mitochondria (T. Takayama and N. Sato, unpublished data). This gene is likely to be acquired by HGT. Except for this, HU is not encoded in any sequenced genomes

of plants or green algae, and therefore, HU is not present in the plastids of the green lineage, as originally proposed by Sato (2001). Such comparison clearly indicates that HU is present in the plastids of the red lineage.

As described above, HU was replaced by SiR as a major DNA-binding protein in the plastid nucleoids during the evolution of green lineage. However, the exact role of the two proteins might be different. HU is necessary for the optimal activity of transcription, while SiR always inhibits transcription activity. The HU-DNA complex is bulky and the quenching of DAPI fluorescence is negligible, whereas SiR-DNA complex is highly condensed and induces strong quenching of DAPI fluorescence due to compaction. Hence, HU is a modulator of nucleoid activity as in *E. coli*, while SiR is a general repressor in plant plastids (Sato *et al.*, 2003).

## 2. Discontinuous Evolution of Plastid Genomic Machinery

The plastid genetic system had long been considered to be similar to that of prokaryotes, notably of cyanobacteria. But the radical difference in the major DNA-binding proteins in the nucleoids of various different plastids and prokaryotes suggests that the evolution of the genetic system of plastid is rather discontinuous. Here I use the word "genomic machinery" to designate the entire machinery that is associated with the plastid genome and supports the function of the plastid genome, such as the machinery for replication, transcription, as well as structural and regulatory DNA-binding proteins. Nucleoids may represent a structural entity of genomic machinery. Figure 4 illustrates current hypotheses on the discontinuous evolution of plastid genomic machinery.

### a. Replication Machinery

The components of the replication machinery of plastids are under heavy investigation. DNA polymerase, helicase and primase have been partially purified from plant plastids, but the molecular nature (DNA or protein sequences) remained obscure. Plastid genome replication is known to proceed by both rolling circle and D-loop mechanisms (Kollodner and Tewari, 1975). The replication origin was mapped near the rDNA region in many plants (Mühlbauer *et al.*, 2002), but no consensus sequence for the plastid *ori* was deduced. The characterization of replication activity using the nucleoid preparation of tobacco (Sakai, 2001) established that the nucleoids contain a DNA polymerase similar to bacterial DNA polymerase I (PolI). Interestingly, the mitochondrial DNA polymerase is also similar to the plastid polymerase with respect to apparent molecular mass and conditions for optimal activity (Sakai, 2001). Genomic sequence of *Arabidopsis* (Arabidopsis Genome Initiative, 2000) suggests that there are two genes that may encode PolI-like proteins. A rice cDNA encoding a PolI-like protein (about 100 kDa) was characterized (Kimura *et al.*, 2002). The protein was found to be enriched in the plastid fraction, but exact localization of the protein has not been established. A 70-kDa DNA polymerase prepared from pea chloroplasts was also similar to PolI, but its template specificity was different from that of rice polymerase (Gaikwad *et al.*, 2002). The PolI-like enzymes found in the plants lack 5′-> 3′ exonuclease domain, and therefore, correspond to the so-called "Klenow fragment" of PolI. A similar enzyme was found to be encoded by the nuclear genome of *C. merolae* (Matsuzaki *et al.*, 2004), and the characterization is being performed in our laboratory (see Moriyama *et al.*, 2003; Sato *et al.*, 2003). Hence, available evidence suggests that the replication of plastid genome is performed by a Klenow-like enzyme. Primase, helicase and single-stranded DNA-binding protein (SSB), which are all necessary for replication, are also found to be encoded by the nuclear genome of *A. thaliana* and *C. merolae*, except that helicase (DnaB) is encoded by the plastid genome in *C. merolae* and other red and chromophyte algae. DNA polymerase III, a highly processive enzyme (namely, a polymerase that synthesizes very efficiently a long DNA strand during a single passage over the template) involved in bacterial replication, and DnaA protein, which is necessary for bacterial replication initiation, are not encoded by any genomes of plants and algae (Sato, 2001).

These new findings suggest that the mechanism and machinery for the replication of plastid genome are fundamentally different from those of cyanobacteria. Hence, the replication system of endosymbiont must have been replaced by the one offered by the host cell at the initial phase of endosymbiogenesis (Fig. 4, Phase 1). The most probable source of such replication system present in the host cell was the mitochondrial replication system encoded by the nucleus. This explains the similarity of plastid and mitochondrial DNA polymerases. It should be noted that the $\gamma$-type DNA polymerase, known as the mitochondrial replicase in fungi and animals, is not present in plants (Fliée *et al.*, 2002).

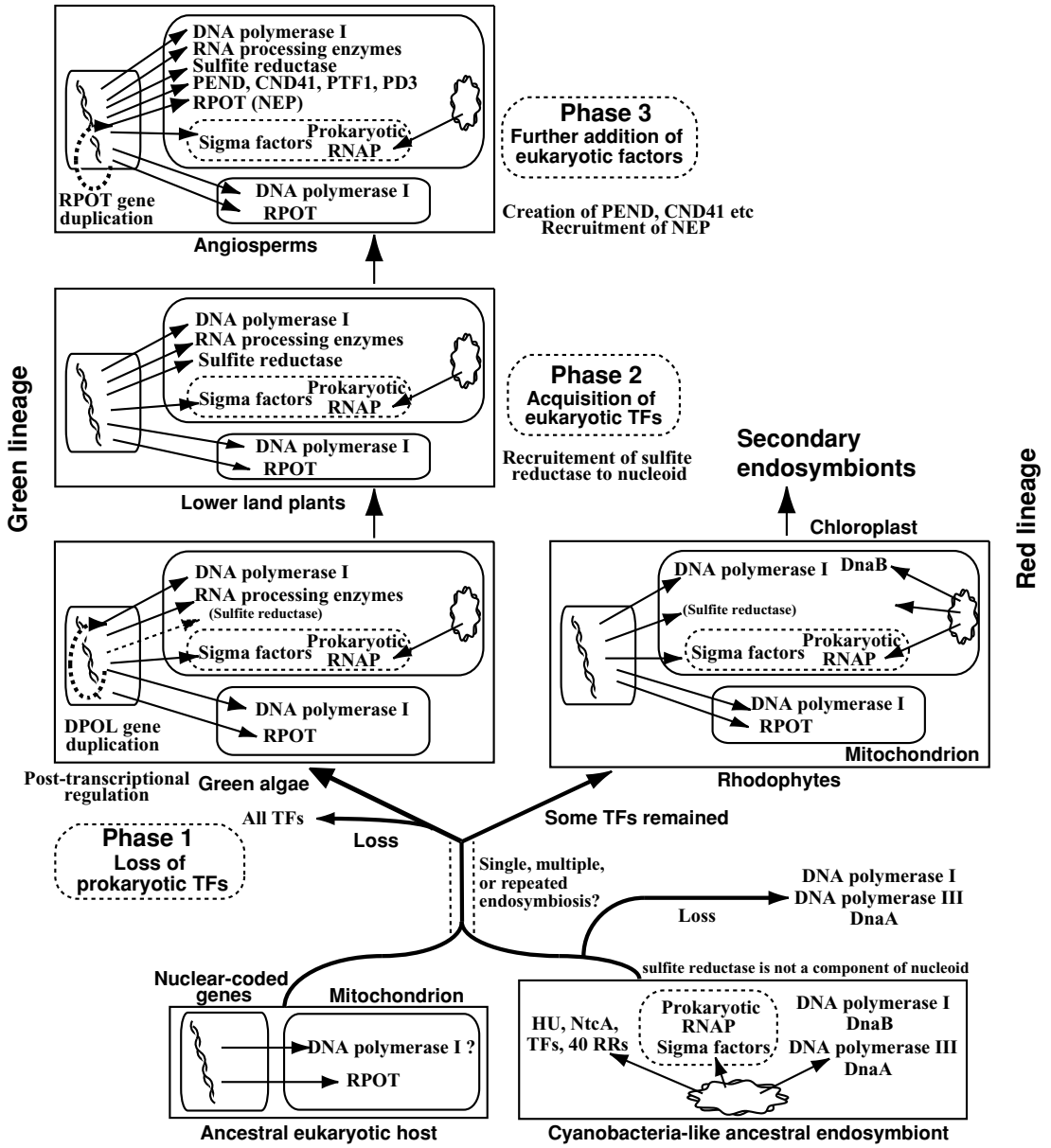



*Fig. 4.* Hypothesis on the discontinuous evolution of plastid genomic machinery. After the initial endosymbiosis by a cyanobacterial endosymbiont into an ancestral eukaryotic host, the replication system was rapidly replaced by the existing mitochondrial system. At the same time, many of the transcription factors and DNA-binding proteins were lost, except some that are still encoded by the plastid genome of red lineage. This is Phase 1. In Phase 2, green algae acquired some eukaryotic factors for the regulation of plastid genome to become land plants, such as sulfite reductase that compacts plastid nucleoids and represses transcription and replication activities. In the evolution of angiosperms (flowering plants), novel DNA-binding proteins such as PEND, CND41 and PTF1 were created from eukaryotic proteins. In addition, mitochondrial RNA polymerase was recruited to plastids (NEP) mainly for the transcription at the beginning of plastid development (Phase 3). For details, see text, as well as Sato (2001) and Sato *et al.* (2003). This figure was reproduced and modified from Fig. 7 of Sato *et al.* (2003) by permission.

The origin of the PolI-type polymerase of plants and algae has not been identified, because phylogenetic analysis suggested that they are not similar to cyanobacterial or α-proteobacterial PolI (Sato *et al.*, 2003). In contrast, the fact that similar DNA polymerases are present in both *A. thaliana* and *C. merolae* again supports the single origin of green and red lineages of plastids.

### b. RNA-Polymerases

The presence of plastid-encoded and nuclear-encoded RNA polymerases (called PEP and NEP, respectively) in angiosperm plastids is well documented (Maliga, 1998; Sato *et al.*, 2003). The PEP, consisting of α, β, β′, β″ and σ subunits, is similar to cyanobacterial RNA polymerase (Fig. 4). The former four subunits are encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes, respectively, in the plastid genome. The sigma subunit is encoded by the nuclear genome (Tanaka *et al.*, 1997). The β′ and β″ subunits correspond to the N- and C-terminal halves, respectively, of the β′ subunit of RNA polymerase (encoded by the *rpoC* gene) in *Escherichia coli*, and this splitting (at the levels of both protein and gene) characterizes the similarity of cyanobacterial and plastid RNA polymerases. However, in a red alga, *Cyanidioschyzon merolae*, a single gene encoding a fusion polypeptide including the β′ and β″ parts is present in the plastid genome. This is likely to be a result of fusion, but not a result of HGT from a bacterial *rpoC* gene, because phylogenetic analysis using *rpoC1* part also supports relatedness of *C. merolae* and other red algae, and the fusion region is significantly shorter than the corresponding region in bacterial β′ sequences. The details of plastid transcription are described in Chapter 8. Here, I only point out that the *rpoA* gene is transferred to the nucleus in a moss *Physcomitrella patens* (Sugiura *et al.*, 2003). In a parasitic plant *Epifagus*, none of the PEP subunits are encoded by the plastid genome (Wolfe *et al.*, 1992).

The NEP is a single-polypeptide enzyme similar to the one in bacteriophages T3, T7 and SP6 (Hedtke *et al.*, 1997). A similar enzyme (product of a nuclear gene *RpoT*) is present in the mitochondria of all eukaryotic organisms analyzed, including fungi, animals and plants, except possibly in a primitive protist *Reclinomonas americana*, which possesses mitochondrially encoded bacteria-type RNA polymerase. NEP is known in various angiosperms, but this type of enzyme is not reported in gymnosperms, lower plants and algae. Detailed phylogenetic studies indicated that NEP was created by gene duplication during the evolution of angiosperms (Kabeya *et al.*, 2002). This is consistent with the organization of promoter region of NEP-dependent genes, such as *atpB* and *accD*. In lower plants and algae, the 5′ upstream regions of these genes are different from the 5′ regions of angiosperm genes. There are two copies of *RpoT* genes (*RpoT1* and *RpoT2*) in a moss *P. patens*, and both have two potential initiator methionines. The protein product translated from the 5′-most methionine is targeted to chloroplasts, while the translation product from the second methionine is targeted to mitochondria (Richter *et al.*, 2002; Sato *et al.*, 2003). Identical results were obtained for both RpoT1 and RpoT2. However, RpoT proteins are not detected in the chloroplasts of *P. patens*. Absence of NEP in chloroplasts is confirmed by the complete sensitivity of transcription to tagetitoxin in chloroplasts (Kabeya *et al.*, 2004). A plausible explanation is that the 5′-most methionine is not recognized as the initiation codon *in vivo*, namely, in the natural context with the native 5′ upstream sequence of mRNA (Sato *et al.*, 2003; Kabeya and Sato, 2005). Detailed discussion on this issue will be published elsewhere, but we should be cautious about possible artifacts of experiments using GFP. Here, it is sufficient to note that gene duplication of the *RpoT* gene occurred several times during the evolution of plants, and also the plastid targeting sequence was added to RpoT proteins several times independently. In mosses, RpoT genes are duplicated and both copies possess a potential plastid targeting sequence (Kabeya *et al.*, 2002). However, actual plastid targeting was achieved only in angiosperms, in which a duplicated RpoT with a plastid targeting sequence lost the original methionine that directs synthesis of a mitochondrially-targeted polypeptide (Fig. 4, Phase 3). This example illustrates the complexity of evolutionary history of a gene against our naïve assumption of minimal evolution. Actual gene evolution occurs only rarely starting from a particular event among many similar events that occurred many times.

### c. DNA-Binding Proteins

The expression of plastid genes is regulated at both transcriptional and post-transcriptional levels. Various DNA-binding proteins are known to regulate transcription of plastid genes, such as CDF2 that binds to the upstream region of the rRNA operon (Bligny *et al.*, 2000), and PTF1 that binds to the AAG box located within the light-responsive *psbDC* promoter (Baba *et al.*, 2001). The PEND protein is a DNA-binding protein present in the envelope membrane of developing pea chloroplasts (Sato *et al.*, 1998). The MFP1, originally thought to be a nuclear protein, was found to be a thylakoid-associated, nucleoid-binding protein (Jeong *et al.*, 2003). All these proteins are considered to be of eukaryotic origin (Sato, 2001; Sato *et al.*, 2003). On the other hand, prokaryotic regulators, such as OmpR homologues (Ycf27 and Ycf29), RbcR (Ycf30) and NtcA (Ycf28), which are encoded by the plastid genomes of red lineage, are not

present in the plastids of green lineage, since none of them are encoded by the plastid or nuclear genomes (including ESTs) of the green lineage sequenced to date (Sato, 2001; Sato *et al.*, 2003; see Fig. 4). Such differences mark a clear contrast between the plastids of green lineage and the plastids of red lineage, as well as contrast between plastids and cyanobacteria.

### *E. Co-evolution of Mitochondria and Plastids*

Plastids and mitochondria are both present in a single algal or plant cell. Accumulating evidence suggests that similar proteins are involved in various functions of the two organelles. In the case of enzymes of carbon metabolism such as lipoamide dehydrogenase subunit of pyruvate dehydrogenase and glycine dehydrogenase, different isoforms originating from eukaryotic host (or α-proteobacterial endosymbiont) and cyanobacterial endosymbiont, respectively, are located in mitochondria and plastids (Lutziger and Oliver, 2000). This is not co-evolution but parallel endosymbiotic evolution. In other cases, such as division and replication, identical proteins, or proteins of single origins, function in the two compartments. The similarity of DNA polymerases in plastids and mitochondria is described above. There are different forms of RNA polymerases (RpoTs) that are targeted to plastids and mitochondria, respectively. The similarity of division mechanism, especially involvement of dynamin-like proteins (Miyagishima *et al.*, 2003; Osteryoung and Nunnari, 2003) has been documented (see Chapter 5). Recently, a homologue of Toc64, a chloroplast translocon component, was found to be present in the mitochondrial outer membrane (Chew *et al.*, 2004). These are examples of different isoforms present in the two organelles. Such isoforms are closely related and of a single origin. Some may be of plastid origin (e.g. Toc64), while some are of mitochondrial origin (DNA polymerase, RpoT). However, various proteins are known to be targeted to both mitochondria and plastids (for a review, see Peeters and Small, 2001). Some may be dual targeting because of ambiguous signal, while some possesses two distinct signals. Aminoacyl transferases are examples of dual (or triple) targeting enzymes. In fact, the total number of genes encoding this family of crucial enzymes essential in loading amino acids onto tRNA is rather limited (40 in *C. merolae* and 42 in *A. thaliana*, but 36 in budding yeast and 35 in fruit fly). These data suggest that mitochondria and plastids share some important proteins, either resulting from dual targeting, or from gene duplication. The co-evolution of mitochondria and plastids has not been discussed as a major principle of organellar evolution, probably because the cross-contamination of plastid and mitochondrial proteins that has not been resolved by biochemical analysis, made it difficult to assess correct localization of organellar proteins. However, molecular data are now becoming available with complete genomic sequences that should make a full assessment possible. In addition, phylogenetic genomics is a powerful tool that can be used to trace evolutionary history of proteins present in both mitochondria and plastids. We are therefore ready to study co-evolution of the two organelles.

## IV. Conclusion: Opposing Principles in the Evolution of Plastids

The notion "plastid" is a major principle that unifies the diversity of various plastids present in various types of cells in diverse photosynthetic eukaryotes. Endosymbiogenesis is another potent principle that ensures the continuity of all plastids and cyanobacteria. However, we still do not know exactly what the progenitor of plastids looked like or how the three plastid lineages diverged from the initial photosynthetic eukaryotes. The occurrence of secondary and tertiary endosymbiogenesis is becoming supported with higher confidence by comparative genomic studies. Comparative genomics with expanding genomic data are providing clues to resolve problems that had previously been difficult to attack, such as comparison of cyanobacteria and plastids, and of plastids of different lineages. One of the consequences of such comparative genomics is recognition of fundamental differences in plastid genomic machinery in different lineages and differences in genomic machinery of plastids and cyanobacteria. The photosynthetic apparatus is highly conserved in different plastids and cyanobacteria, but, with respect to other functions, plastids were diversified during evolution, either as organelles of different functions in various algae, or as plastids having potentials of differentiation in flowering plants. The plastids of apicomplexans represent a special case, in which plastids lost photosynthetic functions but retain biosynthetic capabilities. In other cases such as *Trypanosoma* (Hannaert *et al.*, 2003) in which plastids were lost as organelles, some biosynthetic genes remain encoded by the nuclear genome and continue to function as a memory of presence of plastids. Opposing principles are continuously unifying and diversifying the plastids to provide the Earth with rich photosynthetic products.

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Note Added in Proof: As a consequence of recent development of Bayesian inference of phylogeny, I reanalyzed the phylogeny of plastids and cyanobacteria (Fig. 2b) in 2005. The results of analysis of both rRNA and protein-coding DNA sequences became consistent and suggested that the plastids diverged from the *Synechocystis-Anabaena* clade of cyanobacteria (N. Sato, unpublished results). This is illustrated in the inset of Fig. 3 as an alternative.

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# Chapter 5

# The Mechanism of Plastid Division: The Structure and Origin of The Plastid Division Apparatus

Shin-ya Miyagishima
*Department of Plant Biology, Michigan State University, East Lansing, MI 48824, U.S.A.*

Tsuneyoshi Kuroiwa*
*Department of Life Science, College of Science, Rikkyo (St. Paul's) University, 3-34-1 Nishi-ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*

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*Author for correspondence, email: tsune@rikkyo.ne.jp

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## Summary

Chloroplasts were derived from a free-living cyanobacterium which was engulfed by a primary non-photosynthetic eukaryotic host cell and subsequently evolved into a plastid. Plastids are never synthesized *de novo* therefore, as with bacteria, their continuity is maintained by the division of preexisting plastids. Although plastids have their own genome, plastid division is performed by host cell nuclear-encoded proteins. Consistent with their bacterial origin, plastids use the bacterial FtsZ ring and related factors, the genes of which were transferred to the host eukaryotic nucleus over evolutionary time. Recent genome sequencing projects show that most other proteins once involved in bacterial division have been lost. It was recently suggested that another ring structure called the plastid-dividing ring, which was found before FtsZ, is of host eukaryotic origin. Moreover, recent studies have revealed that the rings of the eukaryote-specific dynamin-related family of GTPases are involved in the final stage of plastid division. These results suggest that plastid division is mediated by the coordinated action of a prokaryote-derived division system and a system added by the host eukaryotic cell. During plastid division, the FtsZ, plastid-dividing (PD) and dynamin rings form in this order. The PD ring is a double (or triple) ring structure located both inside and outside the plastid envelope, the FtsZ ring forms in the stroma and dynamin functions at the cytosolic side of the division site. Recent studies also showed that primitive mitochondria use mechanisms very similar to those of plastids, suggesting that the host cell used almost the same strategy to regulate the division of the cyanobacterial endosymbiont as it did for mitochondria.

## I. Introduction

Plastids are of fundamental importance in the living world, and life on Earth is maintained largely by the energy trapped in plastids (chloroplasts). In addition, certain vitamins and essential fatty acids are also derived from plastids. Since their establishment in eukaryotic cells about two billion years ago (see Chapter 4), plastid continuity has been maintained by the multiplication of existing plastids. Consequently, investigations as to how plastids arose and how they reproduce are vital for understanding plant cells.

It is widely believed that plastids and mitochondria arose from bacterial endosymbionts related to cyanobacteria and α-proteobacteria, respectively (Margulis, 1970; Gray, 1992, 1999; Cavalier-Smith, 2000; McFadden, 2001; Chapter 4; Fig. 1), and although most of their genes have been lost or transferred to the host nuclear genome, they still have several features similar to bacteria. Both organelles contain nucleoids and ribosomes, and neither are synthesized *de novo*. Instead, their continuity is maintained by the division of pre-existing organelles and the distribution of daughter organelles to daughter cells during cytokinesis (Boffey and Lloyd, 1988).

Although the concept of plastid continuity by the division of pre-existing plastids was first put forward by Schimper (1885) on the basis of morphological

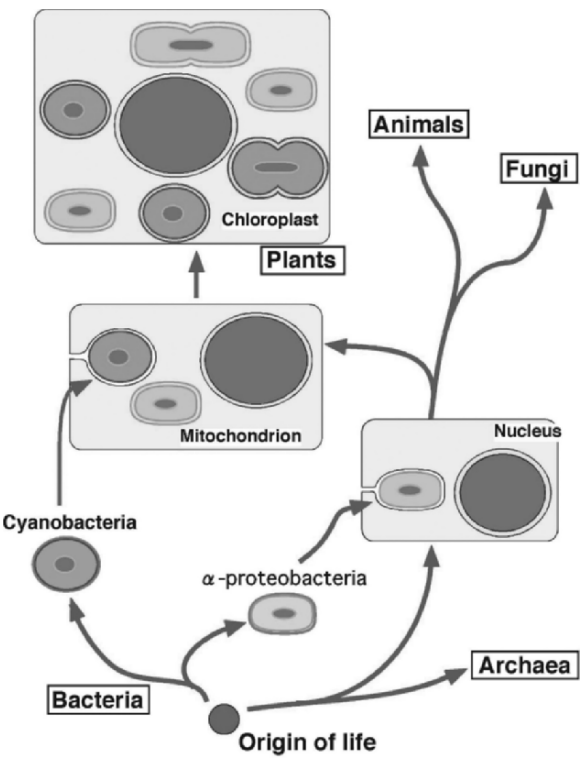


*Fig. 1.* (See also Color Plate 2, p. xxxv.) Mitochondria and plastids are the descendants of serial endosymbiotic events. Mitochondria arose first from an α-proteobacterial ancestor that was acquired by primitive eukaryotic host evolved from archea. Chloroplasts later arose from a cyanobacterial ancestor acquired by a eukaryote in which mitochondria were already established.

*Abbreviations:* FtsZ – Filamenting temperature sensitive Z; MD ring – mitochondrion-dividing ring; PD ring – plastid-dividing ring.

observations, alternative theories suggest that plastids might arise *de novo* or by septation. In 1951 Chiba, based on Feulgen staining results, suggested that chloroplasts of *Selaginella* and two flowering plants contained DNA; however, a decade passed before reports of DNA in chloroplasts and mitochondria became common (Gillham, 1994). The multiplication of plastids by division was confirmed in the 1960s based on cytological and biochemical evidence of the presence of DNA and ribosomes in plastids and mitochondria (Swift and Woltenholme, 1969).

It is now well established based on extensive structural studies that plastids multiply by division along with duplication and separation of their nucleoids (DNA-protein complex) (T. Kuroiwa and Suzuki, 1981; T. Kuroiwa *et al.*, 1981; T. Kuroiwa, 1982, 1991), and that different plastid types, including proplastids, etioplasts, chloroplasts, and amyloplasts, are capable of division (T. Kuroiwa *et al.*, 1981; T. Kuroiwa, 1982; Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Gillham, 1994). Although many earlier studies clarified plastid division in several taxa and the several circumstantial conditions affecting the frequency of division (T. Kuroiwa *et al.*, 1981; Possingham and Lawrence, 1983), the mechanisms of plastid division were poorly understood. It is only recently that structural and biochemical studies have started to provide an understanding of the mechanisms and origin of this division at the molecular level. Findings of ring structures around the plastid division site (Mita *et al.*, 1986; Mita and Kuroiwa, 1988; T. Kuroiwa, 1989) and identification of the proteins involved in the division process have raised the concept that plastid division is accomplished by a division apparatus (plastid-dividing rings; PD rings) (Mita *et al.*, 1986; Mita and Kuroiwa, 1988; T. Kuroiwa *et al.*, 1998) composed of a bacterial FtsZ-based complex (Osteryoung and Vierling, 1995) and host eukaryote-derived complex that includes dynamin (Miyagishima *et al.*, 2001c, 2003a, b; Gao *et al.*, 2003). Based on this hypothesis, the factors involved in chloroplast division are now being identified, and by using identified structures and proteins as markers, detection of the division process even before constriction of the division site or in pleomorphic plastids has become possible (T. Kuroiwa *et al.*, 2001; Momoyama *et al.*, 2003).

This chapter describes where and when plastids divide (Section II) and then focuses on recent studies on the mechanism of plastid division at the structural and molecular levels (Section III). Because recent studies have shown strong similarities between the mechanisms of plastid and mitochondrial division, the known features of mitochondrial division are also mentioned where relevant. For earlier detailed reviews please refer to the following: Leech (1976, 1986), T. Kuroiwa (1982), Possingham and Lawrence (1983), Boffey and Lloyd (1988), Mita and Kuroiwa (1988), T. Kuroiwa (1989), T. Kuroiwa (1991) and Pyke (1997). The following recent reviews are also helpful: T. Kuroiwa (1998), T. Kuroiwa *et al.* (1998), Osteryoung (2001), Osteryoung and McAndrew (2001), Osteryoung and Nunnari (2003), McFadden and Ralph (2003) and Miyagishima *et al.* (2003b).

## II. Regulation of Timing and Mode of Plastid Division

Some primitive algae such as *Cyanidium caldarium* and *Cyanidioschyzon merolae* have only one chloroplast per cell and chloroplast division is coupled with the cell cycle, thus giving a simple system to investigate plastid division. In higher plants, some of which offer useful molecular genetic systems, plastids divide independently of cell division and the copy number of plastids within an organism varies considerably depending on the cell type, developmental stage, and environmental conditions. Furthermore, plastid division in higher plants occurs non-synchronously even in the same multiplastidic cell. It seems therefore that the system regulating the timing of plastid division has diversified during evolution.

Although the basic division system was likely established long ago in primitive algae, since then an additional controlling system has been added. Therefore, the system of plastid division must be understood by comparing algae and higher plants to distinguish which mechanisms are basic and which are additional.

### *A. Relationship Between Plastid and Cell Division and Differentiation*

During cell multiplication, plastids multiply to supply the needs of the resulting daughter cells. To maintain the number of plastids per cell, plastid and cell division must occur at the same rate. Therefore, the host eukaryotic cell must control the rate of plastid division relative to that of cell division.

Many unicellular algae have only one chloroplast per cell, and it is obvious that a direct and precise relationship must exist between the cell cycle and timing of plastid division (Fig. 2a and 3). Although the timing of chloroplast division relative to cell cycle stage varies depending on species, chloroplasts in these algae divide

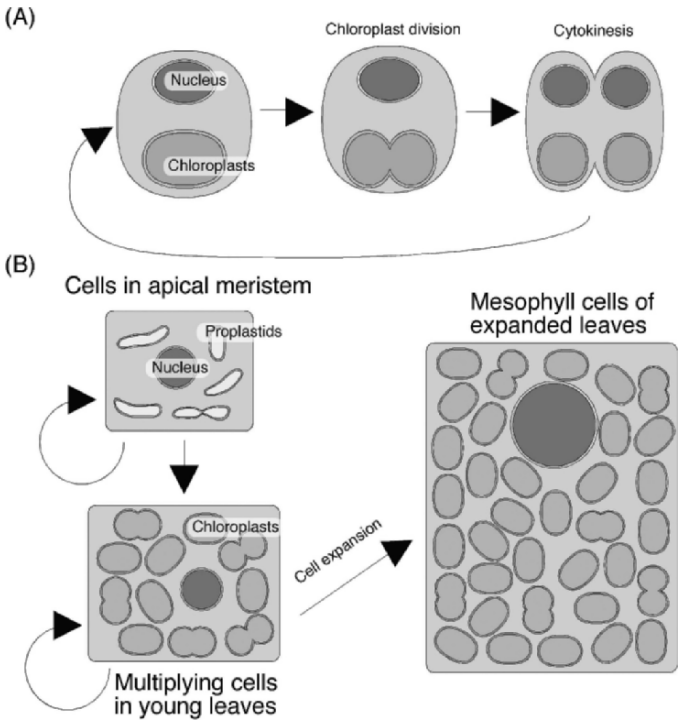


*Fig. 2.* (See also Color Plate 2, p. xxxv.) Schematic representation of the timing of chloroplast division relative to the cell cycle. (a) In unicellular algae, which have one chloroplast per cell, chloroplast division occurs once per one cell cycle prior to cytokinesis, although the timing of chloroplast division varies depending on species. (b) In meristematic cells in higher plants, the rate of proplastid and cell multiplication is almost the same, thereby allowing daughter cells to receive nearly the same number of proplastids as the mother cell. After leaf cells start to differentiate, proplastids are converted to chloroplasts. During the early proliferative stage in which mitosis takes place, chloroplast replication keeps pace with cell division even though chloroplasts divide nonsynchronously. During late expanding stage, cell division ceases but chloroplast division continues for two or three more cycles and nuclear DNA replication also continues, resulting in enlarged cell of high ploidy.

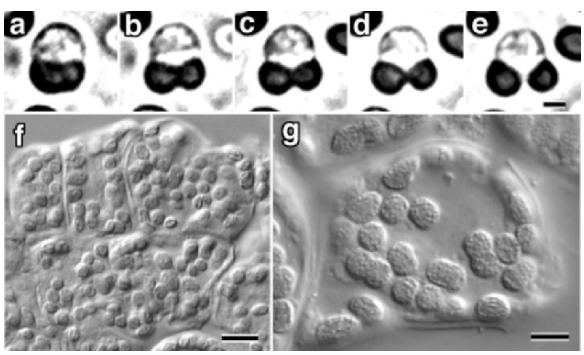


*Fig. 3.* Chloroplast division in a primitive alga and a higher plant **. (a–e)** Time-lapse observation of a single cell of the unicellular red alga *C. merolae* during the chloroplast division. The chloroplast (black part) divides once at particular point of the cell cycleoplast. From **a** to **e** was about 2 hs. **(f, g)** Micrographs of young leaf mesophyll cells of spinach. Chloroplasts (granulated structures) divide nonsynchronously even in the same cell (dividing chloroplasts indicated with arrows). Bars = 1 μm (e), 10 μm (f) and 5 μm (g).

at a particular point of the cell cycle before cytokinesis is completed (e.g. Mita and Kuroiwa, 1988; K. Suzuki *et al.*, 1994; Ogawa *et al.*, 1995). Generally, in algae that have two or more chloroplasts per cell, chloroplast division occurs non-synchronously but the approximate number of chloroplasts per cell is still maintained (e.g. Butterfass, 1979; exceptions are listed in Possingham and Lawrence, 1983).

In higher plants, the relationship between the cell cycle and plastid division is more flexible and complex. In meristematic cells (and cultured cells at the log phase), proplastids in the same cell divide non-synchronously, however, proplastid and cell division occur at similar rates so that newly formed cells have almost the same number of proplastids (T. Kuroiwa *et al.*, 1981). The number of plastids in other tissues changes depending on developmental stage; the well known processes occur during leaf (T. Kuroiwa *et al.,* 1981; Possingham and Lawrence,

1983; Miyamura *et al.*, 1990; T. Kuroiwa *et al.*, 1992; Fig. 2b and 3) and root (Miyamura *et al.*, 1986; T. Suzuki *et al.*, 1992) development. For example, in spinach, apical meristem cells contain about twelve proplastids and this number slightly increases during earlier stages of leaf development when leaf growth due to increases in cell numbers occurs. In the subsequent stage, when leaf growth due to cell expansion occurs, cell division stops but chloroplast division continues and the number of chloroplast per cell reaches about 200 (Possingham and Lawrence, 1983). This expansion stage is mimicked in leaf disc cultures, where the number of chloroplasts per cell reaches more than 1000 in the presence of cytokinin (Yagisawa *et al.*, 2003). In higher plants, plastids do not multiply to their final number in meristematic cells. Instead a large proportion of the final population in mature cells is derived from the division of differentiated plastids, such as chloroplasts and amyloplasts, in cells where ploidy multiplication occurs without mitosis (Possingham and Lawrence, 1983; Yagisawa *et al.*, 2003).

Another complexity of the mode of plastid multiplication in higher plants is in the duplication and separation of nucleoids. In meristematic tissues and young developing tissues, plastid nucleoids are duplicated at a high rate, while in subsequent expanding leaf or root tissues, plastid division occurs without significant synthesis of DNA and nucleoids (T. Kuroiwa *et al.*, 1981; Possingham and Lawrence, 1983; T. Suzuki *et al.*, 1992).

In higher plants, the mode of plastid division is a highly complex system. Proplastid division is responsible for plastid continuity because plastids are inherited from generation to generation or from meristems as proplastids. Nevertheless, because the origin of plastids lies with an ancestor of extant cyanobacteria and since in unicellular algae chloroplasts are usually the only type of plastids, chloroplast division is considered the basic mechanism of plastid division from an evolutionary standpoint. The complex system in higher plants should therefore be understood as a mechanism gained by higher plants along with the differentiation system of plastids.

### *B. Mode of Plastid Division*

Using optical and electron microscopy and time-lapse observations, many studies have shown that plastids divide by binary constriction division (T. Kuroiwa *et al.*, 1981; T. Kuroiwa, 1982; Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Gillham, 1994). Although binary division was common to the plastids of all species examined, chloroplasts with multiple constrictions have been frequently observed in many multiplastidic green algae such as *Bryopsis* and in the embryonic tissues of land plants. For example, in the ferns *Ophioglossum* and *Hymenophyllum* chloroplasts have two or more constriction sites associated with the PD ring, especially in the vascular parenchyma (Duckett and Ligrone, 1993b).

Observations of shape only make it difficult to determine the exact location of division in pleomorphic plastids. However, recent studies using the PD ring and FtsZ ring as indicators of the division site have made detection of the exact plastid division site possible. For example, tobacco BY-2 cells, which are not photosynthetic, have pleomorphic plastids, the shape of which changes during culture (T. Suzuki *et al.*, 1992). Recent immunofluorescence studies have shown that these plastids have two or more FtsZ rings (Momoyama *et al.*, 2003). Similarly, the pleomorphic chloroplasts in the embryonic cap cells in the non-photosynthetic organs of *Pelargonium zonale* have multiple FtsZ rings (H. Kuroiwa *et al.*, 2001). This kind of plastid division might be required for fast multiplication which might also be the case for other tissues. Further studies concerning the mechanisms in other tissues are therefore required. Although the above two cases are similar, the plastids in the BY-2 cells show constriction at only one FtsZ ring site (Momoyama *et al.*, 2003) whereas the embryonic cap chloroplasts constrict at two or more FtsZ ring sites simultaneously (H. Kuroiwa *et al.*, 2001). Therefore there are at least two kinds of multiple fission in plastids.

## III. Structural and Molecular Mechanisms of Plastid Division

Although the concept that plastids, like cells, multiply by fission along with nucleoid division was established by the early 1980s (T. Kuroiwa *et al.*, 1981; T. Kuroiwa, 1982), and although electron micrographs have shown that plastids divide by simultaneous constriction of the inner and outer envelopes, few studies concerning the mechanism responsible for plastid division were available until recently. Of the characterized plastid genomes, none had a complete set of genes sufficient for self-replication. Plastids that lack ribosomes (the barley mutant, albostrians; Hashimoto and Possingham, 1989) can still divide. These results indicated that nucleus-encoded factors are exclusively responsible for plastid division. A few early studies reported electron dense structures in the constriction zone

of dividing plastids (K. Suzuki and Ueda, 1975; Chaly and Possingham, 1981; Leech *et al.*, 1981) but, as described elsewhere in detail (T. Kuroiwa, 1989), the interpretation of the structure and location were incorrect.

## *A. Strategies Used to Examine the Mechanism of Plastid Division*

### *1. The Red Alga* Cyanidioschyzon merolae *as a Tool for Studying Organellar Division Apparatuses*

As mentioned above, plastids in higher plants divide non-synchronously even in the same cell and the division frequency changes depending on the tissue. Consequently, it is difficult to observe numerous dividing chloroplasts at the ultrastructural level or dissect them from the tissues. In contrast, in some unicellular algae, which have only one chloroplast per cell, chloroplast and cell division are typically synchronized. Among these species, the cells of *Cyanidioschyzon merolae* (Fig. 3a–e and 4) offer unique advantages for characterization of the PD ring and further analysis of organellar division. *C. merolae* is a small (2 gμm in diameter), unicellular organism that inhabits sulphate-rich hot springs (pH 1.5–2.5, 45°C) (De Luca *et al.*, 1978). They lack a rigid cell wall and contain a nucleus, one mitochondrion and one plastid, the divisions of which can be tightly synchronized by light/dark cycles (K. Suzuki *et al.*, 1994; T. Kuroiwa *et al.*, 1995; Terui *et al.*, 1995). This alga also has a minimal set of small membrane-bound compartments, e.g., a microbody (peroxisome), a single Golgi apparatus with two cisternae, coated vesicles, a single endoplasmic reticulum, and a few lysosome-like structures, as well as a small volume of cytosol (T. Kuroiwa *et al.*, 1994). Recently, the complete genome sequences of the mitochondria (Ohta *et al.*, 1998), chloroplasts (Ohta *et al.*, 2003) and nuclei (Matsuzaki *et al.*, 2004) were sequenced and the results showed that of all free-living eukaryotes *C. merolae* has the smallest set of genes sequenced to date, thus making it suitable for examining the basic system required for organellar biogenesis.

Since the identification of plastid dividing rings (PD rings) in the chloroplasts of *C. caldarium* (Mita *et al.*, 1986), similar rings have been observed in many plants. *C. merolae* has the largest PD rings so far examined and they can be detected clearly just before the onset of constriction (T. Kuroiwa, 1998; T. Kuroiwa *et al.*, 1998). A structure similar to the PD ring was also identified in the mitochondria of *C. merolae* (Fig. 4). This structure, called the MD ring, appears to consist of two rings, an outer ring on the cytosolic side of the outer membrane

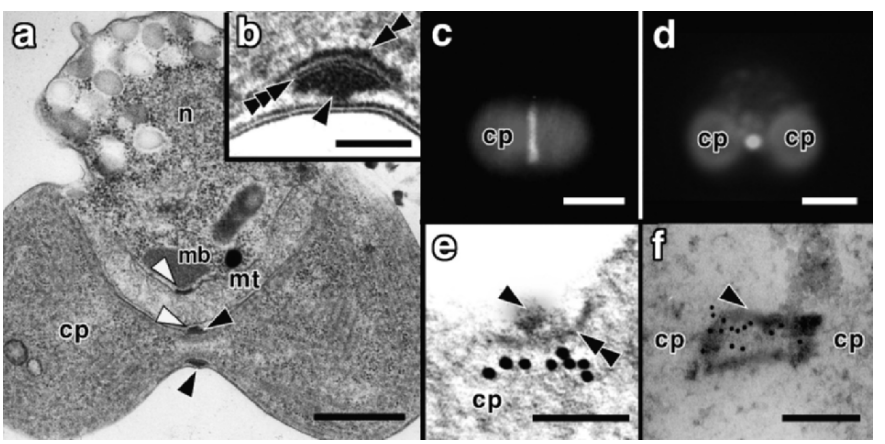


*Fig. 4.* (See also Color Plate 2, p. xxxv.) The PD, FtsZ, and dynamin rings in the red alga *C. merolae*. (a) Electron micrographs of a *C. merolae* cell containing a dividing chloroplast and mitochondrion. (b) Magnified cross-section of the PD ring. The PD ring is composed of an outer ring (on the cytosolic side of the outer envelope), a middle ring (in the intermembrane space), and an inner ring (on the stromal side of the inner envelope). The MD ring, structure similar to the PD ring, is also observed at mitochondrial division site. **(c, d)** Immunofluorescence images of the FtsZ (CmFtsZ2; **c**) and dynamin (CmDnm2; **d**) rings during chloroplast division. Bright fluorescence shows localization of each protein and transparent fluorescence is autofluorescence of the chloroplasts. **(e, f)** Immunoelectron micrographs showing localization of the FtsZ and the dynamin at the chloroplast division site. The FtsZ ring localizes in stroma and faces the inner plastid-dividing ring at the far side from the inner envelope while the dynamin ring localizes between the outer PD ring and the outer envelope. Gold particles indicate location of each protein. Black arrows, arrowheads and double arrowheads indicate the outer, inner, and middle PD rings, respectively. White arrows indicate the MD ring. *cp*, chloroplast; *mb*, microbody; *mt*, mitochondrion; *n*, nucleus; Bars = 500 nm (**a**), 50 nm (**b, e**), 1 μm (**c, d**), and 100 nm (**f**).

and a thin inner ring on the matrix side of the inner membrane (T. Kuroiwa *et al.*, 1993). This was a key factor in clarifying the similarities between plastids and primitive mitochondrial division.

Recently, Minoda *et al.* (2004) developed a method for nuclear transformation in *C. merolae* using exogenous DNA with homologous recombination. In the future, this system should facilitate further studies at the molecular level.

## 2. Arc (Accumulation and Replication of Chloroplasts) Mutants

Mesophyll cells in expanded *Arabidopsis thaliana* leaves usually contain >100 uniformly sized chloroplasts, the size and number of which are accurately regulated. To identify the genes that regulate chloroplast multiplication, twelve recessive *A. thaliana* mutants with altered numbers of chloroplasts per mesophyll cell were collected and named *arc* (accumulation and replication of chloroplasts) mutants (Marrison *et al.*, 1999; Pyke, 1999). For example, the *arc3* (Pyke and Leech, 1992), *arc5*, *arc6* (Pyke and Leech, 1994) and *arc12* (Pyke, 1999) mutants contain 1 to 15 giant chloroplasts per cell, suggesting that chloroplast division is partially inhibited in these mutants. In the *arc5* mutant, chloroplasts begin to divide but appear to stop when they become centrally constricted, suggesting that the *arc5* gene product is required to complete the separation process (Robertson *et al.*, 1996). Contrary to the above mutants, *arc1* has a larger number of smaller chloroplasts per cell than the wild-type (Pyke and Leech, 1992). In the *arc10* and *arc11* mutants, the mesophyll cell chloroplasts are highly heterogenous in size within a single cell (Pyke, 1999). This size heterogeneity might be caused by the presence of a subpopulation of chloroplasts that do not divide or that divide by some other form of abnormal chloroplast division, such as asymmetric division.

Although the proplastid division in meristematic tissues is also perturbed in the *arc6* (Robertson *et al.*, 1995) and *arc12* mutants (Pyke, 1999), the *arc3* (Pyke and Leech, 1992) and *arc5* (Pyke and Leech, 1994) mutants appear to specifically affect chloroplast division. Some of these mutations were recently mapped; they are described below.

Some of the proteins required for plastid division have been found in *A. thaliana* by genetics and reverse genetics (described later). Since it is difficult to detect the fine structure and characterize the composition and transition of the plastid division apparatus biochemically, examination by other systems including *C. merolae* are required.

## 3. Incorporation of Information About Bacterial Division

Because the ancestor of plastids is a cyanobacterium and since plastids still use part of the bacterial division system, accumulated information about the mechanism of bacterial division can be incorporated into studies of plastid division. Some factors involved in plastid division have been identified by reverse genetics (described later) to allow examination of plant nuclear homologs of bacterial division genes. This section summarizes the main information known about bacterial division. Detailed summaries can be found in other extensive reviews such as Bramhill (1997), Rothfield *et al.* (1999) and Errington *et al.* (2003).

Filamentous temperature-sensitive (*fts*) genes were identified in *Escherichia coli* mutants collected in the late 1960s. These *fts* mutants have a cytokinesis defect and, as a result, elongate to form filaments (Hirota *et al.*, 1968). Among the several Fts proteins, FtsZ is a GTPase structurally similar to tubulin (Lowe and Amos, 1998) that self-assembles into a ring structure beneath the cytoplasmic membrane at the division site (Bi and Lutkenhaus, 1991). FtsZ is conserved among most bacteria and archaea. The formation of the FtsZ ring is the first event at the division site and initiates the recruitment of the other proteins that constitute the bacterial division complex (Bramhill, 1997; Rothfield *et al.*, 1999; Errington *et al.*, 2003). Therefore, of the several proteins involved in division, FtsZ is thought to play a central role in prokaryotic cell division.

Once the FtsZ ring is formed at the division site, FtsA and ZipA bind directly to FtsZ. After FtsA and ZipA have joined the septal ring, the remaining proteins, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsW, FtsI and FtsN, localize in this order (the most current review can be found in Schmidt *et al.*, 2004). Some of these latter proteins are thought to be involved specifically in cell wall growth at the division site, which is also essential for cell division in many bacteria (Nanninga, 1998).

In *E. coli*, placement of the FtsZ ring is governed by the *minB* operon, which encodes three gene products, MinC, MinD and MinE (de Boer *et al.*, 1989). MinC is a division inhibitor that suppresses the formation of the FtsZ ring (Hu *et al.*, 1999). Its activity is dependent on the membrane-bound MinD protein, which is thought to form a heterodimer with MinC (Huang *et al.*, 1996). MinE prevents MinC/D from acting at the cell

center, thereby allowing FtsZ ring assembly and hence cell division, but only at that position (de Boer *et al.*, 1989). Time-lapse observations of *E. coli* have shown that MinE oscillates from a mid cell point to both cell tips, sweeping MinE and MinD away from the division site (Hale *et al.*, 2001). The *min* locus is so-named because mutations in *minC* or *minD* allow the FtsZ ring to assemble at aberrant sites near the cell poles, resulting in the formation of minicells that lack chromosomes (de Boer *et al.*, 1989). Mutations in *minE*, on the other hand, permit MinCD to act ectopically midcell, thereby preventing FtsZ ring assembly at all sites and resulting in the formation of bacterial filaments (de Boer *et al.*, 1989).

Although FtsZ localization at the division site and FtsZ polymerization were shown *in vitro*, the bacterial division apparatus cannot be observed directly on electron microscopic sections and the exact nature of the FtsZ ring has never been detected *in vivo*.

## B. The Plastid Dividing (PD) Ring

### 1. Structure and Universality of the PD Ring

Before the bacterial FtsZ ring was discovered in 1991, ultrastructural studies showed electron-dense ring structures encircling the constriction furrow of dividing plastids. This ring structure, called the plastid-dividing ring or PD ring (later referred to as the outer PD ring), was first identified on the cytosolic face of the outer envelope in the red alga *C. caldrium* (Mita *et al.*, 1986). Subsequently, electron microscopy in *Avena sativa* identified an inner PD ring on the stromal face of the inner envelope in addition to the outer PD ring (Hashimoto, 1986). The outer and inner PD rings have now been detected in *Rhodophyta* (Mita *et al.*, 1986; Mita and Kuroiwa, 1988; K. Suzuki *et al.*, 1994; Miyagishima *et al.*, 1999b) and several species of *Chlorophyta* (green algae and terrestrial plants) (Hashimoto, 1986; Tewinkel and Volkmann, 1987; Hashimoto and Possingham, 1989; Oross and Possingham, 1989; Chida and Ueda, 1991; Duckett and Ligrone, 1993a, b; Ogawa *et al.*, 1995; Robertson *et al.*, 1996) and it is thought to be ubiquitous throughout the plant kingdom (summarized by T. Kuroiwa, 1998; T. Kuroiwa *et al.*, 1998; Fig. 4). In the red alga *C. merolae*, a middle PD ring was also identified in the intermembrane space between the inner and outer envelopes (Miyagishima *et al.*, 1998a) (Fig. 4). Although the *C. merolae* PD ring seems to be composed of inner, middle and outer rings, the middle PD ring has not been observed in land plants.

The PD ring has also been seen in chloroplasts. In *Heterosigma akashiwo*, a raphidophyte alga, which acquired chloroplasts by secondary endosymbiosis with red algae (Cavalier-Smith, 2000; McFadden, 2001), the chloroplasts are enveloped by four membranes (two pairs of tightly oppressed double membranes); the inner pair constrict in advance of the outer pair. At the division site of the inner pair of these four surrounding membranes inner and outer PD rings have been observed, consistent with the hypothesis that the inner pair is derived from a red algal endosymbiont (Hashimoto, 1997).

Recently, a structure similar to the PD ring was identified in the Glaucophyta *Cyanophora paradoxa*, which has peptidoglycan between the outer and inner envelopes. In this alga, a structure similar to the inner PD ring was observed but the outer PD ring was not (Hashimoto, 2003). Based on this result, Hashimoto (2003) hypothesized that peptidoglycan ingrowths as found in bacteria still play a role during constriction, thereby dividing chloroplasts without an outer PD ring. However, the location of the PD ring was unclear in the electron micrographs used, and therefore more detailed observations of the PD rings in *C. paradoxa* are required.

Although the components of the PD rings are still unknown, the outer ring appears to be composed of a bundle of fine filaments about 7 nm in diameter in *C. caldarium* (Mita and Kuroiwa, 1988; T. Kuroiwa, 1991; T. Kuroiwa *et al.*, 1998) and about 5–6 nm in diameter in *C. merolae* (T. Kuroiwa *et al.*, 1998; Miyagishima *et al.*, 2001b). Since the chloroplasts of *C. caldrium* do not divide during cytochalasin B treatment and hence become giant chloroplasts (T. Kuroiwa *et al.*, 1998), it is possible that the fine filaments of the PD ring might be actin-like components. However, actin signals appeared on the contractile ring for cytokinesis but not on the PD ring using immunofluorescence and immunoelectron microscopy (Takahashi *et al.*, 1998). Analysis of the genome sequence of *C. merolae* revealed the absence of a myosin gene and cDNA clones for actin genes (Matsuzaki *et al.*, 2004) suggesting that the PD ring filaments are not actin filaments although they are similar. Identification of the PD ring components is on going using dissected PD rings.

### 2. Behavior of the PD Ring

The morphology and behavior of the two (or three) PD rings differ and their details throughout the division cycle were clarified in the unicellular red algae *C. caldarium* (Mita and Kuroiwa, 1988; T. Kuroiwa,

1989) and *C. merolae* (Miyagishima *et al.*, 1998b, 1999a, 2001a) and then in the dicot *Pelargonium zonale* (H. Kuroiwa *et al.*, 2002). However, the behavior of the PD ring has not been observed in *A. thaliana*.

In a series of studies on *C. merolae* using synchronized organelles and electron microscopy, the mode of formation, contraction and disassembly of the PD ring was clarified. It was revealed that formation of the inner ring precedes that of the middle and outer rings (Miyagishima *et al.*, 1998b). As the outer PD and MD rings contract, they grow thicker and maintain a constant volume, while the thicknesses of the middle and inner PD rings do not change and their volumes decrease at a constant rate with contraction (Miyagishima *et al.*, 1999a). Since the densities of each ring at all stages of contraction are constant, the disassembly of the inner and middle rings parallels their contraction (Miyagishima *et al.*, 1999a). Just before the completion of chloroplast division, the inner and middle rings disassemble completely and disappear. The outer ring, on the other hand, exists throughout chloroplast division and remains in the cytosol between the daughter chloroplasts. The remnants of the outer ring start to disassemble and disappear from the surface just after chloroplast division (Miyagishima *et al.*, 2001a).

Similar results have been partially obtained in green algae. Although the middle PD ring has yet to be reported in other species, the morphology of the inner and outer PD rings is similar between *C. merolae* and the green alga *Nannochloris bacillaris* (Ogawa *et al.*, 1995). In *Nannochloris* and another green alga *Trebouxia potteri* (Chida and Ueda, 1991), the two rings widen in the same manner as in *C. merolae.* Recently, fine structural analysis of the PD ring throughout plastid division was performed in the dicot *Pelargonium zonale* revealing behavior similar to that of the PD ring in algal species (H. Kuroiwa *et al.*, 2002). Because the two or three PD rings behave differently throughout the plastid division cycle, the components and functions of these rings are probably also different.

## C. FtsZ and the Related Factors That Descended From Cyanobacteria and α-Proteobacteria

### 1. Involvement of FtsZ in Chloroplast and Mitochondrial Division

The first protein shown to play a role in plastid division was a plant nuclear homolog of the key bacterial division protein, FtsZ. In 1995, chloroplast-targeted FtsZ was found in the *A. thaliana* nuclear genome (Osteryoung and Vierling, 1995) and FtsZ homologs have been reported in several photosynthetic eukaryotes, including organisms with chloroplasts that originated from secondary endosymbiosis (Beech and Gilson, 2000; Gilson and Beech, 2001). These eukaryotic FtsZ proteins are related most closely to cyanobacterial counterparts (Beech and Gilson, 2000; Gilson and Beech, 2001) indicating an endosymbiotic origin. Gene disruption experiments in the moss *Physcomitrella* (Strepp *et al.*, 1998) and the expression of anti-sense RNA in *A. thaliana* (Osteryoung *et al.*, 1998) inhibited chloroplast division generating giant chloroplasts. Although these results raised the possibility that plant FtsZ forms a ring structure at the plastid division site, two hypotheses were suggested. Based on topology, T. Kuroiwa *et al.* (1998) hypothesized that FtsZ forms a ring structure inside the chloroplast. While Osteryoung *et al.* (1998), on the other hand, proposed that FtsZ1 and FtsZ2 form the inner and outer PD rings, respectively, based on the fact that higher plants have two types of FtsZ, one of which is not transported into chloroplasts *in vitro*. However, using immunoelectron microscopy, T. Kuroiwa *et al.* (1999) showed that FtsZ signals appeared not on the outer PD ring but on the stromal side at the constriction region of the dividing chloroplast. Subsequently, GFP-tagged FtsZ was introduced into the moss showing FtsZ-containing cytoskeletal-like networks in the chloroplasts (Kiessling *et al.*, 2000). Nevertheless, as shown by Vitha *et al.* (2001), these were probably artifacts of excess amounts of GFP-tagged FtsZ. Recent immunocytochemical studies have, however, demonstrated that plant FtsZs form ring structures at the chloroplast division sites (Mori *et al.*, 2001a, b; Vitha *et al.*, 2001; Fig. 4).

It was initially hypothesized that FtsZ1 functions on the cytosolic side of the division site (Osteryoung *et al.*, 1998), but experiments in *Lilium longiflorum,* showed that not only does FtsZ1 have transit peptides but it also localizes at the stromal side of the division site (Mori *et al.*, 2001b). Subsequently, in *A. thaliana* both FtsZ1 and FtsZ2 were shown to have transit peptides (McAndrew *et al.*, 2001; Fujiwara and Yoshida, 2001) and in P. *zonale* both proteins were shown to be localized at the stromal side of the division site by immunoelectron microscopy (H. Kuroiwa *et al.*, 2002).

The discovery of chloroplast FtsZ focused attention on the search for the FtsZ protein that might be involved in mitochondrial division. However, no sequences that resemble *ftsZ* were found in the genomes of *Saccharomyces cerevisiae* or *Caenorhabditis elegans* or in the recently sequenced genomes of other

non-photosynthetic eukaryotes. It is now clear that *A. thaliana* has no FtsZ proteins that are related to those of α-proteobacteria, however, both α-proteobacteria- and cyanobacteria-type FtsZs were isolated from the heterokont alga *Mallomonas* (Beech *et al.*, 2000) and primitive red alga *C. merolae* (Takahara *et al.*, 2000). Furthermore, the α-proteobacteria-type FtsZ is localized in the mitochondria (Beech *et al.*, 2000; Takahara *et al.*, 2000) and a very recent study showed that this protein forms a ring on the matrix side at the division site (Takahara *et al.*, 2001; Nishida *et al.*, 2003). Similar FtsZ sequences have also been found in some other protists including the slime mold *Dictyostelium* (Gilson *et al.*, 2003; Kiefel *et al.*, 2004; Miyagishima *et al.*, 2004). These results indicate that primitive mitochondria use FtsZ in the same manner as chloroplasts and that this protein was lost from higher eukaryotes during evolution. Recently completed genome projects have demonstrated that FtsZ was also lost from some plastids that originated from secondary endosymbiosis, such as the plastids (apicoplasts) of apicomplexan parasites, which cause malaria (McFadden *et al.*, 1996; see Chapter 24).

## 2. The Two Types of FtsZ Proteins Required for Organelle Division in Eukaryotes

Although FtsZ in chloroplasts has a cyanobacterial origin, chloroplasts might also have developed a paralogous FtsZ protein. Whereas most bacteria (including cyanobacteria) have only one *ftsZ* gene, green algae and higher plants have several *ftsZ* genes that are clustered into two phylogenetic groups, FtsZ1 and FtsZ2 (Osteryoung *et al.*, 1998; Osteryoung and McAndrew 2001; Wang *et al.*, 2003). Although the functional differences between FtsZ1 and FtsZ2 are still not known, depletion of either protein in *A. thaliana* disrupts plastid division, suggesting that instead of being redundant, each of these proteins has a distinct function (Osteryoung *et al.*, 1998). A comparison of their primary structures revealed that FtsZ2 proteins contain a short conserved sequence at the C-terminus (C-terminal core domain), much like bacterial FtsZs, but this is not the case for FtsZ1 (Osteryoung and McAndrew, 2001).

Recently, two types of FtsZ proteins were also found in red algae and heterokonts (algae evolved by engulfing red algae during secondary endosymbiosis) (Miyagishima *et al.*, 2004). Although, like green lineage (green algae and plants) FtsZ proteins, one FtsZ has a C-terminal core domain and the other does not, phylogenetic analyses suggest that the FtsZ without the C-terminal core domain emerged independently in green lineage organisms, red algae and heterokonts. The *C. merolae* and *Dictyostelium* genomes also encode two types of mitochondrial FtsZ proteins, one of which lacks the C-terminal sequence (Miyagishima *et al.*, 2004).

These results suggest that acquisition of additional FtsZ proteins that lack the C-terminal core domain might have been a crucial event in the establishment of plastids and mitochondria. The C-terminal core domain binds to FtsA and ZipA (Ma and Margolin, 1999), neither of which is found in the genomes of *C. merolae* or *A. thaliana*. However, conservation of the C-terminal core domain suggests that these FtsZ proteins likely have protein partners that are, as yet, unknown. Given that FtsZ1 and FtsZ2 co-localized in plastids even when the expression level and assembly pattern of each was altered experimentally (McAndrew *et al.*, 2001), the additional FtsZ present in organelles likely confers an additional function of the FtsZ ring.

## 3. The Factors Around the FtsZ Ring

The existence of FtsZ proteins implicated in plastid division suggests that plastids divide using a mechanism similar to that of bacteria. This proposal led to the identification of additional nucleus-encoded homologs of bacterial division proteins such as MinD and MinE in *A. thaliana* using reverse genetics.

In *AtMinD1*-antisense transgenics, chloroplasts divide asymmetrically resulting in chloroplasts of variable size (Colletti *et al.*, 2000). In addition, the *arc11* mutation, which causes a similar phenotype (described above), was mapped to the *AtMinD1* gene (Fujiwara *et al.*, 2004). Similarly, *AtMinE1* overexpression in *A. thaliana* resulted in giant chloroplasts (Itoh *et al.*, 2001; Reddy *et al.*, 2002) or in division site misplacement giving rise to multiple constrictions along the length of the plastids (Maple *et al.*, 2002). These results are readily explained if the MinD and MinE proteins retain a function analogous to that of the bacterial counterparts in higher plants.

Another known protein that regulates plastid FtsZ ring formation is ARC6. The *arc6* mutation was mapped to *A. thaliana* using a nuclear homolog of the *Ftn2* gene (Koksharova and Wolk, 2002), which was identified by transposon mutagenesis in *Synechococcus* sp. PCC 7942. Like the *E. coli fts* mutants (Bramhill, 1997; Rothfield *et al.*, 1999), the *ftn2* mutants displayed a filamentous morphology resulting from cell division defects (Koksharova and Wolk, 2002). The *ARC6 gene* encodes a DnaJ-like protein localized in

the inner envelope at the division site (Vitha *et al.*, 2003). Based on the FtsZ filament morphology observed in the *arc6* mutant and in plants that overexpress *ARC6,* it is hypothesized that ARC6 functions in the assembly and/or stabilization of the plastid-dividing FtsZ ring.

In addition to MinE, MinD and Ftn2, three other proteins derived from cyanobacteria, ARTEMIS (Fulgosi *et al.*, 2002), Crl1 (Asano *et al.*, 2004), and Gc1 (Maple *et al.*, 2004), were suggested to be involved in plastid division based on observations that disruption of the genes generated giant chloroplasts in the cells. These proteins were not localized at the division site and the functions of these proteins during plastid division are still unknown. It is possible that the observed phenotype might be the result of secondary defects.

The above results suggest that the mechanism of bacterial division still regulates plastid division. Although the mechanism of cyanobacterial division probably differs from that of *E. coli* and *Bacillus* as in the case of ARC6/Ftn2, the cyanobacterium *Anabaena* sp. PCC 7120 encodes FtsK, FtsW, FtsI, FtsQ and FtsZ among the proteins localized at the division site. The chloroplast genomes of the green algae *Nephroselmis* and *Mesostigma* encode FtsW and FtsI. However, other than *ftsZ*, the organelle and nuclear genomes of *A. thaliana* and the red alga *C. merolae* lack sequences related to these genes, suggesting that these proteins have been lost from the chloroplasts and mitochondria without being transferred to the host nuclear genomes. It is also notable that MinC is also missing in plant and algal genomes in contrast to MinD and MinE, which have been retained for plastid division after endosymbiosis.

In summary, plastids have retained the basic bacterial division system, but this retention was partial. Of the bacteria-derived proteins that have been localized at the division site, only FtsZ and its regulatory proteins have been retained. The loss of bacterial division proteins, most of which are recruited after FtsZ ring formation, probably accompanied the loss of cell walls and the acquisition of an outer envelope in plastids. Some proteins involved in the bacterial division apparatus are thought to act in the periplasm, and one (FtsI) acts in peptide glycan synthesis (Bramhill, 1997; Rothfield *et al.*, 1999) and would not be needed once the bacterial cell wall was lost. The complete genome of the red alga *C. merolae* and other sequence databases lack $\alpha$-proteobacterial homologs of bacterial division genes other than FtsZ; therefore, the loss of a large portion of proteins was probably also the case in primitive mitochondria that still use the FtsZ ring.

### 4. The Relationship Between the FtsZ and PD Rings

The cytoskeletal nature of FtsZ and localization of plant FtsZs in the chloroplast implicated these proteins as components of the inner PD ring (T. Kuroiwa *et al.*, 1998), or both the inner and outer PD rings (Osteryoung *et al.*, 1998) as described above. However, biochemical and immunocytochemical studies on *C. merolae* have indicated that this is not the case. A series of experiments using isolated dividing chloroplasts demonstrated that the FtsZ and PD rings in chloroplasts exhibit differential stabilities under a variety of biochemical conditions, indicating they are distinct structures (Miyagishima *et al.*, 2001c). In addition, the FtsZ ring assembles and disassembles prior to the PD ring. Immunoelectron microscopy showed that the FtsZ ring is positioned on the interior side of the inner PD ring (the far side from the inner envelope) *in C. merolae* (Miyagishima *et al.*, 2001c; Fig. 4); similar results were also observed in the dicot *P. zonale* (H. Kuroiwa *et al.*, 2002) by immunoelectron microscopy.

No electron-dense structures like the PD ring have been observed at the division sites in cyanobacteria, as seen in other bacteria (i.e. the bacterial division apparatus based on FtsZ has not been observed directly using electron microscopic sections). In addition, the 7 nm filaments in *C. caldarium* (Mita and Kuroiwa, 1988; T. Kuroiwa *et al.*, 1998) and 5 nm filaments *in C. merolae* (Miyagishima *et al.*, 2001b) that compose the outer PD ring are most likely novel filamentous structures. Taken together, these results suggest that the three PD rings were probably added by the host eukaryotic cell after cyanobacterial endosymbiosis.

## D. Dynamin: A System From the Host Eukaryotic Cell

### 1. Involvement of a Dynamin-Related Protein in Mitochondrial Division

The dynamin family of proteins are eukaryote-specific, large GTPases involved in the fission of several membrane systems (van der Bliek, 1999; Hinshaw, 2000). Dynamin was first purified from a microtubule-rich fraction of bovine brain (Shpetner and Vallee, 1989). Subsequently, it was linked to endocytosis by the discovery that the paralyzed *Drosophila* mutant *shibire* (Grigliatti *et al.*, 1973) had a mutation in the dynamin

gene (van der Bliek *et al.*, 1991) that caused the pool of releasable synaptic vesicles to be depleted as a result of a blockade of recycling via clathrin-mediated endocytosis (Kosaka and Ikeda, 1983; van der Bliek *et al.*, 1993; Damke *et al.*, 1994). Thereafter, dynamin was shown to assemble into a ring at the neck of clathrin-coated pits, where it appears to play a role in pinching off vesicles from the plasma membrane (Hinshaw and Schmid, 1995; Takei *et al.*, 1995). Several proteins have been included in the dynamin family due to their structural similarities (van der Bliek, 1999; Hinshaw, 2000).

Among the *Saccharomyces* mutations that cause defects in the distribution and morphology of mitochondria (*mdm* mutants), which were first described in the 1990s (McConnell *et al.*, 1990; Yaffe, 1999), *mdm29* has been mapped to *DNM1*, one of three dynamin-related yeast genes (Otsuga *et al.*, 1998). Expression of dominant-negative forms of Drp1 (the human ortholog of Dnm1p) resulted in the aggregation of mitochondria (Smirnova *et al.*, 1998). These results suggest that one of the dynamin family proteins is required for mitochondrial division.

Analogous to the function of conventional dynamin at the plasma membrane, DRP-1 (*C. elegans* ortholog of Dnm1p) (Labrousse *et al.*, 1999) and Dnm1p (Bleazard *et al.*, 1999; Sesaki and Jensen, 1999) are found on the cytosolic side of the mitochondrial division sites. After mitochondrial FtsZ was identified in lower eukaryotes and the apparent loss of mitochondrial FtsZs in higher eukaryotes was revealed by various genome sequencing projects, it was suggested that dynamin evolutionarily replaced the function of FtsZ during mitochondrial division (Erickson, 2000; Margolin, 2000; Arimura and Tsutsumi, 2002). One of the reasons given was that FtsZ and dynamin are both self-assembling GTPases, although they have opposite positions with respect to the organelle membranes: dynamin is located outside, whereas FtsZ is located inside the mitochondria at the division site. Recently, fluorescent microscopy with GFP-FtsZ was used to show that a dynamin-like protein in *A. thaliana*, namely ADL2b, which is related to Dnm1p, was localized at the mitochondrial division sites (Arimura and Tsutsumi, 2002). This suggested the acquisition of dynamin for mitochondrial division occurred in an ancestor that is common to animals, fungi and plants. In agreement with this proposal, mitochondrial division in *C. merolae* uses a dynamin (CmDnm1) ring in addition to the FtsZ and MD rings (Fig. 1) (Nishida *et al.*, 2003).

## *2. Involvement of a Dynamin-Related Protein in Plastid Division*

Contrary to the hypothesis that dynamin replaced FtsZ during evolution, mitochondrial division in *C. merolae* exhibits crucial differences with respect to the timing of the formation and disassembly of the FtsZ and dynamin rings. In *C. merolae* mitochondria, the MD ring forms after the FtsZ ring and before constriction at the division site, in the same manner that chloroplast FtsZ and PD rings form, whereas the dynamin ring forms at a later stage during mitochondrial division (Nishida *et al.*, 2003). The FtsZ ring is split and distributed into the two daughter mitochondria during the final stage of constriction, although remnants of the MD and dynamin rings persist for a short time after division has been completed (Nishida *et al.*, 2003). The formation of the dynamin ring during the later stages of division was also suggested for *C. elegans* (Labrousse *et al.*, 1999) and was recently demonstrated in *S. cerevisiae* (Legesse-Miller *et al.*, 2003). These results suggest that the role of dynamin in mitochondrial division is probably restricted to the final phase of division and that the function of dynamin is not redundant with respect to FtsZ.

The role of dynamin is distinct from that of the FtsZ and MD rings, and mitochondrial division in lower eukaryotes and chloroplast division are similar processes in that they both use FtsZ and have similar PD and MD ring structures. These observations led to the assumption that dynamin functions in the latter stages of chloroplast division. The *C. merolae* genome only contains two dynamin-like sequences: *Cmdnm1* (involved in mitochondrial division as described above) (Nishida *et al.*, 2003) and *Cmdnm2* (Miyagishima *et al.*, 2003a). Recently, immunofluorescent and immunoelectron microscopy were used to show that CmDnm2 forms a ring at the chloroplast division site in *C. merolae* (Miyagishima *et al.*, 2003a; Fig. 4). Simultaneously, ARC5, a dynamin-like protein in *A. thaliana*, was shown to form a ring structure at the division site of chloroplasts using a GFP-tagged protein (Gao *et al.*, 2003). CmDnm2 is recruited to the cytosolic side of the chloroplast division site during the middle stage of constriction, but it does not form a continuous ring structure under the outer PD ring until a later stage of division, much like mitochondrial dynamin (Fig. 1) (Miyagishima *et al.*, 2003a). The chloroplasts in *arc5* mutants are frequently dumbbell-shaped (Pyke and Leech, 1994; Robertson *et al.*, 1996; Marrison *et al.*, 1999) and associated with the PD ring

(Robertson *et al.*, 1996). This phenotype, together with the localization of this protein, suggests that dynamin is required in the later stage of chloroplast division, in contrast to the FtsZ and PD rings.

### *3. Sequential Transition of the FtsZ, PD and Dynamin Rings*

The dynamin-related protein CmDnm2 is a cytosolic protein that localizes at the cytosolic side associated with the outer PD ring of the plastid division site (Miyagishima *et al.*, 2003a; Fig. 4). Although this location is similar to that of the outer PD ring, a study using *C. merolae* showed that CmDnm2 is not the major component of the outer PD ring (Miyagishima *et al.*, 2003a). In contrast to the outer PD ring, CmDnm2 forms a continuous ring only at the final phase of division at which point it localizes between the outer PD ring and outer envelope (Miyagishima *et al.*, 2003a; Fig. 4). Therefore, the plastid division apparatus is composed of at least FtsZ, three PD, and dynamin rings and these rings form in this order at the division site.

The FtsZ ring forms first, inside the chloroplast (Fig. 5, stage 2), and then the PD ring forms from the inner to the outer rings before division site constriction (Fig. 5, stage 3). The FtsZ ring then localizes at the stromal side of the inner PD ring. During contraction a patch-like structure including dynamin is recruited to the cytosolic surface of the division site (Fig. 5, stages 3 and 4). During the late constriction stage, the dynamin ring forms on the cytosolic side of the outer PD ring from these cytoplasmic patches (Fig. 5, stage 5), and then migrates to the space between the outer PD ring and outer envelope (Fig. 5, stage 6). In addition, during this stage when constriction is still in progress, the FtsZ ring disassembles and FtsZ is excluded from the division site and the two future daughter chloroplasts (Miyagishima *et al.*, 2001c) (Fig. 5, stage 6). The middle and inner PD rings disappear just before the completion of division, while remnants of the outer PD ring remain between the daughter chloroplasts, and remnants of the dynamin ring remain clinging to each daughter chloroplast (Fig. 5, stage 7). After completion of division, the remnants of the PD and dynamin rings disappear in the cytosol (Miyagishima *et al.*, 2001a, 2001c, 2003a).

It is still unclear which ring or combination of rings is directly responsible for bringing about constriction; the association of rings with the constriction site does not necessarily imply that these structures produce

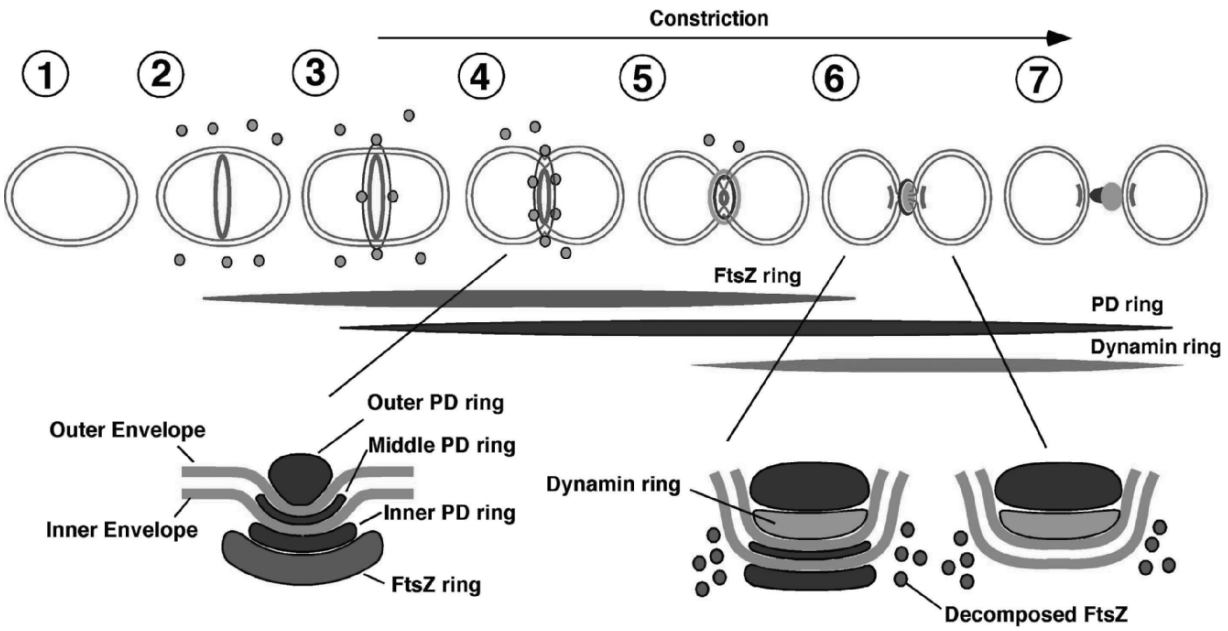


*Fig. 5.* (See also Color Plate 2, p. xxxv.) Sequential transition of the plastid division apparatus composed of chloroplast the FtsZ, PD, and dynamin rings. Sequential events during the chloroplast division are illustrated in the upper side. A cross section of the division site is shown on the lower side. Red, FtsZ ring; green, patches or ring of dynamin-related protein; black, PD ring (only the cytosolic outer PD ring is shown in the upper illustration). See text for details.

the force required for constriction. Although the conformation of assembled FtsZ (Lu *et al.*, 2000) and dynamin (Marks *et al.*, 2001) is altered by the hydrolysis of GTP, it is premature to conclude that these proteins produce sufficient force to constrict the organelle. A recent study suggested that dynamin is merely a regulatory GTPase, rather than a mechanochemical protein (Sever *et al.*, 2000). Of the five rings, only the outer PD ring exists throughout division site constriction. Constriction starts just after formation of the outer PD ring (Miyagishima *et al.*, 2001a). Consequently, the outer PD ring and 5 to 7 nm filaments composing the ring (Mita and Kuroiwa, 1988; T. Kuroiwa *et al.*, 1998; Miyagishima *et al.*, 2001b) are most likely associated with the generation of the constriction force by sliding the filaments in the initial stages (T. Kuroiwa *et al.*, 1998; Miyagishima *et al.*, 2001b).

### *E. The Evolutionary Relationship Between Plastid and Mitochondrial Division*

In chloroplast division, the FtsZ, PD and dynamin rings form in that order. This system of plastid division must have been established long ago in primitive algae because, generally, the same mechanism is observed in primitive red alga and higher plants.

After endosymbiosis, FtsZ and some factors regulating FtsZ ring formation were retained by the plastids, while most of the other proteins recruited by FtsZ were lost. The dynamin ring (and probably also the PD ring) was then added after endosymbiosis. At least the PD and FtsZ rings have been conserved in the chloroplasts subsequent to secondary endosymbiosis, although apicomplexan parasites have lost FtsZ (Osteryoung, 2001).

The most striking suggestion given by recent studies is that almost the same event occurred during the establishment of mitochondria. Some protists have retained α-proteobacterial FtsZ for mitochondrial division. The MD ring, which has a structure very similar to the PD ring, was found in red algae (T. Kuroiwa *et al.*, 1993, 1998) and in the true slime mold *Physarum polycephalum* (T. Kuroiwa *et al.*, 1977; T. Kuroiwa, 1986). A dynamin-related protein is also involved in mitochondrial division in diverse eukaryote species (Bleazard *et al.*, 1999; Labrousse *et al.*, 1999; Sesaki and Jensen, 1999; Smirnova *et al.*, 2001; Arimura and Tsutsumi, 2002; Nishida *et al.*, 2003). During mitochondrial division in the red alga *C. merolae*, the FtsZ, MD and dynamin rings form in this order like in chloroplast division (Nishida *et al.*, 2003). These facts suggest that mitochondria divided by almost the same mechanisms as plastids soon after they were established in lower eukaryotes. Mitochondria were established before plastids suggesting that the host cell used the same strategy to regulate the division of the cyanobacterial endosymbiont as it did for the α-bacterial endosymbiont.

Most of the eukaryotes examined thus far lack mitochondrial FtsZ and the MD ring has never been observed in higher eukaryotes. Based on these results it is suggested that during evolution FtsZ was lost from the mitochondria and the MD ring was lost or simplified during eukaryotic evolution. The loss (or simplification) of these structures occurred independently in plants and in a common ancestor of fungi and animals because algae retained these structures after they branched from fungi and animals. It is likely that there are unknown mechanisms that cause constriction in the mitochondria at least until dynamin localizes at the division site during the late stage of constriction.

The fact that dynamin-related proteins associate with both the chloroplast and mitochondrion during division suggests that factors related to these proteins are conserved in chloroplast and mitochondrial division. In *S. cerevisiae*, two proteins that bind to Dnm1p have been identified and shown to be involved in mitochondrial division. One protein, called Mdv1p/Fis2p/Gag3p/Net2p, is a WD-repeat protein that colocalizes with Dnm1p, and the other, Fis1p/Mdv2p, is an integral component of the mitochondrial membrane and is evenly distributed on the surface of the mitochondria (Osteryoung and Nunnari, 2003). There are no obvious plant and algae specific paralogs of these proteins in *C. merolae* or *A. thaliana* suggesting that these partners are not involved in chloroplast division, although other common partners might await discovery.

## IV. Conclusions and Future Research Directions

Recent studies identified some proteins involved in the plastid division apparatus and gave significant insights into the evolutionary origin of the plastid division machinery: 1) Plastid division is accomplished by a division apparatus composed of nucleus-encoded proteins, 2) plastid division machinery is a combination of both cyanobacterial-derived and eukaryote-specific systems, and 3) the division machinery is very similar between plastids and primitive mitochondria.

Components of the division machinery identified thus far, except for the dynamin-related protein, are

derived from cyanobacterial-ancestor and these proteins were identified mainly based on incorporation of information of bacterial division system. Now, however, it seems that analyses based on bacterial information have been saturated. Because many of cyanobacterial cell division proteins were missing from photosynthetic eukaryotes, there should be several factors, including the PD rings, which are derived from eukaryotic host. Identification of factors derived from eukaryotic host should give us further insights into how eukaryotic host had regulated division of the cyanobacterial endosymbiont and then turned it into plastid.

Since plastids contain DNA-protein complexes (nucleoids) as do bacteria, plastid division consists of nucleoid division and division of membrane as described earlier. Although, the membrane division has been studied and the study was reviewed in this chapter, mechanism of nucleoid division and relationship between nucleoid and membrane division have not been studied well in molecular level. Division machinery of membrane have been studied and now being clarified in detail whereas it is still unknown how the timing and frequency of formation of the division apparatus are regulated by eukaryotic host. Recent molecular studies have revealed only a few aspects of plastid division, in which many systems should be involved based on many earlier cytological studies. Although there are still several issues that should be addressed, conclusion of this chapter should give significant hints to address those issues.

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# Section II

## The Plastid Genome and its Interaction with the Nuclear Genome

# Chapter 6

# Expression, Prediction and Function of the Thylakoid Proteome in Higher Plants and Green Algae

Klaas van Wijk*
*Department of Plant Biology, Emerson Hall 332, Cornell University, Ithaca, NY 14853, U.S.A.*

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## Summary

The thylakoid membrane system contains not only the photosynthetic electron transport chain, with about 100 proteins, but also many additional peripheral and integral thylakoid proteins, most of which do not have a known function. Many of these additional thylakoid proteins are expected to be involved in binding and stabilization of cofactors, or folding and turnover of thylakoid proteins. The thylakoid proteome forms a functional network, with expression levels of individual proteins that vary dependent upon chloroplast function and age as well as environmental conditions. The thylakoid proteome of chloroplast of higher plants, green algae and photosynthetic bacteria have received a significant amount of attention in recent years. Various fractionation and mass spectrometry

*Author for correspondence, email: kv35@Cornell.edu

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techniques have been applied to catalogue the thylakoid proteome. Neural Network and Hidden Markov Models, in combination with experimentally derived filters, have been used to try to predict the lumenal and thylakoid membrane proteome. Some of the many protein–protein interactions, as well as post-translational modifications, have been characterized. Nevertheless, our understanding of the thylakoid proteome and its dynamics is very incomplete. Rapid improvements and wide-scale implementation of mass spectrometry and new tools for comparative proteomics, in particular those based on stable isotope labeling, will undoubtedly accelerate this understanding in the near future. The Plastid Proteome Data Base (PPDB at http://ppdb.tc.cornell.edu/) and other web resources for thylakoid proteome data are discussed. This chapter will briefly summarize recent experimental and theoretical efforts concerning different aspects of thylakoid proteomics and outline future challenges and possibilities.

## I. Introduction

Characterization of the proteins of the thylakoid membrane system is essential to understand thylakoid function, its biogenesis and breakdown during senescence, and its dynamic responses to changing developmental states and abiotic conditions. This characterization not only includes the identification of proteins but also determination of protein expression levels, protein–protein and protein–ligand interactions, post-translational modifications and sub-thylakoid localization. Advances in proteomics have been driven by rapid advances in mass spectrometry (MS) using the soft ionization techniques of matrix assisted laser desorption ionization (MALDI) and electro spray ionization (ESI) and the exponentially increasing amount of genome and expressed-sequence tag (EST) data from different species. Despite continuous improvements in proteomics techniques and tools, it remains challenging to identify proteins that are expressed only under particular conditions (e.g. adverse growth conditions, developmental stage, etc.), or with very low expression levels. However, it may be possible to predict these low abundant or transient proteins using prediction strategies. Focused approaches can then be used to experimentally identify these candidate proteins. Successes and shortcomings of such theoretical thylakoid proteome predictions will be reviewed. The increasing amounts of thylakoid and other plant 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 essential and will be discussed.

A significant number of reviews have been published in recent years on the topic of proteomics and we refer to these for general aspects of proteomics and mass spectrometry (e.g. Aebersold and Mann, 2003; Patterson and Aebersold, 2003; Steen and Mann, 2004). The topics of plant proteomics, chloroplast proteomics and thylakoid proteomics were recently summarized in special issues of the journals Photosynthesis Research (volume 78:3 in 2003) and Phytochemistry (volume 65:11 in 2004) and by van Wijk (2000, 2004). The current chapter summarizes the most recent published efforts and strategies concerning experimental and theoretical characterization of the thylakoid proteome in higher plants and green algae.

## II. Experimental Identification of the Thylakoid Proteome in Chloroplasts of Plants and Algae

### *A. Challenges and Fractionation*

Compared to the total plant proteome, with tens of thousands of proteins, the thylakoid proteome is relatively simple. However, complete identification of the thylakoid proteome is nevertheless challenging, in particular because of the wide range in levels of protein expression, with ratios of molar concentrations between different protein species ranging from 1 to 1,000,000 or more. In addition, some proteins are likely to be only transiently expressed, such as expression of the high-light stress response proteins (e.g. Elips). This wide expression range and dynamic nature make thylakoid proteome analysis quite challenging. Given the current status of proteome technology, it is quite clear that no single approach will be optimal to capture all proteome information. In general, cataloging proteomes clearly

*Abbreviations:* cICAT – cleavable isotope coded affinity tags; cTP – chloroplast transit peptide; ESI – electro-spray ionization; EST – expressed sequence tag; FTICR – Fourier transform ion cyclotron resonance; GRAVY – grand average of hydropathy values; HPLC –high performance liquid chromatography; IMAC – immobilized-metal-affinity chromatography; LC – liquid chromatography; LHC – light-harvesting chlorophyll a/b-protein complex; lTP –lumenal transit peptide; MALDI – matrix assisted laser desorption ionization; MS – mass spectrometry; MS/MS – tandem mass spectrometry; PPO – polyphenol oxidase; SPP – stromal processing peptidase; TMD – trans-membrane domain; TMHMM – hidden Markov model transmembrane predictor; TOF – time of flight; TPP – three-phase partitioning.

benefits from fractionation into subcellular compartments, because knowing the exact subcellular localization(s) of a protein is often important to determine its functional role. In the case of the thylakoid proteome, fractionation based on membrane affinity and solubility will help to determine the nature of the protein–thylakoid interactions. These different protein fractions or sub-proteomes, can then be directly analyzed by MS, or further fractionated using one-dimensional (1-D) or two-dimensional (2-D) on-line chromatography (e.g. multidimensional protein identification technology or MUDPIT) and/or different off-line fractionation techniques (e.g. 1-D and 2-D gel electrophoresis, off-line chromatography, organic solvent extraction, etc.). Parallel use of different ionization techniques (MALDI and ESI) to bring peptides as ions into the gas phase leads to improved coverage of simple and complex proteomes. Connecting data from different approaches will bring about a coherent picture of where proteins are located, and whether they are of high or low abundance.

The thylakoid proteome can be divided into the lumenal, integral and stromal side peripheral thylakoid proteome. Alternatively, the thylakoid system can be divided, based on the level of membrane stacking, into granal lamellae and stromal lamellae. Each of these thylakoid sub-fractions has its own subset of proteins, or sub-proteomes. In addition, proteins can be present at multiple sub-chloroplast locations, e.g., associated with thylakoid and envelope membranes. These different purified chloroplast sub-proteomes have been investigated using a variety of approaches and plant species, in particular, *Arabidopsis thaliana*, pea, spinach, maize, rice, and the green alga *Chlamydomonas reinhardtii*.

## *B. Identification of Lumenal and Peripheral Thylakoid Membrane Proteins*

A significant number of lumenal thylakoid proteins and proteins associated with the stromal side of the thylakoid membrane have been identified over the past decades. Many of these proteins are fairly abundant and are part of the photosynthetic apparatus. With improvements in mass spectrometry of proteins and peptides, and the increasing amount of genome-sequence information, it has been possible to more systematically try and identify proteins associated with the thylakoid system.

Systematic experimental analyses of protein fractions enriched for lumenal and/or peripheral proteins of the thylakoids, first from pea, spinach and subsequently *A. thaliana*, were carried out by protein separation on 2-D electrophoresis gels with immobilized pH gradient (IPG) strips as the first dimension and SDS-PAGE as the second dimension. This was followed by protein identification using peptide mass finger-printing using MALDI-TOF MS and off- or on-line ESI-MS/MS and Edman degradation sequencing (Kieselbach *et al.*, 1998; Peltier *et al.*, 2000; Peltier *et al.*, 2002; Schubert *et al.*, 2002; Friso *et al.*, 2004). One of the most striking discoveries was the large number of lumenal peptidyl-prolyl *cis-trans* isomerases and the extensive family of lumenal proteins with similarity to OEC23, a well-known component of the water-splitting complex of photosystem II (PSII). The functions of most of these proteins are still unknown. The N-termini of many proteins were sequenced by Edman degradation to validate localization prediction, taking advantage of the observation that lumenal proteins have a cleavable lumenal transit peptide (lTP), located directly downstream of the cleavable chloroplast transit peptide (cTP) (see below for further discussion on localization predictions).

To extract soluble peripheral proteins that very tightly interact with the thylakoid membrane, salt-washed and sonicated *A. thaliana* thylakoids were solubilized in Triton X-114 followed by 2-phase separation and resolution of the water-soluble proteins on 2-D gels (Friso *et al.*, 2004). The Triton X-114-extracted population represented only ~7% of the total extracted peripheral proteome and was dominated by OEC33 and the family of fibrillins. These fibrillins are typically associated with lipid particles or plastoglobules that interact with the thylakoid membrane or are present in the stroma (Kessler *et al.*, 1999; Rey *et al.*, 2000). The affinity of the lumenal and peripheral proteins for the thylakoid membrane was assessed by cross-correlating the different extracted protein populations (Friso *et al.*, 2004).

Collection of all confirmed lumenal proteins from these proteomics papers, and other published literature (e.g. Gupta *et al.*, 2002a; Weigel *et al.*, 2003), showed that so far 50 lumenal proteins have been experimentally identified in *A. thaliana.* A complete list can be found in Sun *et al.* (2004) or downloaded from the plastid proteome database, PPDB (http://ppdb.tc.cornell.edu/). Orthologs for these lumenal proteins typically exist in other plant species and more than over 200 lumenal proteins sequences have been collected (Peltier *et al.*, 2002; Westerlund *et al.*, 2003). Interestingly, poly-phenol oxidase (PPO) is a fairly abundant, thylakoid lumenal protein found in many plant species, such as tomato, spinach, maize,

clover and others (Newman *et al.*, 1993; Sommer *et al.*, 1994; Hind *et al.*, 1995), but orthologs are absent in *A. thaliana*. PPOs are copper metalloproteins that catalyze the oxidation of mono- and *o*-diphenols to *o*-diquinones. PPOs are up-regulated upon wounding and herbivore attack, mediated by the octadecanoid wound-signaling pathway (Koussevitzky *et al.*, 2004). Possibly, *Arabidopsis* and other *Brassicacea* have different defense mechanisms, not dependent on PPO.

### C. Identification of Integral Membrane Proteins in Higher Plant Thylakoids

Twenty to thirty percent of the genes in any sequenced genome encode integral membrane proteins that contain one or more alpha-helical trans-membrane domains (TMD) (Krogh *et al.*, 2001). These membrane proteins typically fulfill critical functions in the transport of ions, small organic molecules and proteins, or in intra- and inter-cellular communication. Hydrophobic membrane proteomes are challenging to analyze experimentally because of their hydrophobic nature, resulting in insolubility, adsorption, and incompatibility with ionic detergents with mass spectrometry.

A very significant number of integral thylakoid membrane proteins or proteins that interact tightly with the thylakoid membrane (e.g. via parallel helices or possibly via lipid anchors) have been identified over several decades, prior to emergence of proteomics. Many of those are abundant members of the four major complexes of the photosynthetic apparatus. In addition, several components of the NADH dehydrogenase complex, alternative oxidase (Immutans), and an interesting protein involved in cyclic electron flow (PGR5) (Munekage *et al.*, 2002) have been identified. Others are protein translocation components, such as SecY, SecE, Alb3 and TatA/E (Tha4) and TatB (HCF106) (reviewed in Mori and Cline, 2001) and thylakoid members of the FtsH protease family (Lindahl *et al.*, 1996; Chen *et al.*, 2000). With the current generation of mass spectrometers and sequence information, it is now possible to systematically identify the thylakoid membrane proteome (or least the more abundant members).

In general, different experimental strategies for large scale identification of membrane proteins have been explored (reviewed in Wu and Yates, 2003). Despite extensive efforts to synthesize non-ionic detergents for separation of membrane proteins by 2-D gels, with IPG strips in the first dimension, no significant membrane protein separation with high dynamic resolution and quantitative recovery has been reported (see Santoni *et al.*, 2000). This is particularly true for the more hydrophobic membrane proteins, with positive grand average of hydropathy values (GRAVY) as a measure of hydrophobicity (Kyte and Doolittle, 1982). As will be discussed further below, 2-D gels have been used in studies on abundant light-harvesting proteins (LHCPs), which could be visualized on 2-D gels thanks to their high abundance, although the percentage of recovery is unknown. Organic solvent extraction, using a mixture of chloroform/methanol (Molloy *et al.*, 1999; Seigneurin-Berny *et al.*, 1999; Ferro *et al.*, 2003; Friso *et al.*, 2004), or direct extraction by methanol (Blonder *et al.*, 2002), or chromatography (Tarr and Crabb, 1983; Lew and London, 1997; Gomez *et al.*, 2002) have proven successful for different types of plant and non-plant membrane proteomes, either combined with 1-D SDS-PAGE, followed by in-gel digestion and mass spectrometry (MS) or in-solution digestion and on-line-LC MS/MS. Cyanogen bromide cleavage of the insoluble fraction of yeast cells, combined with extensive 2-D LC-MS/MS, was also successful in identification of yeast membrane proteins (Washburn *et al.*, 2001). Recently, direct methanol extraction of membranes, followed by in-solution trypsin digestion in buffered methanol of membrane fragments, and nano-LC-ESI-MS/MS was reportedly successful in identifying a significant fraction of integral bacterial and human membrane proteins (Blonder *et al.*, 2004a; Blonder *et al.*, 2004b).

In the remainder of this section, I will briefly review proteomics studies on the hydrophobic thylakoid proteome. Whitelegge and his group analyzed PSII-enriched thylakoid membranes from pea and spinach (so-called BBY particles) (Gomez *et al.*, 2002). After extraction of the intact proteins from the lipid bilayer, the intact protein mixture was separated by reverse-phase HPLC and analyzed by on-line ESI-MS. Identification of the corresponding proteins was carried out based on these intact protein mass measurements. This approach worked well for the abundant members of the thylakoid membrane proteome, because these are well-characterized proteins (e.g. N-termini are known) and because many of the proteins are encoded by the small, sequenced plastid genome. Around 90 intact mass tags were detected, corresponding to approximately 40 gene products with different post-translational modifications. Provisional identification of 30 of these gene products was based on coincidence of measured mass with that calculated from genomic sequence and prior identification of the N-terminus (Whitelegge *et al.*, 2003). In a subsequent paper from the same group, the intact mass measurement approach

was combined with chemical cleavage using cyanogen bromide, followed by peptide identification by LC-ESI-MS/MS and MALDI-TOF MS (Gomez *et al.*, 2003). Fifty-eight redundant and abundant nuclear-encoded thylakoid membrane proteins were identified from different plant species (35 non-redundant proteins from *A. thaliana*), and their N-termini were assigned based on the combined information from the intact protein mass measurements and the peptide fragments. These N-termini were compared with processing sites predicted by ChloroP for cleavage by the stromal processing peptidase and SignalP for prediction of proteins with lumenal transit peptides, as done in earlier studies on the thylakoid proteome (Peltier *et al.*, 2000; Peltier *et al.*, 2002). It was concluded that cleavage sites of a small set of integral thylakoid membrane proteins that are inserted via the "spontaneous" pathway (i.e. not assisted by proteins—see Robinson *et al.*, 2001), were correctly predicted by SignalP, whereas the cleavage site of several LHC proteins whose insertion was dependent upon the signal recognition particle (and Alb3—see Eichacker and Henry, 2001) were not accurately predicted (Gomez *et al.*, 2003). It is curious that SignalP predicted the cleavage site of some proteins better than ChloroP. It should be noted that the thylakoid membrane likely contains some 100 to 200 integral membrane proteins, but the mechanism for insertion is not known for the majority of proteins.

Zolla and colleagues followed the intact mass measurement strategy advocated by Whitelegge to address the accumulation of members of the LHC family of PSI and PSII in different plant species (Huber *et al.*, 2001; Zolla *et al.*, 2002; Zolla *et al.*, 2003). Identification and relative quantification of individual members is a challenge because of the high sequence similarity between different members of LHC subfamilies. However, differential accumulation of the LHCI and LHCII proteins appears important in adaptation of plants to different abiotic conditions (see Ganeteg *et al.*, 2004, for discussion). Protein separation and identification were achieved by means of reversed-phase HPLC-ESI-MS.

In a separate study, Schmid and colleagues studied the protein composition of the LHC of PSI of tomato leaves ( *Lycopersicon esculentum)* (Storf *et al.*, 2004). They combined 1-D and 2-D gel electrophoresis with immunoblotting and ESI-MS/MS. This analysis identified Lhca1-5 as well as additional paralogs for Lhca4 and Lhca5.

In an effort to systematically analyze the thylakoid membrane proteome from *A. thaliana* chloroplasts, Friso *et al.* (2004) used acetone/chloroform/methanol fractionation of salt-stripped thylakoid membranes, combined with ESI-MS/MS. The original extraction protocols that Ferro *et al.* (2000) used for the chloroplast envelope proteome were adapted to remove the very abundant hydrophobic chlorophylls and carotenoids and to improve extraction efficiency. The fractions were further separated by different techniques (1-D gels, offline HPLC, and enzymatic and nonenzymatic protein cleavage techniques) to improve dynamic resolution. In addition to the nearly complete set of hydrophobic members of the thylakoid-bound photosynthetic machinery, several low abundant proteins were also identified, such as components of the Sec translocon and PGR5 involved in cyclic electron flow (Munekage *et al.*, 2002). Altogether, 154 proteins were identified, of which 76 (49%) are alpha-helical integral membrane proteins. Twenty-seven new proteins without known function but with predicted chloroplast transit peptides were identified, of which 17 (63%) are integral membrane proteins. These new proteins, likely important in thylakoid biogenesis, include two rubredoxins, a potential metallochaperone, and a new DnaJ-like protein. The data were integrated with the analysis of the lumenal-enriched proteome, as published by Peltier *et al.* (2002) and Schubert *et al.* (2002).

To identify additional integral membrane proteins or proteins that are tightly bound to the thylakoid via lipid anchors or short helical structures parallel to the membrane plane, a new fractionation protocol was developed (Peltier *et al.*, 2004), based on a so-called "three-phase partitioning" (TPP) technique using *t*-butanol, which was originally developed successfully for soluble proteins (Dennison and Lovrien, 1997). These TPP protocols were adapted to membrane proteins, using salt-stripped thylakoid membranes as the target proteome. This adaptation comprises essentially the switch from *t*-butanol to *n*-butanol, addition of solubilization buffer (SDS, urea and tributyl phosphine) to the upper and lower phase, and selection of appropriate $(NH_4)_2SO_4$ concentrations and pH shifts (Peltier *et al.*, 2004). Using this new strategy, 242 proteins were identified in the salt-stripped thylakoid membrane of *A. thaliana*, at least 40% of which are integral membrane proteins. The functions of 86 proteins are unknown and include proteins with tetratricopeptide repeats, pentatricopeptide repeats, rhodanese and DnaJ domains. The TPP analysis allowed visualization of a whole new "layer" of proteins, including nearly 100 proteins without known function, as well as known protein translocation components of the thylakoid membrane system.

### D. Functional Classification of the Thylakoid Proteome in Higher Plants

Together with more classical "gene for gene" studies collected from many published studies, these experimental thylakoid proteomics studies discussed above identified 384 proteins (Peltier *et al.*, 2004). A small number (less than 5%) of these proteins are contaminations from the chloroplast envelope, or (abundant) proteins from other subcellular locations.

All proteins were assigned to functional categories, using a classification developed for MapMan (Thimm *et al.*, 2004), and classification was subsequently simplified into 15 groups (Table 1) (Peltier *et al.*, 2004). As expected, the largest percentage of proteins is involved in photosynthetic electron transport, ATP synthesis and chlororespiration (30%). In addition, 25% of the proteins have no obvious function; many of these can be expected to be involved in biogenesis and maintenance of the photosynthetic apparatus. Proteins involved in folding, processing and proteolysis also represent a very significant fraction (18%), whereas 8% of the proteome is involved in various aspects of direct or indirect defense against oxidative stress (Table 1 and Fig. 1a).

Using the trans-membrane predictor TMHMM and experimental data from the primary literature, we split these 384 proteins into three groups: (i) 50 soluble lumenal proteins, (ii) 143 integral membrane proteins, and (iii) 190 peripheral proteins that do not have a lumenal transit peptide. Some proteins of the latter category are possibly more abundant in the stroma. Thirty-six of the TMD proteins and 9 of the peripheral proteins are chloroplast-encoded. No chloroplast-encoded lumenal proteins are known. Functional classifications of the proteins in each group are shown in Fig. 1b, which suggest that the dominating functions of the known lumenal proteome are mainly in protein folding, processing and degradation in addition to photosynthesis, while about 10% of the lumenal proteins have no known function. Of course, this is a simplification, because the precise functions of the many isomerases and OEC paralogs are still unknown. The predominant function of the known integral thylakoid membrane proteins is photosynthesis (50% of total), while about 25% of the integral membrane proteins have no predicted function and 5 to 7% of the integral membrane proteins are involved in oxidative stress defense. The functions of the peripheral proteome on the stromal side of the thylakoid membrane are most diverse. It shows that the thylakoid membrane functions as a "platform" for DNA and RNA binding and protein translation, in addition to biosynthesis of a number of compounds. A small fraction is directly involved in photosynthetic electron transport. The functions of about 25% of the thylakoid proteome are unknown. In reality, that percentage may be even larger, because the precise function for many proteins that were assigned to a functional class is in fact not known (Fig. 1b).

### E. Thylakoid Proteome Analysis in the Green Alga Chlamydomonas reinhardtii

Sequencing of the *C. reinhardtii* nuclear genome to near completion and the known sequences of the organellar genomes have made the green alga *C. reinhardtii* an even more attractive unicellular model

*Table 1.* Functional classification for the thylakoid and envelope proteomes. Proteins were identified by thylakoid proteome studies (Peltier *et al*., 2002, 2004; Schubert *et al*., 2002, Friso *et al*., 2004) or by more "classical" biochemical tools, forward or reverse genetics. Together this resulted in 384 thylakoid and other (integral, associated or co-purified) proteins. All proteins received a functional classification as described in Thimm *et al*. (2004). Functional classification of thylakoid proteins are expressed in percentage of total.

| Functional category | Thylakoid proteins (% of total) |
|---|---|
| (Cyclic) electron transport, ATP synthesis, chlororespiration | 30.1 |
| Unknown function | 25.2 |
| Protein fate | 18.2 |
| Redox proteins and oxidative defense and stress | 8.1 |
| DNA/RNA/translation | 6.2 |
| Calvin-Benson cycle, photorespiration, starch, OPP, glycolysis | 2.3 |
| Terpenoids and tetrapyrrole metabolism | 2.3 |
| Other developmental functions | 2.1 |
| Hormone and lipid metabolism | 1.6 |
| Other metabolic functions | 1.0 |
| N, S, aa and nucleotide metabolism | 0.8 |
| Mitochondria and others | 0.8 |
| Co-factor and vitamin metabolism, metal handling | 0.8 |
| Transport of ions and other small molecules | 0.5 |

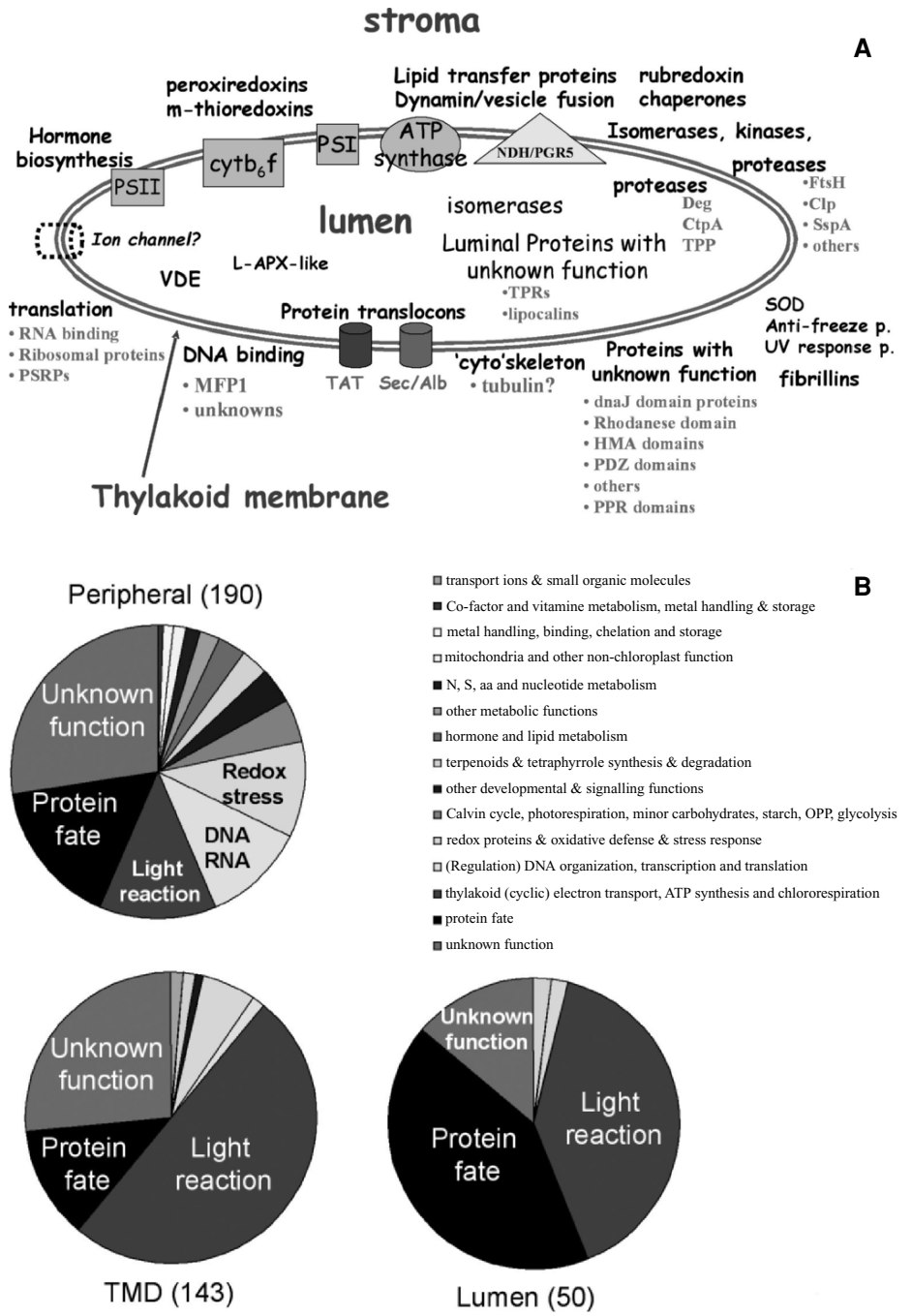


*Fig. 1.* (See also Color Plate 3, p. xxxvi.) Functions of the thylakoid proteome. (a) Overview of the thylakoid proteome and its compartmentalization. (b) Functional classification of the lumenal, peripheral and integral thylakoid proteome from *Arabidopsis thaliana*. Proteins were identified by large scale proteomics studies or with more "classical" biochemical tools or forward or reverse genetics. Thylakoid proteins were collectively identified from experimental thylakoid proteomics studies (Peltier *et al.*, 2002; Schubert *et al.*, 2002; Friso *et al.*, 2004; Peltier *et al.*, 2004). In addition, the literature was carefully screened for additional thylakoid and envelope proteins. Together this resulted in 384 thylakoid proteins.

organism to study chloroplast and thylakoid function (Rochaix, 2002; Gutman and Niyogi, 2004). The newly available sequence information was used to analyze the extensive family of light-harvesting genes in *C. reinhardtii* (Elrad and Grossman, 2004), which includes nine genes encoding polypeptides of the major light-harvesting complex of PSII, two genes encoding the minor light-harvesting polypeptides of PSII, and nine genes encoding polypeptides predicted to comprise the PSI light-harvesting complex. Furthermore, there are five genes encoding early light-induced proteins and two genes encoding L18 polypeptides (Elrad and Grossman, 2004).

Hippler and colleagues experimentally analyzed the LHCI and LHCII family in the thylakoid proteome of the green algae *C. reinhardtii*, particularly in the context of iron deficiency and different photosynthetic mutants. They used 2-D gel electrophoresis, combined with immunoblots and MS (Hippler *et al.*, 2001; Moseley *et al.*, 2002; Stauber *et al.*, 2003; Takahashi *et al.*, 2004). Aspects of comparative proteome analysis are discussed in Section VI. Two-dimensional protein maps of *C. reinhardtii* thylakoids and PSI particles, were generated and abundant LHCI and LHCII proteins identified by ESI-MS/MS (Stauber *et al.*, 2003). Peptides were identified that were unique for specific LHCIIs. Epitope tagging experiments confirmed the presence of differentially N-terminally processed Lhcb6 proteins. The mass spectrometric data revealed differentially N-terminally processed forms of Lhcb3 and phosphorylation of a threonine residue in the N-terminus. Several LHCI proteins were also identified. It is relevant to note that Hippler and colleagues strongly advocated the use of 2-D gels (with IPG strips) for thylakoid membrane proteomics, despite a general consensus that recovery of membrane proteins from 2-D gels is very poor (Santoni *et al.*, 2000; Luche *et al.*, 2003).

## III. Properties and Predictions of the Thylakoid Proteome

### *A. Properties and Predictions of the Integral Thylakoid Proteome*

Using the subcellular localization predictor TargetP in combination with the transmembrane-domain predictor TMHMM, 520 plastid-localized proteins were predicted to have one of more TMDs. When using the consensus prediction listed in the database Aramemnon (http://aramemnon.botanik.uni-koeln.de/), which contains a consensus prediction of membrane proteins, this number is slightly larger. It is important that we can predict which of these 520 TMD proteins are actually located in the thylakoid membrane and which are located in the inner membrane of the chloroplast envelope. With the exception of those proteins that carry a lumenal transit peptide (lTP) for targeting the N-terminus to the lumen (Mori and Cline, 2001; Robinson *et al.*, 2001) or an L18 domain in the case of a subset of chlorophyll-binding thylakoid proteins (Tu *et al.*, 2000), we do not know how these putative integral membrane proteins are sorted within the chloroplast to the inner envelope or to the thylakoid membrane. It is possible that examination of the known thylakoid and envelope proteome could suggest physical-chemical properties that can be then used for predictions in which membrane the less studied TMD proteins are located.

Taking advantage of the accumulated chloroplast proteome information, Sun and colleagues assembled carefully curated proteomes of the inner envelope membrane, the thylakoid membrane, and the thylakoid lumen of chloroplasts from *A. thaliana* from published, well-documented localizations (Sun *et al.*, 2004). These curated proteomes were evaluated for distribution of physical-chemical parameters with the goal to extract parameters for improved subcellular prediction and subsequent identification of additional (low abundant) components of each membrane system. The three curated proteomes differ strongly in average pI and protein size, as well as trans-membrane distribution, with thylakoid membrane proteins being smaller, more acidic and having on average a lower number of TMDs. Unexpectedly, the cysteine content was much lower for the thylakoid proteomes than for the envelope inner membrane, with 60% of processed thylakoid TMD proteins containing only one or no cysteine residues. In contrast, nearly all known inner envelope (and stromal) proteins have multiple cysteines. The low number of cysteine residues in the thylakoid proteome likely relates to the role of the thylakoid membrane in light-driven electron transport and helps to avoid unwanted oxidation/reduction reactions. Based on the different physical-chemical properties between thylakoid and inner envelope TMD proteins, a "rule-of-thumb" for discriminating between the predicted integral inner envelope membrane and integral thylakoid membrane proteins was proposed (Fig. 2).

### *B. Properties and Predictions of the Lumenal Proteome*

As far as it is known, all lumenal thylakoid proteins have a cleavable, N-terminal signal peptide, designated lTP. The lTPs have no conserved sequence motif but do

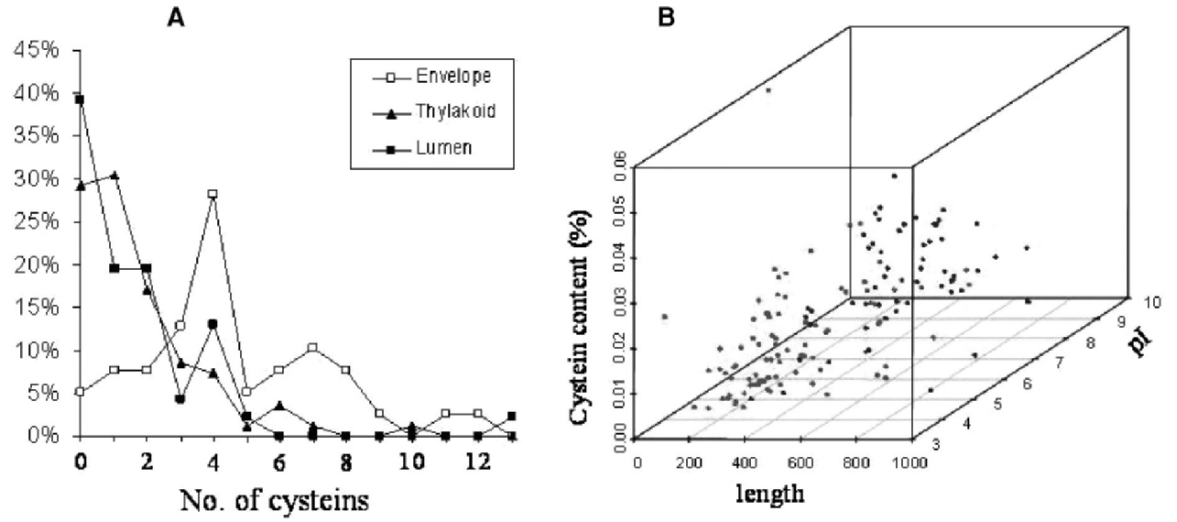


*Fig. 2.* (See also Color Plate 3, p. xxxvi.) Properties of the thylakoid and envelope proteomes. (a) Frequency distribution of proteins in the three curated proteomes based on the number of cysteines. (b) Rule of thumb to distinguish integral thylakoid membrane proteins from integral inner envelope proteins. Scatter plot of the relative cysteine content, protein length and pI for the curated sets of 61 inner envelope and 110 thylakoid integral membrane proteins. The predicted cTPs were removed prior to calculation of these three parameters. The curation procedure and the list of accession numbers can be found in Sun *et al.* (2004). Envelope proteins are symbolized by blue dots and thylakoid proteins by red dots (adapted from Sun *et al.*, 2004).

have a bias in amino acid content that is rather similar to bacterial signal peptides used for translocation of proteins from the cytosol to the periplasm. lTPs can be divided into "n", "a", "h" and "c" domains. The "n" (N-terminal) domain has charged residues, the "a" domain is variable in length, the "h" domain is hydrophobic, and the "c" domain is adjacent to the cleavage site (Keegstra and Cline, 1999). In addition, the cleavage site is quite well conserved and most have the sequence "AxA", but a number of variations were observed (Peltier *et al.*, 2002). Lumenal proteins translocate through the thylakoid membrane either via the so-called "TAT" pathway or via the "Sec" pathway. The lTPs provide most of this pathway specificity, with possible contributions of the "mature" sequence (reviewed in Mori and Cline, 2001; Robinson *et al.*, 2001). In a recent paper, 203 lumenal proteins were collected from different plant species (based on experimental data and ortholog prediction), and experimental parameters were extracted to allow prediction of lumenal proteins (Peltier *et al.*, 2002). It turned out that the TAT substrate pool could be fairly well predicted, but the Sec substrate pool was much more difficult to predict, leading to over-prediction. In an effort to improve the prediction of the lumenal proteome, a specific lTP predictor (LumenP) was developed (Westerlund *et al.*, 2003). Subsequently, the existing predictors, TargetP, SignalP and TMHMM, and LumenP (Westerlund *et al.*, 2003), in combination with different filters, were used to predict the soluble lumenal proteome and thylakoid membrane proteins with lTPs (Sun *et al.*, 2004). These predicted plastid sub-proteomes were compared to the curated experimental sets. This analysis showed that prediction of the lumenal proteome still seems to be difficult, even when including the experimentally derived filters and considering the fairly large training set (>200 proteins) used for developing LumenP (Sun *et al.*, 2004).

## IV. Characterizing Thylakoid Protein Complexes and Protein–Protein Interactions

### *A. Introduction*

Proteins often function in association with other proteins, either transiently or as a stable complex. Identification of interacting proteins is important for establishing the function of newly identified proteins. In past years, tremendous opportunities have arisen to rapidly determine protein interactions, using the strength of modern mass spectrometry combined with complete genome sequence data. Indeed, large scale protein–protein interaction studies using epitope-tagged transgenes were carried out particularly in yeast (Gavin *et al.*, 2002; Ho *et al.*, 2002). In case of thylakoids, most studies concerning protein–protein interactions and

protein complexes used detergent solubilization of the thylakoid lipid bilayer, followed by chromatography, sucrose density fractionation and/or native gel electrophoresis. In some cases, co-immunopreciptation, possibly combined with cross-linkers, was employed. With the emergence of a significant number of thylakoid proteins without obvious functions, determination of protein–protein interactions will be valuable for understanding thylakoid proteome function. In this section we briefly summarize knowledge of protein–protein interactions and protein complexes in the thylakoid system.

### *B. Complexes of the Photosynthetic Apparatus*

The thylakoid proteome is dominated by the four major protein complexes—PSI, PSII, the cytochrome $b_6f$ complex, and the ATP synthase complex. In the last decade, these complexes have been studied in great detail using a range of biochemical and biophysical techniques, frequently combined with (tagged) disruption or anti-sense mutants. In the past 2 years, high resolution X-ray crystal structures were obtained for the cytochrome $b_6f$ complex from the green alga *C. reinhardtii* and from spinach (Stroebel *et al.*, 2003; Zhang and Cramer, 2004). Potentially 53 different proteins were identified in higher plant PSII, when the different OEC paralogs are included, as summarized in Friso *et al.* (2004). It is not clear how many of those are (always) part of the PSII complex. Twenty-three different protein species were found in higher plant PSI complexes. Several of these are present in multiple copies within the complex. The cytochrome $b_6f$ and the ATP synthase complexes are much simpler in protein composition and have, respectively, 8 and 9 different proteins in one or more copies. The predicted members of the NADH dehydrogenase complex in higher plants, forming a fifth complex of much lower abundance, have not all been experimentally observed. These very extensive structural and functional studies are beyond the focus of this chapter, and the reader is referred to previous volumes in this book series, as well as a large number of wonderful reviews. For recent reviews on protein composition, structure and function, I refer to general reviews and books on the photosynthetic apparatus in higher plants and *C. reinhardtii* (Ort and Yocum, 1996; Rochaix *et al.*, 1998; Wollman *et al.*, 1999). For additional reviews, I suggest Shi and Schroder (2004) for the small subunits of PSII, Scheller *et al.* (2001) for a review on the role of the different PSI subunits in eukaryotes, Ben-Shem *et al.* (2003) for a 4.4 Å resolution structure on PSI from spinach, Stroebel *et al.* (2003) for a 3.1 Å structure of the cytochrome $b_6f$ complex from *C. reinhardtii*, and several that describe the composition and function of NADH dehydrogenase complex (Sazanov *et al.*, 1998; Peltier and Cournac, 2002; Casano *et al.*, 2004).

### *C. Other Thylakoid Protein Complexes and Protein–Protein Interactions*

For the many thylakoid proteins that are not part of any of these major complexes, protein interactions partners are unknown. However, in recent years a number of protein–protein interactions have been established. Examples are components of the thylakoid protein translocation machinery, including the TAT complex (Fincher *et al.*, 2003), the SecY/E complex in association with Alb3 (Schuenemann *et al.*, 1999; Klostermann *et al.*, 2002), and interactions between SRP54, FtsY and Alb3 (Moore *et al.*, 2003). The interactions between Alb3 and SecY, the TAT components and FtsY and Alb3 are to some extent transient. In the case of thylakoid-bound FtsH proteases, several complexes were observed (Sakamoto *et al.*, 2003; Yu *et al.*, 2004). Other protein interactions are concerned with biogenesis of photosynthetic proteins, such as the interactions between a lumenal peptidyl-prolyl isomerase (immunophilin, FK506 and rapamycin-binding protein) and the Rieske subunit of the cytochrome $b_6f$ complex (Gupta *et al.*, 2002b). Systematic studies of the protein–protein interactions of the many thylakoid proteins without known functions will undoubtedly help to obtain a more complete overview of the thylakoid proteome.

## V. Post-Translational Modifications of the Thylakoid Proteome

### *A. Introduction*

Stable or transient post-translational modifications can help to anchor proteins to membranes (in case of lipid moieties), regulate activity or protein interactions (as, e.g. phosphorylation), stabilize proteins (e.g. glycosylation and N-terminal formylation), or target proteins for degradation (e.g. ubiquitination). Thus, investigation of post-translational modifications is important but often challenging, requiring a significant amount of protein material, mass spectrometry skills and time. There are many different approaches to determine such post-translational modifications. They can be based on

specific assays (e.g. radio-labeling) or on mass spectrometry, possibly in combination with affinity purification or other purification techniques (e.g. immobilized-metal-affinity chromatography, IMAC) to enrich for a particular type of post-translational modification. In general, it is becoming easier to establish that a protein has been modified, although the precise determination of the site of modification is often quite time consuming, requiring significant amounts of protein material.

Fourier transform ion cyclotron resonance (FTICR)-MS is emerging as a powerful technique to determine post-translational modifications, because many peptide and other bonds can be broken in one MS experiment (especially when using different dissociation techniques) and FTICR-MS instruments have very high mass accuracies. Currently, intact proteins up to ~50kDa can be directly fragmented (Kelleher *et al.*, 1999; Ge *et al.*, 2002). The injection of intact proteins and subsequent fragmentation and characterization within the mass spectrometer has been named "top-down" proteomics (McLafferty *et al.*, 1999; Kelleher, 2004). This stands in contrast to the more widespread "bottom-up" approach, in which proteins are cleaved enzymatically with proteases such as trypsin, chymotrypsin or LysC or non-enzymatically with cyanogen bromide prior to injection into the mass spectrometer. McLafferty and colleagues applied this "top-down" approach on different chloroplast protein fractions and showed that even in a complex protein mixture, intact proteins can be identified and modifications determined (Zabrouskov *et al.*, 2003). In practical terms, the "top-down" approach of complex proteomes is currently only possible in a few specialized labs. However, with further improvements and commercialization, FTICR mass spectrometers in the "top-down" and "bottom-up" approach are likely to become quite central in detailed protein analysis (Patrie *et al.*, 2004).

### *B. N-Terminal Processing and Other N-Terminal Modifications*

As mentioned earlier, the nuclear-encoded thylakoid proteins are imported as precursor proteins with, in nearly all cases, an N-terminal chloroplast transit peptide or cTP. These N-terminal cTPs are removed by a ~140kDa stromal processing peptidase (SPP) that was initially cloned from pea (Oblong and Lamppa, 1992) and is found not only in chloroplasts but also in non-green plastids (VanderVere *et al.*, 1995). This SPP belongs to the family of M16 proteases and is characterized by a conserved His-Xaa-Xaa-Glu-His zinc-binding motif (VanderVere *et al.*, 1995). Only one ortholog of the pea SPP was found in the genome of *A. thaliana*. Recent antisense lines using a full-length cDNA construct from pea SPP in tobacco showed a strong mutant phenotype, with a large percentage of the plants dying as seedling-lethals. Surviving plants exhibited slower shoot and root growth, grossly aberrant leaf morphology, often green and white sectors, and purple pigmentation. In cells where chloroplasts could be identified, they were fewer in number by at least 40%, thylakoids were not fully developed, and starch granules accumulated (Zhong *et al.*, 2003). Together with *in vitro* processing assays using over-expressed SPP and precursors of LHC, ferredoxin and other proteins (Richter and Lamppa, 1998), this one SPP is apparently responsible for most, if not all, processing of cTPs. The cleaved cTPs are only released from SPP after a second cleavage in the C-terminal part of the cTPs. These data are in conflict with suggestions that chloroplasts contain more than one SPP (Su and Boschetti, 1993). However, given the observation of different N-termini of a number of LHCs (e.g. Stauber *et al.*, 2003), it is possible that additional proteases remove amino acid residues from the processed N-terminus of imported nuclear-encoded chloroplast and thylakoid proteins.

A physiologically important excision of N-terminal methionine by peptide deformylase and methionine aminopeptidase seems to occur for many of the chloroplast-encoded proteins of *A. thaliana* and *C. reinhardtii* (Giglione and Meinnel, 2001; Giglione *et al.*, 2003). This process was already shown for several spinach thylakoid proteins by Bennet, Michel and colleagues (Michel *et al.*, 1988; Michel *et al.*, 1991) and recently confirmed by Gomez *et al.* (2003). Pulse-chase experiments revealed that inhibition of peptide deformylase leads to destabilization of a crucial subset of chloroplast-encoded PSII components in *C. reinhardtii*, showing that plastid N-terminal methionine excision is a critical mechanism specifically influencing the life-span of PSII polypeptides (Giglione and Meinnel, 2001; Giglione *et al.*, 2003). However, the mechanisms of proteolysis responsible for the destabilization are unknown.

### *C. Phosphorylation*

Many thylakoid proteins undergo reversible phosphorylation that is dependent on light conditions and chloroplast redox state. This topic has received significant attention as evidenced by many experimental studies and reviews. For recent reviews see Aro and Ohad (2003), Zer and Ohad (2003), and Aro *et al.* (2004).

The first observations of light-dependent phosphorylation of thylakoid proteins dates back to the 1970's and early 1980's by D.R Ort, J. Bennett, C.J. Arntzen, I. Ohad and many others (cited in reviews listed above). Phosphorylation was recognized as being important in state transitions, which regulate the excitation energy balance between the photosystems. In the 1980's several specific thylakoid proteins were shown to be phosphorylated, with Edman degradation sequencing used for identification. Tandem mass spectrometry, in combination with enrichment for phosphopeptides, identified four phosphoproteins, PsbH, D1, D2 and CP43, in PSII core complexes from spinach (Michel *et al.*, 1988). Tryptic digestion of core particles released four phosphopeptides, which were purified by affinity chromatography on $Fe^{3+}$-chelating Sepharose and reverse-phase HPLC. One peptide represented the N-terminus of PsbH. Using tandem mass spectrometry, three peptides were found to belong to the N-terminus of D1, D2, and CPa-2 or CP43. Each began with N-acetyl-O-phosphothreonine, indicating that D1 and D2 had lost their initiating N-formylmethionyl residues. In contrast, CP43 was processed at the N-terminus by removal of the first 14 amino acids. Using both *in vitro* assays and an *in vivo* approach with a mutant of maize that was deficient in cytochrome $b_6f$, it was shown that this complex is needed for LHC phosphorylation but not for PSII phosphorylation (Bennett *et al.*, 1988). In a subsequent study using radiolabeled ATP and light treatments, Michel and colleagues showed light-dependent phosphorylation of several LHCII proteins on their N-termini (Michel *et al.*, 1991). Treatment of the membranes with proteinase K or thermolysin released phosphopeptides that were purified by $Fe^{3+}$-affinity chromatography and reverse-phase HPLC. Sequencing of the phosphopeptides was performed with tandem quadrapole mass spectrometry. Peptides were found to be acetylated at their N-terminal arginine and were phosphorylated on either threonine or serine in the third position. It was concluded that proteolytic processing of pre-LHCII occurs at a conserved methionyl-arginyl bond and is followed by N-terminal acetylation of the arginine and nearby phosphorylation of the mature LHCII. From a comparison of the kinetics of phosphate incorporation into the eight different peptides, it was concluded that basic residues on both sides of the phosphorylation site are important for enzyme recognition. Acetylation of the N-terminus is not required for phosphorylation (Michel *et al.*, 1991).

Vener and colleagues used the so-called "parention scanning", as well as IMAC, to characterize phosphorylated thylakoid proteins more systematically (Vener *et al.*, 2001; Hansson and Vener, 2003). From the ESI-MS/MS analysis of tryptic peptides released from the surface of *Arabidopsis* thylakoids, several new phosphoproteins and phosphorylation sites on well-known photosynthetic proteins were identified. Interestingly, by comparing the levels of phospho- and non-phosphopeptides from thylakoids isolated after different light or temperature treatments, they showed that none of these thylakoid proteins were completely phosphorylated in continuous light nor completely dephosphorylated after a long dark adaptation. Based upon observed +80-Da adducts, D1, D2, CP43, two Lhcbs, and PsbH were confirmed to be phosphorylated, as observed by Vener *et al.* (2001) and in numerous other phosphorylation studies (e.g. Pursiheimo *et al.*, 1998), and a new phosphoprotein was proposed to be the product of *psbT*. The appearance of a second +80-Da adduct on PsbH provided direct evidence for a second phosphorylation site, as was described by Vener *et al.* (2001). Adducts of 32 Da, presumably arising from oxidative modification during illumination, were associated with more highly phosphorylated forms of PsbH, which implied a relationship between the two phenomena (Gomez *et al.*, 2002).

### *D. Palmitoylation and Other Lipid Modifications*

D1, the PSII reaction center protein, with its very high turnover rate, has two post-translational modifications that received significant attention. It was discovered in 1982 that the chloroplast-encoded D1 protein is processed at its C-terminus (Reisfeld *et al.*, 1982). Subsequent studies showed that 10 to 15 amino acids are removed from the C-terminus by a protease present at the lumenal side of the thylakoid membrane and that processing was necessary for formation of an active water-splitting complex (Diner *et al.*, 1988; Nixon *et al.*, 1992). Interestingly, there is a processing enzyme that is specifically expressed for processing the D1 protein. This D1-processing enzyme, assigned CtpA, was first cloned in cyanobacteria (Shestakov *et al.*, 1994) and subsequently in spinach and barley (Inagaki *et al.*, 1996; Oelmuller *et al.*, 1996). The structure of CtpA from the alga *Scenedesmus obliquus* was obtained by X-ray crystallography at 1.8 Å resolution (Liao *et al.*, 2000). CtpA was further characterized by Satoh and colleagues (Yamamoto *et al.*, 2001). Curiously, the *Arabidopsis* genome encodes three proteins that are each annotated as CtpA (1,2,3). It is unclear whether all three CtpA proteins are required. Only one of them, At4g17740.2, was identified by proteomics studies

on peripheral and lumenal thylakoid proteins from *A. thaliana* (Schubert *et al.*, 2002) and studies using IPG strips and native gels (K.J. van Wijk, unpublished data).

The D1 protein, or at least a sub-population, also seems to have a lipid modification as originally described by Mattoo and Edelman (1987). This was recently confirmed by analysis of isolated PSII complexes using chromatography and intact mass measurements, which allowed detection and resolution of a minor population of D1 that was apparently palmitoylated (Gomez *et al.*, 2002).

Water-soluble proteins can often tightly bind to biological membranes by lipid anchors, typically attached at the C-terminal part of proteins. Interestingly, no peripheral thylakoid membrane proteins are known that have lipid anchors, although systematic thylakoid proteome analyses clearly show that a significant population of tightly bound peripheral thylakoid proteins exist that lack any predicted trans-membrane domain (Peltier *et al.*, 2004) (see section IIB). It is possible that these proteins are anchored via strong protein-protein interactions, via short amphipathic helices inserted parallel to the lipid bilayer, or by lipid anchors. There is evidence for protein prenylation in chloroplasts from *in vivo* [$^{3}$H]mevalonate labeling of isoprenoids in spinach combined with biochemical analysis (Parmryd *et al.*, 1997). Approximately 20 prenylated polypeptides were detected by autoradiography after separation by SDS-PAGE. Thermolysin treatment of intact chloroplasts revealed that about 40% of the prenylated polypeptides were associated with the cytoplasmic surface of the envelope outer membrane. The remaining portion was present in thylakoids and/or the envelope inner membrane. The majority of the prenylated polypeptides were associated with larger membrane protein complexes. A farnesyl protein transferase activity was found that was associated with thylakoid membranes (Parmryd *et al.*, 1997).

## VI. Expression Analysis of the Thylakoid Proteome or Comparative Thylakoid Proteomics

Comparative proteomics studies are anticipated to provide insight into the cellular response to abiotic and biotic factors, as well as developmental processes in many different species. In addition, proteomics should help to determine the consequence of gene disruptions. So far, comparative proteomics in plants have mostly involved comparative 2-D gels. Recent examples are studies on seed development in *A. thaliana* and *Medicago trunculata* (Gallardo *et al.*, 2002; Gallardo *et al.*, 2003), the response of the *A. thaliana* nuclear proteome to low temperatures (Bae *et al.*, 2003), and changes in the leaf proteome of pea plants during the seed filling stage (Schiltz *et al.*, 2004). The only published comparative proteomics studies of thylakoid membranes are those by Hippler and colleagues concerning expression of LHC proteins in *C. reinhardtii* under copper deficiency (Moseley *et al.*, 2002; Stauber *et al.*, 2003). In these studies, 2-D gels were used with fully denaturing IPG strips in the first dimension, immuno-blotting and MS. They showed that the loss of LHCI and PSI could be visualized on the 2-D maps. The LHCI and many of the PSI proteins are integral to the membrane (GRAVY indices are mostly between −0.1 and 0). Despite the identification of these hydrophobic membrane proteins on 2-D gels, the recovery of such membrane proteins is typically low and is unlikely to give reliable quantitative results (Santoni *et al.*, 2000).

Several differential stable isotope labeling techniques have emerged and matured sufficiently to carry out meaningful comparative analysis of soluble and membrane proteomes (Goshe and Smith, 2003; Ong *et al.*, 2003). Typically, these isotope labeling techniques are combined with off-line and on-line chromatography of differentially labeled peptide mixtures. Hundreds to several thousands of proteins can be identified and the relative pair-wise concentrations of large numbers of peptides determined. These isotope labeling techniques can be divided between those that are based on addition of the different stable isotopic compounds during growth of the organisms (e.g. $^{13}$N and $^{15}$N in the form of nitrate or [$^{12}$C]arginine and [$^{13}$C]arginine), or those that are based on labeling after extraction of the proteome of interest. When working with plants and other multi-cellular organisms that do not grow naturally in liquid medium, it is often more convenient to use post-extraction labeling. The best example is cysteine-based labeling using cleavable isotope coded affinity tags (cICAT). A newer method is based on a free amine labeling reagent for multiplexed relative and absolute protein quantification (assigned iTRAQ). iTRAQ was commercially launched by Applied Biosystems in the summer of 2004 and has not been rigorously tested in the scientific community (at the time of submission of this chapter in 2004).

The thylakoid proteome is unique in that more than 60% of the known thylakoid proteins in the lumen and integral to the membrane have no or only one cysteine (Sun *et al.*, 2004). In contrast, nearly all chloroplast envelope proteins and also stromal proteins have one or more cysteine. Thus comparative

proteome analysis of the thylakoid proteome cannot be based on cysteine-based isotope labeling methods such as cICAT. Recently a paper was published showing the use of hydrogenated and deuterated formaldehyde in comparative proteomics (Hsu *et al.*, 2003). Under the appropriate conditions formaldehyde reacts specifically with N-terminal amines and the epsilon amine of lysines (Hsu *et al.*, 2003). We have optimized and implemented this labeling technique for the thylakoid proteome (A.J. Ytterberg and K.J. van Wijk, unpublished data). In combination with reverse-phase LC-ESI-MS/MS and quantification from extracted mass chromatograms in MS mode, this differential labeling is accurate and not biased with respect to the isotope. The layout of a typical comparative proteome experiment is shown in Fig. 3a. Examples of MS spectra of the stable isotope pairs of thylakoid membrane proteins are shown in Fig. 3b. The advantage of formaldehyde labeling (deuterated and hydrogenated) is that (i) it is very inexpensive and commercially available, (ii) the derivatization procedure for dimethyl labeling is faster and simpler when compared to some of the other methods, such as cICAT and (iii) the ionic state is not changed significantly by dimethyl modification, and therefore the ionization efficiency of the fragment is more likely to be conserved (Hsu *et al.*, 2003). Finally, the dimethyl modification is a global labeling procedure that labels not only lysine residues but also the N-terminus of the peptide, without significant isotopic effects. In comparison, peptides lacking cysteine residues cannot be labeled by cICAT.

Based on developments of attractive isotope labeling tools for comparative membrane proteomics and the rapid development of software for data interpretation and quantification, it is likely that comparative and truly quantitative thylakoid membrane proteome studies are now within reach. This will open up tremendous opportunities to study the role of the environment on the thylakoid membrane proteome and also study plastid development and senescence in great detail. Finally, chloroplast and thylakoid mutant analysis can be carried out directly at the thylakoid proteome level, without being restricted to only those proteins for which antisera have been generated.

## VII. Bioinformatics Resources for Plastid Proteomics Data

Proteomics studies often generate a large amount of data that are only meaningful when they can be easily accessed via the "world-wide-web" and connected to other types of biological information. The Plastid Proteome Data Base (PPDB) at http://ppdb.tc.cornell. edu/ is a specialized proteome database dedicated to plant plastids. The main objective is to provide a centralized, curated, data deposit for predicted and experimentally determined plastid proteins, their protein-protein interactions and annotated functions, as well as their experimental and predicted molecular and biophysical properties in denatured and native form. Cross-correlation between experimentally identified proteins from *A. thaliana* and maize is available through BLAST alignments. The content of PPDB can be directly accessed through its web interface. Multiple search methods are provided so that the user can retrieve information based on gene identification number, functional annotation and/or various protein properties. Active links to other databases such as TAIR (http://www.arabidopsis.org/) and TIGR (http://www.tigr.org/) are present.

## VIII. Conclusions and Challenges

The composition of the thylakoid proteome is becoming more clear. About 50 non-redundant lumenal thylakoid proteins, ~140 integral thylakoid proteins and over 150 peripheral proteins at the stromal side of the thylakoid membrane have been identified, particularly in *A. thaliana*. About 30% of these proteins are directly involved in the light reactions of photosynthesis, whereas the functions of 25% of the proteins are still unknown. A significant percentage of proteins is involved in different aspects of protein biogenesis, varying from processing and degradation to (un)folding and membrane translocation. Finally, as expected, at least a dozen thylakoid-associated proteins are involved in dealing with active oxygen species. However, based on predictions, many more thylakoid proteins remain to be discovered. Now that so many thylakoid proteins are known, the new challenge is to determine their functional contributions to thylakoid and chloroplast biology. This is likely to include determination of protein-protein interactions as well as protein modifications. The latter might also help to determine the nature of the interactions with thylakoid membranes of proteins lacking trans-membrane domains. Finally, quantitative comparative proteomics based on different stable-isotope labeling techniques will provide insight in the dynamic nature of the thylakoid proteome.

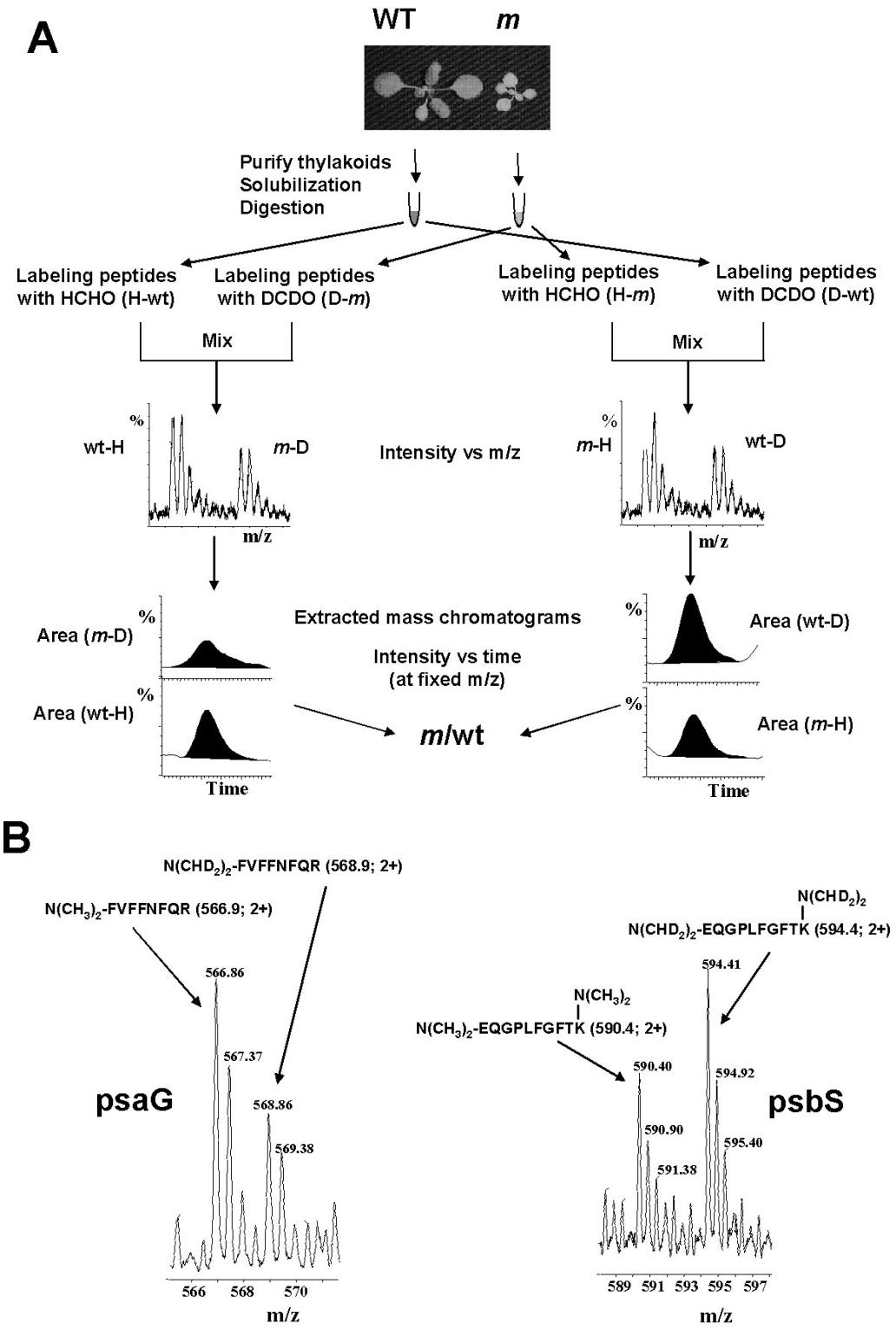


*Fig. 3.* (a) Simplified layout of a comparative proteomics experiment using differentially labeled formaldehyde. (b) Differential labeling of thylakoid membrane proteins psaG (2 TMDs) and psbS (4 TMDs) with [$^1$H]- or [$^2$H]formaldehyde. Total in-solution digests of thylakoid membrane samples (from a cpSRP54 mutant and wild-type *Arabidopsis*) were either labeled with [$^1$H]formaldehyde (four hydrogens) or with [$^2$H]formaldehyde (4 deuteriums). The two labeled samples were mixed and measured by LC-ESI-MS/MS. The two MS spectra show peptide pairs for two different proteins. Peptides were sequenced in MS/MS. Note that the m/z difference is 4/2 for tryptic peptides with C-terminal arginine and 8/2 for tryptic peptides with C-terminal lysine. This result is very reproducible (A.J. Ytterberg, H. Rutschow and K.J. van Wijk).

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# Chapter 7

# The Role of Nucleus- and Chloroplast-Encoded Factors in the Synthesis of the Photosynthetic Apparatus

Jean-David Rochaix*
*Departments of Molecular Biology and Plant Biology, University of Geneva, 30, Quai Ernest Ansermet, 1211 Geneva, Switzerland*

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## Summary

The biogenesis of the photosynthetic apparatus depends on the concerted interactions between the nucleo-cytosolic and chloroplast genetic systems. Combined genetic and biochemical approaches in *Chlamydomonas* and land plants revealed a surprisingly large number of nucleus-encoded factors that are required for the different post-transcriptional steps of chloroplast gene expression. These steps include RNA processing, RNA stability, splicing, translation and assembly of the chloroplast-encoded proteins into functional complexes. The genes of several of these proteins were cloned and their products characterized. A large number of these factors were recruited from enzymes involved in RNA or general metabolism during evolution and integrated into the plastid gene expression machinery. Some of these factors are highly conserved in both prokaryotic and eukaryotic photosynthetic organisms while others appear to have been specifically tailored for a defined task in a way that is organism specific. Most of these factors are part of multimeric protein complexes, some of which interact specifically with chloroplast mRNAs. The activity of several factors is modulated by light and by the redox status of the chloroplast.

*Author for correspondence, email: jean-david.rochaix@ molbio.unige.ch

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## I. Introduction

The beginning of the study of chloroplast gene expression can be traced back to 1962 when Ris and Plaut provided the first unambiguous proof for the existence of chloroplast DNA by detecting Feulgen-positive regions that were DNAse-sensitive in the cytoplasm of the green alga *Chlamydomonas reinhardtii* (Ris and Plaut, 1962). Since that time considerable progress was achieved in characterizing the chloroplast gene expression system and in elucidating some aspects of its regulation. The sequencing of more than 20 chloroplast genomes from land plants, green, red and brown algae revealed that the number of plastid genes ranges between 48 and 200. However, when one considers only land plants and green algae, this number is between 100 and 130. The chloroplast gene expression system is heavily dependent on nuclear gene activity for two reasons. First, many of its basic constituents such as several RNA polymerase subunits, ribosomal proteins and translation factors are encoded by nuclear genes, translated on cytosolic ribosomes and imported into the chloroplast. Second, genetic analysis of many mutants deficient in photosynthetic activity in *Chlamydomonas* and land plants identified a large set of nucleus-encoded factors that are required for specific post-transcriptional steps in plastid gene expression such as RNA processing, RNA stability, editing, splicing and translation. The number of genes of this sort is not known with certainty, but it could well range in the hundreds. In recent years major efforts of several groups has been invested in identifying and in characterizing some of these genes and their products. An important part of this chapter is devoted to this topic with the exception of RNA editing, which will not be considered here because of space limitations.

*Abbreviations:* Caf – Crs-associated factor; CES – control by epistasy of synthesis; Cox – cytochrome oxidase; CRM – chloroplast RNA splicing and ribosome maturation domain; Crp – chloroplast RNA translational factor; Crs – chloroplast RNA splicing factor; EF-Tu – elongation factor Tu; EST – expressed sequence tag; Hcf – high chlorophyll fluorescence; Mca – accumulation/maturation of *petA* mRNA; Mcd – accumulation/maturation of *petD* mRNA; NEP – nucleus-encoded RNA polymerase; PEP – plastid-encoded RNA polymerase; PNPase – polynucleotide phosphorylase; PPR – pentatrico peptide repeat; PSI – photosystem I; PSII – photosystem II; PSRP – plastid-specific ribosomal protein; PTH – peptidyl-tRNA hydrolase; Raa – splicing factor for *psaA* RNA; RRF – ribosome release factor; Rubisco – ribulose 1,5-bisphosphate carboxylase-oxygenase; SRP – signal recognition particle; Tab – translation of *psaB* mRNA; Tat – twin-arginine pathway; Tbc – translation of psbC mRNA; Tca – translation of *petA* mRNA; TIC – translocon of inner chloroplast envelope membrane; TOC – translocon of outer chloroplast envelope membrane; TPR – tetratrico peptide repeat; UTR – untranslated region; ycf – hypothetical chloroplast open-reading frame.

## II. The Basic Chloroplast Gene Expression System

As any other gene expression system, that of plastids includes DNA, RNA polymerase, RNA processing enzymes, ribosomes and translation factors. Because of the endosymbiotic origin of chloroplasts, which involved the invasion of a primitive eukaryotic cell by a cyanobacterial-like prokaryote, it is not surprising that the chloroplast and bacterial gene expression systems are similar in many respects, although they also differ in others.

### *A. Chloroplast DNA*

In land plants and green algae, the size of the chloroplast DNA unit length is comprised between 120- and 200-kb and includes over 100 genes (Sugiura, 1992). Although standard chloroplast DNA preparations revealed mostly genome-sized circular DNA molecules and linear molecules supposed to arise from broken circles, pulse-field electrophoresis indicated that most of the chloroplast DNA is in linear monomers, multimers and complex branched forms with only 3 to 4% as circles (Oldenburg and Bendich, 2003). The chloroplast gene set can be divided into three groups. The first includes 68 to 74 genes coding for components of the plastid gene expression system: 3 subunits of the plastid-encoded DNA-dependent RNA polymerase, 30 to 35 plastid ribosomal proteins, in some cases the elongation factor EF-Tu and the initiation factor InfA, 4 to 5 rRNA genes and 30 to 31 tRNA genes. The second group contains about 30 genes coding for components of the photosynthetic complexes: photosystem II (14 subunits), photosystem I (4 to 5 subunits), the cytochrome $b_6f$ complex (5 subunits), the ATP synthase (6 subunits) and ribulose 1,5-bisphosphate carboxylase (1 subunit). Moreover, *ycf3* and *ycf4* encode PSI assembly factors (Boudreau *et al*., 1997a; Ruf *et al*., 1997) and in green algae and gymnosperms 3 genes encode factors required for the light-independent synthesis of chlorophyll (Li *et al*., 1993). The third group contains genes involved in various metabolic reactions. The *ccsA* gene is required for the attachment

of heme to *c*-type cytochromes (Xie and Merchant, 1996). Eleven genes encoding subunits of NADH dehydrogenase are present in the plastid genomes of land plants but are missing from the chloroplast genome of *Chlamydomonas*. In addition, several chloroplast open reading frames of unknown function were identified. Some of them, such as *ycf1* and *ycf2*, appear to be essential for cell growth and survival (Boudreau *et al*., 1997b; Drescher *et al*., 2000).

### B. Chloroplast DNA-Dependent RNA Polymerases

Given the small size of chloroplast genomes, the existence of at least two distinct RNA polymerases in land plants is rather surprising (Maliga, 1998). The first, called PEP for plastid-encoded RNA polymerase, is similar to the bacterial multi-subunit enzyme and consists of the chloroplast-encoded $\alpha$, $\beta$, $\beta'$, $\beta''$ subunits. Its specificity is conferred by several $\sigma$-type subunits that are nucleus-encoded (Allison, 2000). It represents the major plastid RNA polymerase and is required mainly for the transcription of the photosynthetic genes. The second polymerase, called NEP for nucleus-encoded polymerase, consists of a single subunit that resembles bacteriophage T3/T7 RNA polymerases. This polymerase could not be identified in *Chlamydomonas*. It appears to play an important role during the early steps of plastid development (Lerbs-Mache, 1993; Mullet, 1993). The PEP promoters are similar to the bacterial $\sigma^{70}$ promoters and contain the consensus $-35$ and $-10$ elements. NEP promoters usually contain the YRTA consensus, although some of them lack this element (Liere and Maliga, 2001). Analysis of wild-type and PEP-deficient plants led to the proposal that chloroplast genes or transcription units are transcribed by either PEP, NEP or both polymerases, with PEP and NEP involved mostly in the transcription of photosynthesis-related and housekeeping genes, respectively (Hajdukiewicz *et al*., 1997). However, recent studies using a genomic microarray approach revealed that in the absence of PEP most of the chloroplast genes are transcribed (Krause *et al*., 2000; Legen *et al*., 2002). These results indicate that the levels of the plastid transcripts are not only determined by the type of RNA polymerase but also depend on multiple parameters, including relaxed promoter specificity, transcriptional read-through, RNA processing and stability. Thus the integration of NEP into the chloroplast genetic functional network appears to be rather complex (see Chapter 8).

### C. RNA Processing Enzymes

Because of the cyanobacterial origin of chloroplasts, it is not surprising that the plastid RNA processing and degradation systems share several features with those of eubacteria. As in bacteria, RNA breakdown in chloroplasts starts with an endonucleolytic cleavage of RNA molecules followed by the addition of a polyA or polyA-rich tail (Schuster *et al*., 1999). These polyadenylated products are then digested by polynucleotide phosphorylase (PNPase) that catalyzes the 3′ to 5′ degradation of RNA, possibly together with other exoribonucleases. Under some conditions the enzyme catalyzes processive polymerization (Grunberg-Manago, 1999). Whereas in bacteria polyadenylation is catalyzed by poly-A polymerase, no such enzyme could be found in chloroplasts. Instead, plastid PNPase alone performs both the polyadenylation and the exonucleolytic degradation of the RNA cleavage products (Lisitsky *et al*., 1997; Yehudai-Resheff *et al*., 2001). Chloroplast PNPase exists as a homo-multimeric enzyme complex that is distinct from the *Escherichia coli* degradosome that consists of PNPase, RNase E, a DEAD-RNA helicase and enolase (Baginsky *et al*., 2001).

Most chloroplast endonuclease activities studied are involved in 3′-end formation. The spinach CSP41 enzyme preferentially cleaves stem-loop structures and has specific RNA binding properties (Yang *et al*., 1996; Yang and Stern, 1997). Another endonuclease involved in 3′-end terminal processing of plastid precursor transcripts is a 54-kDa protein of mustard that appears to be regulated by phosphorylation and by the redox state (Nickelsen and Link, 1993; Liere and Link, 1997). The activity of this enzyme is enhanced by phosphorylation and by the oxidized form of glutathione.

### D. Ribosomes

The chloroplast 70S ribosomes resemble those of bacteria by their size, their RNA and protein composition and their sensitivity to the same spectrum of antibiotics. Recent advances in proteomics have provided a very detailed picture of the protein composition of the plastid ribosomes (Yamaguchi and Subramanian, 2000). While most of their proteins have bacterial counterparts, a few polypeptides have been identified that are unique to chloroplasts. In spinach the 30S ribosomal subunit contains 25 proteins, of which 21 are orthologs of *E. coli* ribosomal proteins and 4 are additional plastid-specific ribosomal proteins

(PSRP1 to 4) (Yamaguchi and Subramanian, 2003). The 50S subunit contains 33 proteins, of which 31 are *E. coli* orthologs and 2 are plastid-specific (PSRP5,6) (Yamaguchi and Subramanian, 2000). In addition, the ribosome-recycling factor is associated with the 70S ribosome but not with either of the two purified ribosomal subunits. In *C. reinhardtii* the picture is slightly different. Among the 21 proteins of the 30S ribosomal subunit, 19 are orthologs of the corresponding *E. coli* proteins (Yamaguchi *et al*., 2002). One protein is homologous to spinach PSRP3. Moreover, a novel S1-domain-containing protein, PSRP7 was identified (Yamaguchi *et al*., 2002). Another distinctive feature is that the ribosomal proteins S2, S3 and S5 contain additional domains that are predicted to be located close to each other near the binding site of the S1 protein, based on the crystal structure of the bacterial 30S subunit. The 50S subunit of *C. reinhardtii* contains 27 orthologs of *E. coli* ribosomal proteins, and a homolog to the spinach PSRP6 protein. In addition, two proteins of 38- and 41-kDa, named RAP38 and RAP41 are associated with the 70S ribosome but not with either of the two ribosomal subunits (Yamaguchi *et al*., 2003). They are related to the spinach chloroplast CSP41 RNA-binding protein with endonuclease activity. It was proposed that these PSRP proteins could play a role in the regulation of translation in the chloroplast (Yamaguchi and Subramanian, 2000; Yamaguchi *et al*., 2003).

## III. Genetic Approach: Role of Ancillary Factors in Chloroplast Gene Expression

The biogenesis of the photosynthetic apparatus involves the participation and coordination of the nuclear and chloroplast genetic systems. Most chloroplast proteins are encoded by nuclear genes, translated on cytosolic 80S ribsomes and imported through the TOC/TIC protein translocation system into the chloroplast (Fig. 1; see Chapter 3). A limited number of chloroplast proteins of the photosynthetic apparatus are encoded by the chloroplast genome, translated on plastid 70S ribosomes and assembled with their nucleus-encoded partners into functional complexes. Insertion of proteins into the thylakoid membrane or translocation into the lumen can occur in four different ways, either through the Sec, SRP and Tat pathways or by spontaneous insertion (Cline and Henry, 1996).

Besides biochemical approaches that have provided important insights into chloroplast gene expression, genetic approaches have been particularly helpful in identifying new components of the plastid genetic system.

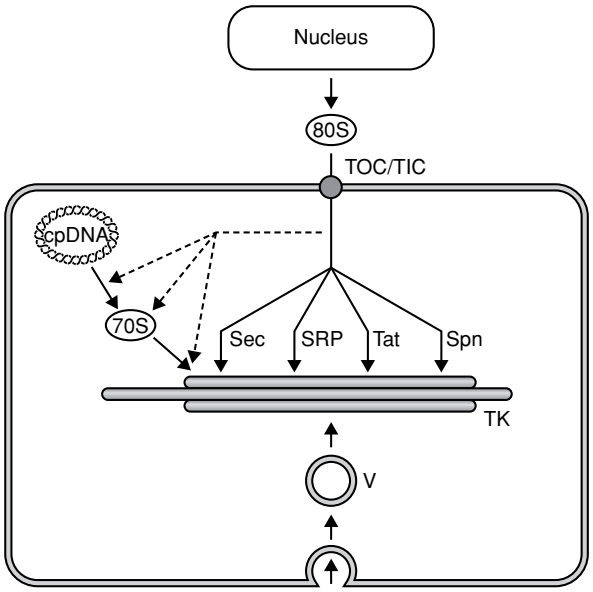


*Fig. 1.* Scheme of biogenesis of the photosynthetic apparatus. This system consists of both nucleus-encoded proteins that are translated on cytosolic 80S ribosomes and chloroplast-encoded proteins that are translated on chloroplast 70S ribosomes. A large set of nucleus-encoded factors are involved in different chloroplast post-transcriptional steps ranging from RNA processing, splicing, translation to assembly (broken lines). Nucleus-encoded proteins are imported through the TOC/TIC chloroplast protein translocation system. Four distinct pathways are used for targeting proteins to the thylakoid membranes (Tk): the Sec pathway (Sec), the SRP pathway, the Tat pathway (Tat) and spontaneous protein insertion (Spn). A vesicle system (V) may also contribute to transport proteins and lipids from the envelope to the thylakoid membrane.

The first interesting mutants were isolated more than forty years ago by Levine and coworkers during a large scale screen of mutants of *C. reinhardtii* unable to incorporate radioactive $CO_2$ (Levine, 1960). More efficient screens based on chlorophyll fluorescence output were developed later by Bennoun and Levine (1967) that are still used today. Besides yielding many mutants deficient in the catalytic functions of the photosynthetic complexes, the majority of these mutants were defective in several plastid post-transcriptional steps such as RNA stability, RNA processing, splicing and translation (Rochaix, 1996). A hallmark of these *Chlamydomonas* mutants is that they are usually specifically affected in the synthesis of a single chloroplast polypeptide. Moreover, a large number of mutants of this sort were identified and most turned out to be nuclear.

*Chlamydomonas* offers several unique advantages for the molecular analysis of these mutants. This is due to several reasons. First, this alga can be manipulated

with ease at the genetic, molecular, biochemical and physiological levels (Harris, 1989). Second, because of the plastid homologous recombination system, chloroplast transformation allows one to perform defined chloroplast DNA manipulations such as specific gene disruptions, exchange of 5′ and 3′ untranslated regions (UTR), site-specific mutagenesis or the insertion of chimeric genes at specific sites in the chloroplast genome (Boynton and Gillham, 1996). Third, the highly efficient nuclear transformation makes it possible to complement nuclear mutants with cosmid libraries containing wild-type genomic DNA (Purton and Rochaix, 1994; Zhang *et al*., 1994). Fourth, the random integration of foreign DNA during nuclear transformation provides an easy way for tagging nuclear mutants (Tam and Lefebvre, 1995). Fifth, the recent establishment of an EST data base and the determination of the nuclear genome sequence of *Chlamydomonas* offer considerable help for identifying new genes involved in chloroplast gene expression (http://genome.jgi-psf.org/chlamy/).

Genetic screens were also used to identify mutants affected in chloroplast gene expression in land plants by screening for pigment or high-fluorescence mutants (Miles, 1982). *Arabidopsis* emerged as a powerful system for this analysis because of its fast growth, its known nuclear genome sequence and the availability of numerous T-DNA insertion lines that provide a powerful tool for reverse genetics (Alonso *et al*., 2003). Another important model system is maize because of its well-developed genetics and because of the availability of numerous tagged lines deficient in photosynthetic activity. Its large size makes it particularly appropriate for biochemical analysis (Stern *et al*., 2004).

### *A. Chloroplast RNA Processing and Stability: Tetratricopeptide and Pentatricopeptide Proteins*

Several nuclear mutants of *C. reinhardtii* were identified that are unable to accumulate specific chloroplast RNAs. As an example, the *nac2* mutant does not accumulate *psbD* RNA whereas the *mbb1* mutant is deficient in *psbB* and *psbH* mRNA accumulation (Kuchka *et al*., 1989; Monod *et al*., 1992). In both cases it was demonstrated that the target sites of the factors affected by these mutations are located within the corresponding 5′-UTRs of these mRNAs (Nickelsen *et al*., 1994; Vaistij *et al*., 2000a). This was shown by inserting into the chloroplast genome chimeric genes consisting of the 5′-UTR of the gene of interest fused to the coding sequence of the *aadA* reporter gene that confers resistance to spectinomycin/streptomycin in the chloroplast (Goldschmidt-Clermont, 1991). The transformed chloroplast strain of mating-type (+) was subsequently crossed with the original mutant of mating type (−). In such a cross all the progeny inherit the chimeric gene from the mating type (+) parent, whereas the nuclear mutation from the mating type (–) parent segregate 2:2. If the 5′-UTR mediates the effect of the nuclear mutation, antibiotic resistance and accumulation of the chimeric RNA should be affected in the mutant nuclear background. This was indeed verified both for the *nac2* and *mbb1* mutations (Boudreau *et al*., 2000; Vaistij *et al*., 2000b). The *psbD* and *psbB* mRNAs exist in two forms, a less abundant long form and a more abundant short form, which differ in the length of their 5′-UTR (Nickelsen *et al*., 1999; Vaistij *et al*., 2000a). In the case of *psbD* these two forms contain 74 and 47 nucleotides, and in the case of *psbB* 135 and 35 nucleotides. In the two mutants the short-form RNA is undetectable whereas the long-form RNA is still detectable, suggesting that the Nac2 and Mbb1 factors could also be involved in processing of these RNAs. Although it was not possible to process these RNAs in vitro, it is unlikely that the two forms are generated by two distinct promoters because small deletions upstream of the processing site do not prevent the formation of the short *psbD* RNA. Site-specific mutagenesis of the *psbD* 5′-UTR revealed that the twelve 5′-terminal nucleotides are critical for *psbD* RNA stability (Nickelsen *et al*., 1999). Moreover, two regions in this 5′-UTR are important for RNA stability. Removal of a long stretch of U residues near the 3′-end had a mild affect on RNA accumulation, but completely abolished translation and photoautotrophic growth. A 40-kDa RNA-binding protein was identified that interacted with the wild-type *psbD* 5′-UTR, but not with the mutant *psbD* 5′-UTR that lacked the U-rich motif (Ossenbuhl and Nickelsen, 2000). RNA-binding was restored in a chloroplast suppressor that grew photoautotrophically and in which the spacing between two *cis*-acting elements in the *psbD* 5′-UTR, important for RNA stability and translation, was re-established (Nickelsen *et al*., 1999). Analysis of several mutant *psbD* 5′-UTRs revealed a clear correlation between photoautotrophic growth and the RNA-binding activity of the 40-kDa protein. This activity was dependent on the Nac2 protein, which may be required for the proper positioning of the 40-kDa protein on the *psbD* 5′-UTR (Ossenbuhl and Nickelsen, 2000). There is an intriguing similarity between the 40-kDa protein and the ribosomal protein S1 of *C. reinhardtii,* CreS1, which was also shown to bind specifically to the U-rich domain of the *psbD* 5′-UTR but considerably less to

*Table 1.* Nuclear mutants of *Chlamydomonas reinhardtii* deficient in chloroplast gene expression.

| | | Site of deficiency | | | |
|---|---|---|---|---|---|
| Mutant | Target | RNA processing and stability | Translation | Assembly | Reference |
| *raa1*, *raa2*, *raa3*, etc.* | *psaA* | + | | | Goldschmidt-Clermont *et al.*, 1990 |
| *F24* | *psaB* | + | | | Girard *et al.*, 1980 |
| *tab1*, *tab2* | *psaB* | | + | | Girard *et al.*, 1980 |
| *F15* | *psbA* | | + | | Drapier *et al.*, 1992 |
| *GE2.10* | *psbB* | + | | | Sieburth *et al.*, 1991 |
| *mbb1* | *psbB* | + | | | Vaistij *et al.*, 2000b |
| *6.2z5* | *psbC* | + | | | Sieburth *et al.*, 1991 |
| *tbc1* | *psbC* | | + | | Kuchka *et al.*, 1989 |
| *tbc2* | *psbC* | | + | | Auchincloss *et al.*, 2002 |
| *nac2*, (*MΦ14*)$^+$ | *psbD* | + | | | Boudreau *et al.*, 2000 |
| *nac1*, *ac115* | | | + | | Lown *et al.*, 2001 |
| *MΦ11* | *petA* | + | | | Girard-Bascou *et al.*, 1995 |
| *MΦ37* | *petB* | + | | | Girard-Bascou *et al.*, 1995 |
| *mcd1* | *petD* | + | | | Drager *et al.*, 1998 |
| *ncc1* | *atpA* | + | | | Drapier *et al.*, 1992 |
| *thm24* | *atpB* | + | | | Drapier *et al.*, 1992 |
| *thm24* | *rbcL* | + | | | L. Mets (unpublished results) |
| *ac-29* | *Lhc* | | | + | Bellafiore *et al.*, 2002 |
| *ac-29* | *PSII* | | | + | Ossenbuhl *et al.*, 2004 |

*Many mutants deficient in *psaA trans*-splicing were identified that fall into at least 14 nuclear complementation groups.
$^+$Allelic mutants are indicated in parenthesis.

the *psbD* 5′-UTR lacking this motif (Merendino *et al*., 2003). However, the size of CreS1, 45-kDa, appears to be distinct from that of the 40-kDa RNA-binding protein, and the relationship between these two proteins needs to be examined.

Besides *nac2* and *mbb1*, other nuclear mutants of *Chlamydomonas* were identified that are deficient in a specific chloroplast mRNA (Table 1). The *petD* RNA is missing in the *mcd1* mutant. Accumulation of this RNA was restored when a polyguanosine tract was inserted in the chloroplast genome in the *petD* 5′-UTR (Drager *et al*., 1998). PolyG sequences are known to block processive exonuclease activity (Caponigro and Parker, 1996). Similar insertions in the *psbD* 5′-UTR in *nac2* and in the *psbB* 5′-UTR in *mbb1* also led to the restoration of *psbD* and *psbB* RNA levels (Drager *et al*., 1998; Nickelsen *et al*., 1999; Vaistij *et al*., 2000a). In all these cases the 5′-ends of the transcripts mapped near the polyG border, suggesting that a 5′ to 3′ exonucleolytic activity was halted at this site. However, it is not possible to exclude that an endonuclease cleaves successively in the 5′ to 3′ direction, and the resulting RNA products are rapidly degraded by a 3′ to 5′ activity.

The genes affected in the *nac2* and *mbb1* mutants were isolated by genomic complementation with a *Chlamydomonas* cosmid library and the proteins characterized. An interesting feature of both proteins is that they contain 9 (Nac2) and 10 (Mbb1) TPR (tetratricopeptide repeat)-like domains (Boudreau *et al*., 2000; Vaistij *et al*., 2000b). TPR domains are present in many proteins involved in distinct cellular functions ranging from transcription, cell cycle control, to mitochondrial protein import. They consist of 34-amino-acid repeats that are loosely conserved. Each TPR repeat consists of a pair of two short antiparallel α-helices A and B. Tandem arrays of TPRs can form a superhelix enclosing a groove that is likely to act as a protein-binding domain (Das *et al*., 1998). Helix A and helix B in the TPR motif are on the inside and outside of the superhelix, respectively, so that most of the side-chains of helix A protrude into the ligand binding site. A mutation in a consensus residue within a single TPR domain of Nac2 abrogates its function (Boudreau *et al*., 2000). Moreover, a deleted version of Nac2 in which nearly all of its N-terminal half was removed, but that contains all TPR domains, is still able to rescue the *nac2* mutant. Taken together, these results indicate that the TPR domains play an important role for the activity of these factors. These proteins belong to a large family of helical repeat proteins. The atomic structure of several of them was determined, including protein phosphatase-5 with its 3 TPR repeats (Das *et al*., 1998), β–catenin with its 12 ARM repeats of 42 amino acids (Huber *et al*., 1997), the A-subunit of protein phosphatase-2A with its 15 HEAT repeats of 39 amino acids (Groves *et al*., 1999), and Pumilio with its 8 Puf repeats of 36 amino

acids (Edwards *et al*., 2001). These tandem helical repeats form an extended surface of the protein that is believed to be involved in protein-protein interactions. However, in the case of Pumilio, a translational regulator of the *hunchback* mRNA in *Drosophila*, this surface binds a specific RNA sequence (Edwards *et al*., 2001).

Recent evidence suggests that TPR-like domains are involved in protein-RNA interactions and may provide RNA-binding specificity. In particular, the TPR domains of the Nac2 protein act as specific RNA-binding sites (F. Barneche and J.D. Rochaix, unpublished results). Nac2 and Mbb1 are part of large molecular weight complexes that are sensitive to RNase treatment, indicating that they contain both proteins and RNA. It remains to be tested whether this RNA includes *psbD* and *psbB* mRNA.

Among the chloroplast transcription units, the *psbB-psbH* operon is one of the few that has been conserved between *Chlamydomonas* and land plants. The *Arabidopsis* mutant *hcf107* is specifically deficient in the processing of this transcription unit (Felder *et al*., 2001). In land plants this transcription unit is processed in a complex way and gives rise to multiple poly- or single-cistronic RNAs. In the *hcf107* mutant all transcripts containing *psbH* at their 5′-terminal end are specifically missing. Interestingly, the Hcf107 protein is a TPR protein and is the homolog of Mbb1 of *Chlamydomonas*. Thus in this case both the transcription unit and one of the processing factors have been conserved between land plants and *Chlamydomonas*.

A large family of proteins related to TPR proteins was identified in land plants (Small and Peeters, 2000). These proteins contain 35-amino-acid repeats referred to as PPRs (pentatricopeptide repeat). In *Arabidopsis*, close to 500 PPR proteins have been found. A large portion of these proteins are targeted to the chloroplast, based on the presence of predicted transit peptides at their N-terminal end (Small and Peeters, 2000) and several PPR proteins are involved in chloroplast RNA stability and processing. Some PPR proteins are required for the synthesis or assembly of components of the plastid translation system. The maize *ppr2* mutant deficient in the PPR2 protein lacks plastid ribosomes (Williams and Barkan, 2003). However, the primary defect in this mutant is not yet known.

Some of the chloroplast PPR proteins are RNA-binding proteins (Table 2). This was first shown by analyzing the *Arabidopsis hcf152* mutant, which is impaired in *petB* mRNA accumulation and in the endonucleolytic cleavage between *psbH* and *petB* that are part of a large precursor RNA (Meierhoff *et al*., 2003). Molecular cloning of the *Hcf152* gene indicated that it codes for a 80-kDa chloroplast protein containing 12 PPR repeats. This protein is a RNA binding protein that binds with high affinity to the *petB* intron-exon junctions and the region between *psbH* and *petB*. Although the Hcf152 sequence does not show any significant sequence identity to other proteins, it displays structural similarity to the maize Crp1 protein that contains 13 PPR domains. Crp1 is required for processing of the *petD* RNA and translation of the *petA* mRNA (see below) (Fisk *et al*., 1999). Another PPR protein is CRR2 that belongs to the plant combinatorial and modular protein family consisting of more than 200 members in *Arabidopsis* (Hashimoto *et al*., 2003). This protein is involved in the processing of the *rps7-ndhB* mRNA and thus essential for the expression of *ndhB*. The PPR protein PGR3 protein is required for the processing of the *petL-petG- psaJ* tricistronic operon and in the stabilization of the *petL* and *petG* transcripts (Yamazaki *et al*., 2004). It is interesting that the two mutant alleles *pgr3-1* and *pgr3-2* change the same conserved threonine in helix A of the 15th and 12th PPR motifs of PGR 3 to isoleucine. Based on the atomic structure of the related TPR repeat one can speculate that the hydrophilic side chain of this threonine could contribute to make the central groove hydrophilic. The positively-charged central groove of PPR domains could accommodate single stranded RNA (Small and Peeters, 2000). The change from threonine to the hydrophobic isoleucine in the *pgr3* mutants would greatly diminish the hydrophilicity of the groove and could affect the RNA binding activity of Pgr3 (Yamazaki *et al*., 2004).

The high chlorophyll fluorescence mutant of *Arabidopsis*, *hcf145*, is specifically deficient in PSI abundance (Lezhneva and Meurer, 2004). All PSI subunits are severely reduced in amount, whereas subunits belonging to the other photosynthetic complexes accumulate normally. The *hcf145* mutant appears to be mainly deficient in the accumulation of the polycistronic *psaA-psaB-rps14* transcript. Run-on analysis of transcriptional activities revealed that the mutant is affected mostly at the level of RNA stability and slightly at the level of transcription. The pale cress (*pac*) mutation in *Arabidopsis* defines a novel nucleus-encoded factor that is required for the maturation of several plastid transcripts (Meurer *et al*., 1998b). However, the gene has not yet been isolated. The *dcl* mutant of tomato is deficient in chloroplast development, palisade cell morphogenesis and embryogenesis (Keddie *et al*., 1996). The *DCL* gene was cloned and found to be required for 4.5S rRNA processing (Bellaoui *et al*., 2003).

*Table 2*. Identified nucleus-encoded factors involved in chloroplast post-transcriptional steps

| Function | Mutant | Target | Factor | Features | Size (aa) | Complex (kDa) | Reference |
|---|---|---|---|---|---|---|---|
| RNA stability/processing | Cr; *nac2* | *psbD* 5′UTR | Nac2 | TPR | 1338 | 1700 | Boudreau *et al.*, 2000 |
| | Cr; *mbb1* | *psbB-psbH* 5′UTR | Mbb1 | TPR | 662 | 300 | Vaistij *et al.*, 2000b |
| | Cr; *MΦ11* | *petA* | Mca1 | PPR | 109 kDa | | Lown *et al.*, 2001 |
| | At; *hcf107* | *psbB-psbH* RNA | Hcf107 | TPR | 744 | | Felder *et al.*, 2001 |
| | At, *hcf152* | *psbH-petB* | Hcf152 | PPR | 778 | 180 | Meierhoff *et al.*, 2003 |
| | At, *crr2* | *rps7-ndhB* | CRR2 | PPR | 657 | | Hashimoto *et al.*, 2003 |
| | At, *pgr3* | *petL-petG* | PGR3 | PPR | 1112 | | Yamazaki *et al.*, 2004 |
| | Zm; *ppr2* | | PPR2 | PPR | 572 | | Williams and Barkan, 2003 |
| | Le; *dcl* | 4.5S rRNA | DCL | | 224 | | Keddie *et al.*, 1996 |
| RNA splicing | Cr; *raa1* | *psaA* exons 2,3 | Raa1 | | 2103 | | M. Goldschmidt-Clermont |
| | Cr; *raa2* | *psaA* exons 2,3 | Raa2 | ψ–uridine synthase | 410 | | Perron *et al.*, 1999 |
| | Cr; *raa3* | *psaA* exons 1,2 | Raa3 | | 1783 | | Rivier *et al.*, 2001 |
| | Zm; *crs1* | *atpF* intron | Crs1 | CRM | 715 | 550 | Till *et al.*, 2001 |
| | Zm; *crs2* | 9 group II introns | Crs2 | PTH | 256 | 500-800 | Jenkins and Barkan, 2001 |
| | | 6 group II introns | Caf1 | CRM | 674 | Caf1-Crs2 | Ostheimer *et al.*, 2003 |
| | | 5 group II introns | Caf2 | CRM | 611 | Caf2-Crs2 | Ostheimer *et al.*, 2003 |
| Translation initiation | Cr; *tbc2* | *psbC* | Tbc2 | PPPEW | 1115 | 400 | Auchincloss *et al.*, 2002 |
| | Cr; *tab1* | *psaB* | Tab1 | lipase | 1040 | | F. Laroche (unpublished results) |
| | Cr; *tab2* | *psaB* | Tab2 | | 358 | 300 | Dauvillee *et al.*, 2003 |
| | Zm: | *petA*, *petD* | Crp1 | PPR | 668 | 340 | Fisk *et al.*, 1999 |
| | | *psbA* | RB47 | polyA binding protein | 623 | | Yohn *et al.*, 1998a |
| | | *psbA* | RB60 | Disulfide isomerase | 532 | | Kim and Mayfield, 1997 |
| Translation elongation | Cr; *ac115* | *psbD* | Ac115 | | 113 | | Rattanachaikunsopon *et al.*, 1999 |
| Translation termination | At; *hcf109* | UAG-transcripts | AtprfB | | 456 | | Meurer *et al.*, 2002 |
| Assembly | At, *hcf136* | PSII | Hcf136 | | 403 | | Meurer *et al.*, 1998a |
| | Cr | PSI | Ycf3* | TPR | 172 | | Boudreau *et al.*, 1997a |
| | Cr | PSI | Ycf4* | | 197 | | Boudreau *et al.*, 1997a |
| | Cr, *ac-29* | Lhc, PSII | Alb3 | | 495 | | Bellafiore *et al.*, 2002 |
| | At, *hcf 101* | 4Fe-4S clusters | Hcf101 | P-loop ATPase | 532 | | Lezhneva *et al.*, 2004 |

Notes: Ycf3 and Ycf4 are encoded by chloroplast genes. Cr, *Chlamydomonas reinhardtii*, At, *Arabidopsis thaliana*, Zm, *Zea mays*, Le, *Lycopersicon esculentum*.

### *B. Chloroplast Splicing Factors*

A conspicuous feature of chloroplast genes is the presence of introns that belong in most cases to the two structurally different families of group I and group II introns. These introns are also found in mitochondrial genes, in the ribosomal RNA genes of protists and in a few bacterial genes. They can be folded into two distinct conserved secondary structures, and in several cases they were shown to self-splice in vitro and to act as catalytic RNAs. The 23S rRNA gene and the *psbA* genes of *C. reinhardtii* contain group I introns. These introns self-splice efficiently in vitro without the requirement for exogenous protein factors (Herrin *et al*., 1990; Durrenberger and Rochaix, 1991; Herrin *et al*., 1991). However, genetic evidence was provided for the existence of nuclear genes that promote efficient splicing of these group I introns in vivo (F. Li *et al*., 2002). Mutations within some of the core helices of the rRNA intron led to slow growth and light sensitivity. Suppressor strains were isolated and genetic analysis revealed that the mutations reside in at least two nuclear genes (F. Li *et al*., 2002). Moreover, the conditions for in vitro self-splicing often require non-physiological concentrations of $Mg^{++}$. The cofactors appear to promote the correct folding of these large catalytic RNAs.

Evidence for light/redox-regulated splicing of the *psbA* pre-mRNAs of *Chlamydomonas* was reported (Deshpande *et al*., 1997). Several of the *psbA* RNA precursors accumulate in wild-type cells in the dark. Exposure of cells to light induces a rapid decrease in precursor levels, which is dependent on photosynthetic electron transport. It is interesting that this regulation by light is only observed for the splicing of the *psbA* pre-RNAs but not for the splicing of 23S pre-rRNA. This light regulation is probably linked to the fact that synthesis of the D1 protein of PSII is strongly light regulated and induced under high light conditions when the protein needs to be replaced at a high rate because of photodamage.

One feature shared by several chloroplast group I introns is that they contain open reading frames that encode endonucleases. These enzymes recognize specifically the intron insertion site within gene copies that lack the intron and introduce a double-strand break, which triggers the subsequent transposition of the intron into this site. This process, called intron homing and initially observed in yeast mitochondria, was demonstrated in *C. reinhardtii* by introducing a short cDNA region of the 23S rRNA gene containing the intron insertion site at an ectopic site in the chloroplast genome of this alga by transformation (Durrenberger and Rochaix, 1991). All transformants examined contained a new copy of the ribosomal intron at this ectopic site indicating that the homing process is very efficient and that it probably played a major role in the spreading of group I introns.

In contrast to group I introns, chloroplast group II introns appear to have lost the ability to self-splice in vitro. A large number of *trans*-acting protein cofactors have been recruited for ensuring efficient splicing in vivo. One of the most intensively studied genes is *psaA* of *C. reinhardtii*, which encodes one of the reaction center polypeptides of PSI. In this alga the *psaA* gene is split into three exons that are dispersed on the chloroplast genome and transcribed independently (Kück *et al*., 1987; Choquet *et al*., 1988). All three exons are flanked by characteristic group II intron sequences. Thus maturation of the *psaA* RNA depends on two *trans*-splicing reactions involving the splicing of the transcripts of exons 1 and 2 and of exons 2 and 3 (Fig. 2). A large number of mutants deficient in *psaA* splicing have been identified and characterized genetically (Goldschmidt-Clermont *et al*., 1990). They fall into three phenotypic classes: class A mutants are deficient in exon 2 to exon 3 splicing, class

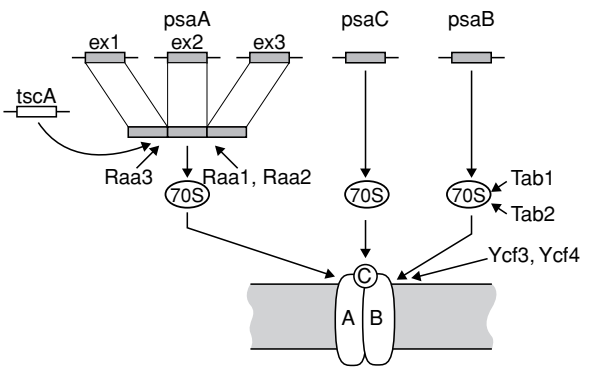


*Fig. 2.* PSI assembly in *C. reinhardtii*. The PsaA (A), PsaB (B) and PsaC (C) proteins that act as ligands of all the PSI redox cofactors are chloroplast-encoded. The *psaA* gene consists of three exons that are widely dispersed on the chloroplast genome. Maturation of the *psaA* mRNA depends on two *trans*-splicing reactions. The chloroplast *tscA* locus encodes an RNA that completes the structure of intron I and that is required for exon1-exon2 splicing. The three phenotypic classes of *trans*–splicing mutants include class A, deficient in exon2-exon3 splicing, class C deficient in exon 2-exon 3 splicing and class B deficient in both *trans*-splicing reactions. Three factors that are deficient in these mutants were cloned and characterized: Raa1 and Raa2 (class A mutants) and Raa3 (class C mutant). Translation of *psaB* mRNA requires the Tab1 and Tab2 factors. Ycf3 and Ycf4 are specifically involved in the assembly of the PSI complex .

C mutants are deficient in exon 1 to exon 2 splicing and class B mutants are deficient in both *trans*-splicing reactions. Mutants of class A, class B and class C fall into 5, 2 and 7 complementation groups, respectively (Goldschmidt-Clermont *et al*., 1990). Thus at least 14 nucleus-encoded factors are required for the maturation of the *psaA* mRNA. This number is probably an underestimate because several complementation groups include a single allele. A further twist in *psaA trans*-splicing is that intron 1 consists of at least three independently transcribed parts (Goldschmidt-Clermont *et al*., 1991). The 5′-end of this intron is co-transcribed with exon 1 and the 3′-end of the intron is cotranscribed with exon 2. Moreover, the middle portion of this intron is encoded by a separate chloroplast locus, *tscA* (Fig. 2). Thus intron 1 is tri-partite. The genes of three of the nucleus-encoded chloroplast factors involved in *psaA trans*-splicing were recently cloned by genomic complementation or through gene tagging. Raa1 and Raa2 comprise 2′103 and 410 amino acids, respectively, and are involved in the splicing of exons 2 and 3 (Perron *et al*., 1999, 2004; M. Goldschmidt-Clermont, unpublished results). Both are part of the same high molecular weight complex. Raa2 is related to pseudouridine synthases. However, pseudouridine synthase activity is not required for *trans*-splicing, because mutations that are known to abolish this activity have no effect on *psaA* mRNA maturation (Perron *et al*., 1999). It is not clear whether Raa2 has pseudouridine synthase activity. Raa3 is a large protein of 1,784 amino acids with a small domain homologous to pyridoxamine 5′-phosphate oxidase. It is involved in the first *psaA trans*-splicing reaction and is part of a high molecular weight complex that also includes *tscA* RNA. Thus at least two distinct RNA-protein complexes are implicated in the *trans*-splicing reactions of *psaA*. These complexes could represent plastid spliceosome-like particles.

In maize, genetic screens identified two genes, Crs1 and Crs2, that are required for the splicing of different subsets of group II introns (Jenkins *et al*., 1997). Based on subtle structural differences, these introns can be classified into subgroups IIA and IIB. Crs1 is specifically required for the splicing of the *atpF* group IIA intron. It is associated with a chloroplast ribonucleoprotein complex, and based on co-fractionation data, it appears that this complex contains *atpF* intron RNA (Till *et al*., 2001). Moreover, Crs1 is also implicated in translation. This protein is the founding member of a family of plant RNA-binding proteins derived by duplication and divergence of a domain of ancient origin named CRM (chloroplast RNA splicing and ribosome maturation) domain, which is conserved in eubacteria and archaea. Crs1 contains three CRM domains. The Crs2 gene is required for the splicing of nine of the ten chloroplast group IIB introns and encodes a protein that is related to peptidyl-tRNA hydrolases (PTH), an enzyme that hydrolyzes the ester bond linking the tRNA to the nascent polypeptide in abortive translation products (Jenkins and Barkan, 2001). It is, however, not clear whether Crs2 has peptidyl-tRNA hydrolase activity, because the Crs2 gene does not complement an *E. coli* $pth^{ts}$ mutant and lacks several amino acids that are important for the activity of the *E. coli* enzyme. The protein is associated with a large RNA-containing complex in the chloroplast stroma that co-sediments with group II intron RNA after sucrose gradient fractionation, suggesting that Crs2 facilitates splicing through direct interaction with intron RNA. A distinctive feature of Crs2 is a short C-terminal extension made of alternating basic and aromatic residues that is absent in the *E. coli* PTH. Crs2 is, therefore, as the *Chlamydomonas* splicing factor Raa2, an example of a factor that has been recruited from enzymes involved in basic RNA metabolism and adapted for facilitating the splicing of group II introns. Similar recruitments have been observed in fungal mitochondria. The splicing factors Cyt18 and Nam2 from *Neurospora* and yeast, respectively, are strongly related to tRNA synthetases (Akins and Lambowitz, 1987; Herbert *et al*., 1988). The fact that, in marked contrast to the mitochondrial group II introns, no plant chloroplast group II intron was shown to self-splice in vitro, raises the possibility that the identified splicing factors are not only involved in intron folding, but that they may also participate together with the RNA in the catalysis of splicing.

Two additional nucleus-encoded splicing factors were identified that are associated with Crs2, called Crs2-associated factors 1 and 2 (Caf1 and Caf2) (Ostheimer *et al*., 2003). They form distinct ribonucleoprotein complexes with Crs2 and are involved in splicing of different subsets of the nine Crs2-dependent group IIB introns. These complexes are bound tightly to their cognate group II introns in vivo as shown by the ability of Caf antisera to immunoprecipitate Crs2 together with the target introns from chloroplast extracts. Both Caf factors contain two repeated domains that are similar to the CRM domains of Crs1. These three factors belong to the same family of splicing factors that evolved through the amplification and diversification of an ancient RNA-binding module (Till *et al*., 2001). The *E. coli* representative, YhbY, which contains a single CRM domain, is a protein that binds to the pre-50S-ribosomal subunit and is most likely involved

in ribosome assembly (Ostheimer *et al*., 2003). It is interesting that this protein was recruited and expanded during evolution of plant genomes to be adapted for the assembly of group II intron RNPs, which, like ribosomes, are catalytic RNPs. The CRM domain family appears to be specific to plants and is absent in animals and fungi.

## C. Chloroplast Translation

### 1. Factors Involved Specifically in the Initiation of Translation

Initiation of translation is a key step and is also important for the coordinate synthesis and assembly of the photosynthetic complexes in the thylakoid membrane. Genetic approaches revealed a surprisingly large number of factors required for this process. Many of these factors appear to act in a mRNA-specific way (Table 1). Thus *Chlamydomonas* mutants specifically deficient in the translation of the mRNA of *petA* (Girard-Bascou *et al*., 1995), *psaB* (Stampacchia *et al*., 1997), *psbA* (Girard-Bascou *et al*., 1992; Yohn *et al*., 1998b), *psbD* and *psbC* (Rochaix *et al*., 1989; Zerges and Rochaix, 1994) mRNA were identified and characterized. In most cases, the nuclear mutations act at the level of translation initiation, as shown by a reduced expression of chloroplast reporter genes driven by the 5′-UTR of these mRNAs in the presence of the nuclear mutant allele. Genetic analysis revealed three nuclear loci, *TBC1*, *TBC2* and *TBC3,* that are required for translation of the *psbC* mRNA. The *psbC* 5′-untranslated region comprises 550 nucleotides and contains target sites that could interact directly or indirectly with Tbc1 and Tbc2, strongly suggesting that these factors act at the level of initiation of translation (Zerges and Rochaix, 1994). A distinctive feature of the *psbC* 5′-UTR is the presence of a large stem-loop structure near the middle of the 5′-UTR that is required for translation. Removal of this structure or mutations within the stem region completely block *psbC* mRNA translation (Rochaix *et al*., 1989; Zerges and Rochaix, 1994; Zerges *et al*., 1997). A nuclear suppressor of these mutations identified a third locus, *TBC3*, involved in the initiation of translation of *psbC* mRNA. This suppressor is also capable of reversing the translational defects resulting from the mutation in *tbc1* but not the *tbc2* mutation (Zerges *et al*., 1997). The factors defined by *TBC1* and *TBC3* appear to interact, directly or indirectly, with the internal stem-loop structure. The *TBC2* gene was cloned by genomic complementation of a *tbc2* mutant (Auchincloss *et al*., 2002). It encodes a protein of 1,115 residues, in which occur nine copies of a degenerate 38- to 40-amino-acid repeat with a quasi-conserved PPPEW motif near its C-terminal end. The middle part of the protein is related in sequence to Crp1, a maize protein involved in the processing and translation of the chloroplast *petA* and *petD* mRNAs (see below). The Tbc2 protein is part of a 400-kDa protein complex in the chloroplast stroma that is likely to play an important role in the intitial steps of *psbC* mRNA translation.

A key step in the assembly of the PSI complex is the coordinate synthesis and assembly of its two core polypeptides, PsaB and PsaA, that form, together with several redox ligands, the main part of the reaction center. Several mutants of *C. reinhardtii* deficient in the synthesis of these two polypeptides were characterized (Girard-Bascou *et al*., 1987; Stampacchia *et al*., 1997). The picture that emerged from their characterization is that mutants deficient in PsaB synthesis are invariably deficient in PsaA synthesis, whereas mutants deficient in PsaA synthesis are still able to transiently produce PsaB, although this protein is unstable in the absence of PsaA. Thus, although these two proteins form the basic symmetrical backbone of PSI, their roles in the assembly of the complex are clearly different. These studies suggest that PsaB is an anchor protein during PSI assembly, which needs to be synthesized and assembled first in the thylakoid membrane before the other PSI subunits are integrated into the PSI complex. The two mutant strains *tab1* and *tab2* are deficient in the synthesis of both PsaA and PsaB as judged from protein pulse-labeling experiments (Stampacchia *et al*., 1997; Dauvillee *et al*., 2003). Moreover, studies with chimeric genes consisting of the *psaB* 5′-UTR fused to the *aadA* reporter gene that confers spectinomycin resistance revealed that this chimeric gene is no longer expressed in the presence of either of these two mutant alleles. These results indicate that at least one target site for the Tab1 and Tab2 factors is comprised within the *psaB* 5′-UTR. For Tab1 the interaction could occur directly or through other factors. *Tab1* encodes a membrane protein of 1,040 amino acids with 5 putative *trans*-membrane domains in its N-terminal part (F. Laroche and J.D. Rochaix, unpublished results). The C-terminal part displays significant sequence identity with lipases, although it is not clear whether Tab1 has lipase activity. Tab2 represents a novel type of RNA-binding protein of 358 amino acids that binds specifically to the *psaB* 5′-UTR (Dauvillee *et al*., 2003). It is localized in the chloroplast stroma and associated with a large RNA protein complex that contains *psaB* RNA. The sequence of Tab2 displays similarity with conserved but uncharacterized proteins

from both prokaryotic and eukaryotic organisms, from cyanobacteria to land plants, capable of oxygenic photosynthesis. One possibility is that Tab2 acts early in the PSI assembly pathway by binding to *psaB* mRNA and perhaps already to the nascent RNA chain. The Tab2 complex could play a role in targeting the *psaB* mRNA to the thylakoids. T-DNA insertion lines with disruptions in the Tab2 ortholog of *Arabidopsis* are deficient in PSI accumulation and also partially deficient in PSII (F. Barneche and J. D. Rochaix, unpublished results). Thus Tab2 appears to have a slightly wider role in the expression of chloroplast proteins in land plants in comparison to *Chlamydomonas*.

The apparent absence of PsaA translation in the absence of PsaB synthesis is reminiscent of bacterial autoregulatory feedback repression in which a polypeptide represses its own translation by interacting with the 5′-UTR of its mRNA (Gold, 1988). An expansion of this model, called control by epistasy of synthesis (CES), was proposed (Choquet *et al*., 1998). In this model the concerted accumulation of the subunits of the photosynthetic complexes is not only achieved through the rapid degradation of the unassembled subunits but also through the regulation of the rate of synthesis of some chloroplast-encoded subunits, called CES subunits, by the availability of their assembly partners, called dominant subunits, from the same complex (Choquet, 2001) (Fig. 3). The strongest support for this model arises from studies of the assembly of the cytochrome $b_6f$ complex of *C. reinhardtii.* Translation of cytochrome *f* is diminished in mutants that lack cytochrome $b_6$ or subunit IV (Choquet *et al*., 2003). This repression of cytochrome *f* translation is mediated through the interaction between the *petA* 5′-UTR and a repressor motif

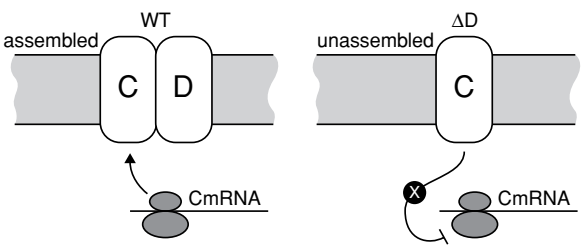


*Fig. 3.* CES (Control by Epistasy of Synthesis) model for the coordinate synthesis of the subunits of the photosynthetic complexes (Choquet *et al*., 1998). Some of the subunits of the photosynthetic complexes act either as dominant (D) or CES (C) subunits. In the absence of the dominant subunit (ΔD) the CES subunit is unassembled. An autoregulatory process leads to the inhibition of translation of the CES subunit through the interaction of the CES subunit with a ternary factor (X) and the 5′-UTR of the CES subunit mRNA (shown with a ribosome). Only the CES (C) and dominant (D) subunits are shown.

within the carboxyl-terminal domain of cytochrome *f* (Choquet *et al*., 2003). It is unlikely that this interaction is direct because no RNA binding site could be detected in cytochrome *f* and because critical residues mediating the CES effect are within the membrane domain of cytochrome *f*. It appears more likely that the CES effect is mediated by a ternary membrane factor, which could either be a translational repressor that is activated upon binding to the repressor motif or an activator that is inactive when bound to the repressor motif. Possible candidates include Mca1 required for the accumulation of *petA* mRNA, which could interact with the *petA* 5′-UTR (Gumpel, 1995; Choquet, 2003) and Tca1 which is required for *petA* mRNA translation and acts through its 5′-UTR (Wostrikoff *et al*., 2001). It is noteworthy that some mutations in the 3′-region of *petA* that abolish the CES have little or no effect on the phototrophic growth of the mutants, suggesting that CES is mostly involved in the fine-tuning of the assembly process (Choquet *et al*., 2003). It will be interesting to test the growth of these mutant strains under various stress conditions and to examine whether the availability of CES provides a clear growth advantage under these conditions.

Recent results indicate that CES may also underlie the biogenesis of other complexes in *Chlamydomonas*, in particular PSI (Wostrikoff *et al*., 2004). Here a CES cascade follows PSI assembly steps: PsaB is required for significant PsaA synthesis and PsaA is required for PsaC synthesis. Thus PsaA acts both as a CES subunit with regard to the dominant subunit PsaB and as the dominant subunit with regard to the CES subunit PsaC. (Choquet *et al*., 2001; Wostrikoff *et al*., 2004). In this model of autoregulation, which occurs through the unassembled CES subunits, a given subunit can only be synthesized when the previous steps in the sequential assembly of PSI have been accomplished. Whether CES also occurs in the chloroplasts of land plants is not yet clear (see below).

In maize the nuclear gene Crp1 is required for the translation of the chloroplast *petA* and *petD* mRNAs as well as for the processing of the *petD* mRNA from a polycistronic precursor (Fisk *et al*., 1999). Crp1 displays sequence similarity to the fungal nuclear gene products Pet309p (Manthey *et al*., 1998) and Cya5p (Coffin *et al*., 1997), which are required for accumulation of the *Cox1* mRNA and its translation. These proteins belong to the PPR protein family. It is, therefore, likely that they act mechanistically in a similar way. However, in contrast to Pet309p, which is associated with the mitochondrial inner membrane, the Crp1 protein is localized in the chloroplast stroma

and associated with a multisubunit complex that is not bound to chloroplast ribosomes. Moreover, a short region of Crp1 is related to yeast threonyl-tRNA synthetase (Fisk *et al*., 1999). Analysis of double mutants lacking both Crp1 and plastid ribosomes indicates that Crp1 influences *petD* RNA processing independently of its role in *petD* mRNA translation. However, it cannot be excluded that *petD* mRNA processing affects its translation. It is possible that the reduction of *petA* mRNA translation results from the deficiency in *petD* mRNA translation as would occur in CES. However, the deficiency in *petA* translation is more severe than the defect in *petD* translation in the *crp1* mutant. Moreover, deletion of *petB* or *petD* in tobacco chloroplasts leads to a drastic reduction of cytochrome *f* with only a mild effect on the association of *petA* mRNA with polysomes (Monde *et al*., 2000). Therefore, it appears more likely that the *petA* translation defect in the maize *crp1* mutant does not result from the reduced rate of *petD* translation.

A nuclear mutant of maize deficient in the accumulation of the ATP synthase was shown to be affected in the translation of the *atpB*/*E* bi-cistronic mRNA, based on the observation that the *atpB/E* mRNA accumulates normally but is associated with only few ribosomes (McCormac and Barkan, 1999).

### 2. Elongation

While numerous mutants from *Chlamydomonas* and vascular plants are deficient in the initiation of chloroplast translation, only a few mutants have been characterized that are affected at the level of chloroplast translation elongation. One of them, *ac115*, is specifically deficient in the translation elongation of the *psbD* mRNA, based on several observations. First, protein pulse-labeling experiments revealed that synthesis of the D2 polypeptide was specifically blocked (Kuchka *et al*., 1988). Second, *psbD* mRNA was still associated with polysomes in this mutant. Third, a chimeric gene consisting of the *psbD* 5′-UTR fused to the *aadA* reporter gene was still expressed in the chloroplast in the presence of the nuclear mutant allele, indicating that the 5′-UTR is not the main target site for the Ac115 factor (J.D. Rochaix and J. van Dillewijn, unpublished results). The gene deficient in *ac115* was cloned and shown to encode a protein of 113 amino acids with no obvious sequence similarity to any known protein (Rattanachaikunsopon *et al*., 1999)

Chloroplast translation elongation can occur in a discontinuous way. Translation intermediates of 15- to 25-kDa were detected for the PSII protein D1 in chloroplasts from barley by protein pulse-labeling (Kim *et al*., 1991). These intermediates were associated with membrane-bound polysomes and could be chased into full-length D1. Toe print analysis revealed specific pausing sites for the translating ribosomes on the mRNA, which were consistent with the translation intermediates. It appears that ribosome pausing during elongation may facilitate the co-translational binding of chlorophyll and the integration of D1 into the thylakoid membrane. Interestingly, the *vir115* nuclear mutant of barley, which is deficient in D1, lacks these D1 intermediates, although the *psbA* mRNA accumulates and is associated with polysomes (Gamble and Mullet, 1989). It is thus possible that the factor deficient in this mutant is involved in ribosome pausing and/or chlorophyll binding.

### 3. Translation Termination

Translation termination occurs when the translating ribosome encounters any of the three stop codons UAA, UAG or UGA. In eukaryotic translation systems, the termination process requires a single release factor, eRF, and the ribosome-recycling factor, RRF, which is needed for the dissociation of the ribosome from the mRNA and tRNAs. In contrast, eubacteria contain three release factors: RF1 (prfA) for decoding UAA and UAG, RF2 (prf B) for decoding UAA and UGA, and RF3. Homologs of prfA and prfB have been identified in *Arabidopsis* that encode chloroplast proteins (Meurer *et al*., 2002). The *Arabidopsis* mutant *hcf109* is deficient in AtprfB. This mutation causes decreased stability of UGA-containing mRNAs. The analysis of this mutant suggests that only AtprfB can terminate translation of plastid transcripts with the UGA stop codon and that this factor is involved in the control of both mRNA stability and translation (Meurer *et al*., 2002). AtprfB expression is highest in photosynthetic tissues and in flowers and low in stems, siliques and roots and thus appears closely linked to chloroplast and flower development and function.

## D. Membrane-Associated Factors Involved in Chloroplast Gene Expression

Localization studies revealed that several of the newly identified factors that participate in chloroplast post-transcriptional steps are associated with a low-density membrane system. These membranes appear to be distinct from the mature thylakoid membranes, although it cannot be excluded that they form a thylakoid precursor compartment (Zerges and Rochaix, 1998). It is also not

clear whether they are related to the inner chloroplast envelope membrane. The factors associated with these low-density membranes are involved in several post-transcriptional steps of plastid gene expression. They comprise a small set of chloroplast RNA-binding proteins, including RB47 (Zerges and Rochaix, 1998), the ribosome recycling factor RF4 (Rolland *et al*., 1999) and the splicing factor Raa2 (Perron *et al*., 1999). Several of the components of the photosynthetic complexes are chlorophyll-binding proteins. Because chlorophyll synthesis is known to occur on the inner membrane of the chloroplast envelope, it is conceivable that coordinate synthesis of the proteins and insertion of the newly synthesized chlorophylls could occur on the inner envelope membrane.

Even polypeptides that are localized in the stroma, such as the large subunit of Rubisco, appear to be synthesized on membrane-bound ribosomes (Kim *et al*., 1994). This could allow for direct translational control by factors that sense the thylakoid proton gradient. Further support for the role of thylakoids in plastid translation stems from the observation that maize mutants deficient in SecY, the chloroplast homolog of the major subunit of the *E. coli* protein translocation system, are affected in the translation of both thylakoid and stromal proteins (Roy and Barkan, 1998). The general view is that the nucleus-encoded thylakoid proteins are imported into chloroplasts by the TOC and TIC protein import apparatus and subsequently transferred directly through the stroma to the thylakoids (Cline and Henry, 1996). This view is based on the identification of precursor intermediates in the stroma (Smeekens *et al*., 1986) and on the chasing of these intermediates into their mature forms both in vivo (Howe and Merchant, 1993) and in vitro (Reed *et al*., 1990) using a thylakoid protein insertion reconstitution system (Cline, 1986). However, evidence for a chloroplast vesicle system operating between envelope and thylakoids has recently emerged (Fig. 1). Earlier studies indicated that chloroplast vesicles are detectable by electron microscopy when leaves are subjected to low temperature (Morré *et al*., 1991) or in *Chlamydomonas* when rapid thylakoid development was induced by transferring *y-1* mutant cells, which are deficient in light-independent chlorophyll synthesis, from the dark to the light at 38°C (Hoober *et al*., 1991). Moreover, chloroplast proteins were identified that are related to proteins involved in vesicle trafficking such as animal NSF, bacterial Ftsh protein (Hugueney *et al*., 1995) and dynamin (Park *et al*., 1998). Recently a mutant of *Arabidopsis* was isolated that is unable to form vesicles and thus is deficient in thylakoid biogenesis (Kroll *et al*., 2001). It is deficient in a gene encoding an inner envelope, lipid transferase protein. This protein is evolutionarily related to the phage shock protein of prokaryotes, a protein involved in membrane repair upon phage infection. The chloroplast vesicle system was further characterized by the use of specific inhibitors of vesicle trafficking (Westphal *et al*., 2001). Microcystin C, known to block the phosphatase involved in vesicle docking, induces the accumulation of chloroplast vesicles. The possibility that these vesicles are involved in both lipid and protein transfer from the inner plastid envelope to the thylakoid membrane must be seriously considered. A challenge will be to determine the exact relationship between the low-density membranes, the thylakoids and the vesicle system and their respective roles in chloroplast gene expression and biogenesis of the photosynthetic apparatus.

### *E. Assembly Factors*

Once chloroplast proteins have been synthesized, they need to be assembled into functional photosynthetic complexes. Only few assembly factors have been identified. One of the key proteins involved in this process is Alb3, which belongs to a widespread family of proteins involved in the integration and assembly of membrane proteins (Kuhn *et al*., 2003). Loss of Alb3 in *Arabidopsis* results in an albino phenotype with a strong reduction in thylakoid membranes (Sundberg *et al*., 1997). Treatment of thylakoid membranes with an Alb3 antibody blocked the integration of the major light-harvesting chlorophyll-binding protein (Moore *et al*., 2000). The corresponding phenotype in *Chlamydomonas* cells lacking Alb3 is less severe, with accumulation of LHCP amounting to 10% relative to wild-type (Bellafiore *et al*., 2002). This may be due to the presence of a second Alb3-like protein in *Chlamydomonas*, whose function may partially overlap with that of Alb3. Although a major function of Alb3 is the integration and assembly of LHCP, it is also involved in the assembly of PSII in *Chlamydomonas*. In the absence of Alb3 the rate of assembly of the D1 protein into the PSII reaction center is reduced (Ossenbuhl *et al*., 2004). Thus Alb3 has a dual role and is involved in the assembly of both nucleus- and plastid-encoded subunits of photosynthetic complexes in *Chlamydomonas*.

The Ycf3 and Ycf4 factors are chloroplast-encoded and specifically required for the accumulation of PSI in *Chlamydomonas* and land plants (Boudreau *et al*., 1997a; Ruf *et al*., 1997). Homologs of Ycf3 and Ycf4 also exist in cyanobacteria. Inactivation of *ycf3* leads to a complete loss of PSI in *C. reinhardtii*, in plants and

in cyanobacteria. Inactivation of *ycf4* has also a strong effect on PSI accumulation in *C. reinhardtii* but affects PSI accumulation only mildly in cyanobacteria (Wilde *et al*., 1995). The *ycf3* and *ycf4* genes of *C. reinhardtii* are part of a large transcription unit that includes the ribosomal protein genes *rps9* and *rps18*. Both proteins are extrinsic thylakoid membrane polypeptides. Although they are specifically required for the accumulation of the PSI complex, they do not co-fractionate with PSI.

Ycf3 contains three TPR (tetratricopeptide repeat) domains. As mentioned above, these domains are usually involved in protein-protein interactions. Mutations in the second and third TPR domain lead to a modest decrease of PSI, but the mutants are unable to grow photoautotrophically and are light-sensitive (Naver *et al*., 2001). This phenotype can be reversed by growing the mutants under anaerobic conditions, which suggests that the light sensitivity results from photo-oxidative damage. Use of a temperature-sensitive *ycf3* mutant in temperature-shift experiments showed that Ycf3 is indeed required for PSI assembly but not for its stability. Further studies revealed that Ycf3 interacts directly with PsaA and PsaD but not with subunits of other photosynthetic complexes (Naver *et al*., 2001). Ycf3 could act as a chaperone that interacts directly with specific subunits of PSI during its assembly.

In contrast to Ycf3, which is not part of a stable complex, Ycf4 is associated with a large molecular weight complex (Boudreau *et al*., 1997a). Sucrose density gradient centrifugation revealed that the Ycf4 complex co-migrates with a minor portion of PSI that exceeds in size the bulk of PSI. Protein pulse-labeling experiments showed that during short pulses, labeled PSI proteins appeared in the Ycf4 region and were subsequently transferred to the bulk PSI after a chase with unlabeled amino acids (Y. Takahashi and J.D. Rochaix, unpublished results). These observations suggest that the Ycf4 complex may play a role in the initial steps of PSI polypeptide integration into the thylakoid membrane.

PSI contains three 4Fe-4S clusters, $F_X$, $F_A$ and $F_B$, which are involved in electron transfer within PSI. Analysis of the PSI deficient *hcf101* mutant provided new insights into the assembly process of these clusters (Lezhneva *et al*., 2004). This mutant is specifically unable to accumulate PSI subunits and is also partially deficient in the accumulation of ferredoxin-thioredoxin reductase, which contains one 4Fe-4S cluster. In contrast, assembly of chloroplast 2Fe-2S centers is not affected in *hcf101*. The Hcf101 protein is localized within the plastids and belongs to an ancient and universally conserved family of P-loop ATPases (Lezhneva *et al*., 2004). It is likely to be involved in the assembly and/or stability of 4Fe-4S clusters.

The *Arabidopsis* nuclear *hcf136* mutant is specifically deficient in PSII (Meurer *et al*., 1998a). Because the plastid-encoded PSII subunits are synthesized normally in this mutant, but do not accumulate, it is likely that the Hcf136 protein is required for the assembly and/or stability of the PSII complex. This protein is located in the thylakoid lumen. It has homologs in cyanobacteria and in the plastome of *Cyanophora paradoxa*, an indication of its prokaryotic origin. However, it is not yet known how this protein functions.

### *F. Modulation of Chloroplast Gene Expression by Light*

Light stimulates several post-transcriptional steps of chloroplast gene expression, particularly translation (Deng and Gruissem, 1987; Malnoe *et al*., 1988). One of the plastid proteins whose translation rate is particularly strongly enhanced by light is the PSII reaction center polypeptide D1. Because of the photochemistry of PSII, photodynamic damage to D1 is unavoidable and the protein needs to be constantly replaced by newly synthesized D1. A protein complex that binds to the *Chlamydomonas psbA* 5′-UTR was characterized. RNA binding of this complex is stimulated by light and the RNA-binding activity correlates with translation of the *psbA* mRNA (Danon and Mayfield, 1991). The complex comprises four proteins, RB38, RB47, RB55 and RB60, of which only RB47 interacts directly with the RNA (Danon and Mayfield, 1991). The RNA-binding activity of this complex is regulated by two light-responsive mechanisms. First, ADP-dependent phosphorylation of RB60 inactivates the binding activity at ADP levels that occur in chloroplasts only in the dark (Danon and Mayfield, 1994b). Second, the RNA binding activity is inactivated by oxidation of the complex and reactivated by reduction of the complex, indicating that this binding activity is under redox control in vitro (Danon and Mayfield, 1994a). The redox regulatory site consists of vicinal dithiols on RB60 (Trebitsh *et al*., 2000), which is related to protein disulfide isomerase and contains two redox-active, thioredoxin-like domains, each with a vicinal dithiol site (Kim and Mayfield, 1997; Trebitsh *et al*., 2001). The RNA-binding subunit of the complex, RB47, belongs to the polyA-binding protein family (Yohn *et al*., 1998a). Proteins of similar size were shown to bind to several chloroplast mRNAs in *C. reinhardtii*, but it is not clear whether they represent one or several

distinct polypeptides of the same size (Nickelsen *et al*., 1994; Zerges and Rochaix, 1994; Fargo *et al*., 2001). It was suggested that a series of oxidation-reduction reactions involving ferredoxin, ferredoxin-thioredoxin reductase and thioredoxin reduce the regulatory vicinal dithiol site of RB60 and thereby activate RNA binding and translation of *psbA* mRNA in response to light-induced photosynthetic electron flow (Danon and Mayfield, 1994a). However, this model is mostly based on in vitro protein-RNA binding studies and will need to be supported by in vivo experiments.

## IV. Perspectives

Since many years, research on chloroplast gene expression has used a wide range of approaches including biochemistry, genetics, molecular biology, biophysics and physiology. Major technological advances in these areas, such as improved nuclear and chloroplast transformation, high through-put DNA and protein sequencing, microarray technology and improved tools for large-scale nuclear reverse genetics, have greatly accelerated the pace of this research.

The genetic analysis of chloroplast gene expression has revealed several surprising features of this system. First, chloroplast gene expression is dependent on a large set of nucleus-encoded factors. Second, these factors act mostly at post-transcriptional steps and are usually required for the expression of a single plastid gene or transcription unit. This was first observed by studying photosynthetic mutants of *Chlamydomonas*. In several cases, expression of a single chloroplast protein gene from this alga requires specifically one factor for RNA processing and stability, and 2 to 3 factors for translation. In extreme cases, as for the maturation of the *psaA* mRNA of *C. reinhardtii*, which depends on two *trans*-splicing reactions, at least 14 nucleus-encoded factors are needed. Thus, given that there are about 100 chloroplast protein genes, one can estimate that several hundred nucleus-encoded factors are involved. Similar findings were also made with mutants of maize and *Arabidopsis*, although in most cases these mutants display more pleiotropic phenotypes. The reason for the existence of this large set of ancillary nucleus-encode factors is not understood. However, the recent molecular identification and characterization of several of these factors has provided new insights into this problem. At this time over twenty of these factors have been characterized through either genetic or biochemical approaches (see Tables 1 and 2) and can be grouped into several classes. The first includes factors that clearly evolved from prokaryotic proteins. Representatives of this class are the splicing factors Crs1, Caf1 and Caf2, which all contain the prokaryotic CRM RNA-binding module and form a new family of RNA-binding proteins. The translation factor Tab2 was also derived from a prokaryotic protein, although it does not contain a known RNA-binding domain. The second class is the widespread PPR protein family. At least one member of this class, Hcf152, was shown to be an RNA-binding protein. The chloroplast TPR proteins form the third class. Some of its members are also RNA-binding proteins. The fourth group is composed of proteins that have been recruited from pre-existing proteins involved in general RNA metabolism and adapted to their new function. Examples of this class are Raa2 derived from pseudouridine synthase, Crs2 derived from peptidyl tRNA hydrolase and RB47, which resembles polyA-binding proteins. In the case of Raa2, the activity of pseudouridine synthase is no longer required for its new function in splicing. A fifth class contains factors of which parts are derived from enzymes involved in general metabolism. Examples are Tab1, which contains a lipase domain; Raa3, which contains a domain resembling pyridoxamine 5′-phosphate oxidase; and RB60, a protein disulfide isomerase that appears to be implicated in redox control of the RNA-binding activity of RB47. A sixth group includes factors that are not related in any obvious way to proteins of known function.

Sequencing the genomes of several photosynthetic organisms has provided a very comprehensive picture of their genetic informational content. Sequences have been determined of several cyanobacterial genomes, of numerous chloroplast genomes, and of the nuclear genomes of *Arabidopsis*, rice and *Chlamydomonas*. Moreover, large EST collections have been established for several land plants and algae. These large sequence data sets have not only been invaluable for the identification of genes, they have also provided new important insights into the evolutionary forces that have shaped these genomes.

It is now possible to rapidly identify genes encoding proteins with known or related functions or new genes with roles in chloroplast gene expression. However, the availability of predicted amino acid sequences does not always provide clues as to their function. In this respect, an impressive effort with *Arabidopsis* has generated large collections of T-DNA insertion lines that are publicly available (Alonso *et al*., 2003). It is thus possible to use these lines to study the function of specific genes. Moreover, it allows one to study the function of a given gene not only in one organism in

which the corresponding mutant exists, but it also allows one to analyze the phenotype of the inactivation of the orthologous gene in a different organism. This approach is very powerful and has been used successfully for studying genes involved in photosynthesis in different organisms thereby providing a comprehensive picture of their function (Swiatek *et al*., 2001).

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# Chapter 8

# Plastid Transcription: Competition, Regulation and Promotion by Plastid- and Nuclear-Encoded Polymerases

A. Bruce Cahoon*
*Department of Biology, Middle Tennessee State University, Box 60, Murfreesboro, TN 37132, U.S.A.*

Yutaka Komine
*Department of Plant & Microbial Biology, University of California-Berkeley, 111 Koshland Hall, Berkeley, CA 94720, U.S.A.*

David B. Stern
*Boyce Thompson Institute at Cornell University, Tower Road, Ithaca NY 14853, U.S.A.*

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## Summary

Complete plastid function, including gene expression, is necessary for photosynthesis. Furthermore, plastid genomes have been retained in non-photosynthetic plants (Wolfe *et al.*, 1992) and even in the distantly related Apicomplexans

*Author for correspondence, email: acahoon@mtsu.edu

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(Wilson *et al.*, 2003; see Chapter 24), which suggests that they have key functions outside of photosynthesis. Recently published reverse genetic experiments from *Arabidopsis* and tobacco (Ahlert *et al.*, 2003; Kuroda and Maliga, 2003; Yao *et al.*, 2003) provided the first direct evidence that plastid-encoded or plastid-localized proteins are necessary for embryogenic tissues to grow or develop.

Plastid transcription has been somewhat overlooked because of a (warranted) focus on post-transcriptional regulatory mechanisms (Goldschmidt-Clermont, 1998), and because under at least some growth conditions, plastids accumulate an over-abundance of transcripts, i.e., far in excess of what is required for translation (Eberhard *et al.*, 2002). This hyper-accumulation of transcripts, in this case a result of standard culture conditions (*Chlamydomonas* cells grown in rich medium), would appear to supplant the physiological pressure to produce only needed mRNAs. In other words, we may have domesticated our model organisms, causing them to suffer from an "obese pet" syndrome. In the wild, plastid physiology may be very different and indeed, plastid transcription is not under a simple binary on/off global regulation.

In this review we paint plastid transcription as a dynamic process involving multiple RNA polymerases (perhaps as many as three in higher plants), an interplay of nuclear- and plastid-encoded factors, several classes of promoters, and layers of environmental regulation, all working together in a developmentally significant manner. Although what we cite is limited due to space, we recognize and appreciate the numerous reviews and detailed studies published by others.

## I. Introductory Remarks

### *A. Discovery of Plastid RNA Polymerases*

Plastid transcription activity was first detected biochemically from maize chloroplast extracts in the early 1970's and characterized as a DNA-dependent RNA polymerase(s) with a polypeptide complement similar to that of eubacteria (Bottomley *et al.*, 1971; Smith and Bogorad, 1974). Complete or targeted sequencing of chloroplast genomes in the mid 1980's (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Sijben-Muller *et al.*, 1986) strongly suggested that plastid genomes encoded subunits of a DNA-dependent RNA polymerase homologous to the multi-subunit RNA polymerase used by eubacteria. This enzyme was eventually dubbed the plastid-encoded RNA polymerase, or PEP.

Although the existence of PEP was established early on, various data hinted, and later proved, that it was not the sole RNA polymerase in the chloroplast. Some organisms appeared unable to produce PEP because they either lacked one or more of the PEP genes (parasitic plants such as *Epifagus virginiana*) or lacked functional plastid ribosomes (the barley *albostrians* mutant). Although *Epifagus* cannot photosynthesize, it does retain some plastid transcriptional activity (Morden *et al.*, 1991) and in the case of *albostrians*, which is variegated, the albino sectors were found to still transcribe a subset of plastid genes (Hess *et al.*, 1993). A directed knockout of one of the PEP subunits in tobacco provided conclusive proof that the absence of PEP only selectively affects plastid transcription (Allison *et al.*, 1996), and thus a nuclear-encoded RNA polymerase(s) (NEP) must also exist.

At that time the most likely candidate for the NEP was a 110-kDa protein from spinach chloroplasts that could recognize and transcribe from a T7 bacteriophage promoter. The size of the protein and its activity suggested that the NEP might resemble phage single-subunit polymerases (Lerbs-Mache, 1993). Phage-like RNA polymerases (RpoT's) had been known to transcribe the mitochondrial genomes of human and yeast cells for some time (reviewed in Shoubridge, 2002), but there was no precedent for their occurrence in plastids. The first plant *RpoT* gene was cloned from *Chenopodium album*, a dicot plant of the family *Chenopodiaceae*, and its product was predicted to be localized to mitochondria (Weihe *et al.*, 1997). However, a screen of an *Arabidopsis* genomic library with that clone, and eventually the complete nuclear genome sequence, revealed three distinct *RpoT* genes. Subcellular localization analysis using GFP fusions was employed to demonstrate that the phage-like RNA polymerases have distinct localization patterns, either to mitochondria, to plastids, or to both organelles (Hedtke *et al.*, 1997, 2000).

*Abbreviations*: ESI-MS/MS – electrospray ionization-tandem mass spectrometry; EST – expressed sequence tag; GFP – green fluorescent protein; MALDI-TOF-MS – matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; NEP – nuclear-encoded polymerase; PCR – polymerase chain reaction; PEP – plastid-encoded polymerase; RpoT –RNA polymerase T7-like; YFP – yellow fluorescent protein.

Taken together, these findings paint a picture of two very different RNA polymerase types serving one small genome. The questions of why and how beg to be answered, and indeed are still only beginning to be answered. The first comprehensive model of plastid transcription suggested that NEP was responsible for transcribing PEP and housekeeping genes early in plastid development, and that the importance of NEP diminished as the cells mature and PEP takes over the majority of transcription (Hajdukiewicz *et al.*, 1997). This model still stands, although some of its details have been challenged within the past few years, as discussed below.

## II. Plastid-Encoded Polymerase (PEP)

### A. Subunit Composition

True to their cyanobacterial origins, PEPs have a catalytic core comprised of RpoA (homologous to the *Escherichia coli* α subunit), RpoB (β subunit homolog), and RpoC1 and RpoC2 (β′ subunit homologs). In *E. coli* the β′ subunit is a single polypeptide, whereas in plants the homolog is split into two proteins; the amino-terminal moiety is encoded by *rpoC1* while the carboxy-terminal portion is encoded by *rpoC2*. In *Chlamydomonas* the *rpoC2* gene has apparently been split again into two pieces (Maul *et al.*, 2002).

The core enzyme subunits are not, however, universally encoded in the plastid genome. Recently, *rpoA* was shown to be encoded in the nucleus with a plastid-specific transit peptide in the moss *Physcomitrella patens* (Sugiura *et al.*, 2003). Plastid DNAs from some non-photosynthetic plants such as *Epifagus virginiana* (discussed above) and from the malarial parasite *Plasmodium falciparum* also lack *rpoA*. However, they also lack all other *rpo* genes, which suggests that rather than a functional nuclear transfer, they may have lost the PEP altogether in favor of a transcriptional mechanism that is solely reliant upon NEP.

Although PEP activity can be readily assayed in stromal fractions, with a method used to dissect chloroplast promoter structure *in vitro* (Gruissem and Zurawski, 1985b), purifying PEP has proven more challenging. In dicotyledonous higher plants, attempts to isolate a transcriptionally-active protein fraction comprised of the critical number of proteins yielded preparations containing all of the expected holoenzyme subunits but also up to 13 other co-purifying proteins. As discussed below, the composition of the active fractions varied depending upon the species and the developmental stage of the plastid (reviewed in Liere and Maliga, 2001). However, a much simpler composition was reported from maize, essentially identical to the *E. coli* enzyme (Hu and Bogorad, 1990).

In mustard (*Sinapis alba* L.), two different protein fractions with *in vitro* PEP activity have been identified. One, from etiolated plastids, contains four recognizable proteins corresponding to the $\alpha\beta\beta'\beta''$ core subunits, whereas one from mature plastids possesses the four core proteins and an additional nine. Four of these putative accessory proteins have been identified by microsequencing and MALDI-MS as a 36-kDa RNA-binding protein, a 29-kDa annexin-like protein, a 26-kDa Fe-superoxide dismutase, and a 100- to 110-kDa putative RNA processing protein (Pfannschmidt *et al.*, 2000; Loschelder *et al.*, 2004). Without reconstitution or reverse genetic experiments, it is difficult to evaluate the significance of these co-purifying proteins. The RNA-binding protein, however, is homologous to CSP41 of spinach and tobacco. Depletion of tobacco CSP41 by antisense-expression was accompanied by a decrease in chloroplast transcription activity (Bollenbach *et al.*, 2003). Additionally, a CK2-type plastid localized kinase (PTK) co-purifies with the core enzyme in transcriptionally active fractions from mustard (Ogrzewalla *et al.*, 2002). The possible significance of this kinase is discussed below.

Suzuki *et al.* (2004) linked a poly-histidine tag to the α subunit of the tobacco PEP core polymerase, isolated protein complexes on nickel columns, and attempted to identify all the components by MALDI-TOF MS mass fingerprinting and sequencing by ESI-MS/MS. A ~900-kDa complex containing 13 proteins was purified from isolated chloroplasts. Four of the thirteen proteins were core enzyme subunits and five of the remaining nine were deemed very likely to be functional components based on their abundance. Interestingly, none of these PEP-associated proteins resembled components isolated from the mustard complex and none of them have a clearly discernable function in transcription or RNA processing. The isolated complex could, however, initiate transcription from the *psbA* promoter when mixed with purified maize sigma factors.

### B. Physiology of Cells That Lack PEP

PEP-deficient plants/cell lines and a series of directed transplastomic PEP knockouts have been isolated and characterized. These lines established that PEP activity is required for photosynthesis but not for cell survival.

Deficient plastids remain in an immature state, and not all transcription is abolished in these lines. Specific transplastomic *rpoA*, *B* and *C1* knockouts were produced in tobacco. These plants had to be grown in sucrose-supplemented culture medium due to a total lack of photosynthetic capability (Allison *et al.*, 1996; DeSantis-Maciossek *et al.*, 1999; Krause *et al.*, 2000; Legen *et al.*, 2002). Other examples of PEP-deficient cells have come from less direct mutations such as the clonal absence of plastid ribosomal proteins in barley *albostrians* and maize *iojop*, which cause leaves to produce non-photosynthetic stripes (Walbot and Coe, 1979). Lack of translation in these sectors precludes plastid protein production, including that of PEP (Han *et al.*, 1993; Hess *et al.*, 1993; Silhavy and Maliga, 1998b). Numerous tissue culture lines such as tobacco BY-2, rice and maize BMS were also demonstrated to lack a functional PEP either as the result of nuclear mutations or the loss of *rpo* genes caused by plastid genome instability (Vera and Sugiura, 1995; Vera *et al.*, 1996; Kapoor *et al.*, 1997; Silhavy and Maliga, 1998b; Liere and Maliga, 1999; Cahoon *et al.*, 2003). We must note, however, that loss of PEP does not inevitably result in the complete loss of photosynthetic activity. Two hemiparasitic *Cuscuta* species, *C. gronovii* and *C. subinclusa*, have lost PEP activity but retain photosynthesis, apparently by acquiring NEP promoter motifs (discussed below) upstream of photosynthesis-essential genes such as *rbcL*, which typically have PEP promoter motifs (Berg *et al.*, 2004).

## C. Sigma Factors

The eubacterial RNA polymerase holoenzyme includes the core enzyme and a transiently associated fourth protein (sigma factor), which is essential for promoter recognition and transcriptional specificity in some bacteria (reviewed in Borukhov and Severinov, 2002; Borukhov and Nudler, 2003). Presuming that PEP follows the bacterial model of transcription, and ignoring for the moment possible accessory subunits, we would expect sigma factor homologs to regulate its activity. To date, no sigma factor genes have been found in chloroplast DNA. However, a number of nuclear genes that encode sigma-like proteins have been identified in dicot (e.g. *Arabidopsis*—Table 1) and monocot species (e.g. maize—Table 1), as well as in algae. Experimental evidence relevant to plant sigma-like factors was thoroughly reviewed by Allison (2000). To briefly highlight the state of knowledge at that point, 21 genes encoding sigma-like factors had been cloned from nine species by screening libraries with a conserved prokaryotic sigma-factor region or degenerate PCR primers, or by conducting EST database searches using conserved amino acid sequences. Subsequently, sigma factor genes were isolated from *Nicotiana tabacum* (Oikawa *et al.*, 2000), *Physcomitrella patens* (Hara *et al.*, 2001), and *Sinapis alba* L. (Homann and Link, 2003) using *Arabidopsis* clones or in silico screens. In each case, two or more plastid sigma factors are implied by the available data. In contrast, available genome sequence as well as DNA filter hybridizations strongly suggest that the alga *Chlamydomonas reinhardtii* possesses only a single chloroplast sigma factor (A. V. Bohne, A. Weihe and D. B. Stern, unpublished results). This, combined with evidence that *Chlamydomonas* lacks NEP (Eberhard *et al.*, 2002), hints at an unusually simple chloroplast transcriptional apparatus in this organism.

### 1. Sigma Factor Localization

All sigma-like factors isolated so far are nuclear-encoded, with divergent N-terminal sequences that could include plastid transit peptides. Therefore, determining their subcellular localization has been an important facet of their analysis. Three methods have been used, *in vitro* import of radiolabeled precursors into purified chloroplasts and mitochondria; usage of specific antibodies coupled to cell fractionation and immunoblot analysis; and most popularly, fusion of putative transit peptides to green fluorescent protein (GFP) or yellow fluorescent protein (YFP), followed by transient or stable expression and confocal microscopy.

Use of these techniques for maize (Lahiri *et al.*, 1999) and *Arabidopsis* (Tanaka *et al.*, 1997; Kanamaru *et al.*, 1999), e.g., revealed that most sigma factor genes indeed encode plastid proteins (Table 1), although GFP/YFP experiments obviously do not demonstrate actual localization of the native protein. Thus, in many cases, it remains to be demonstrated whether all sigma-like proteins are indeed expressed *in vivo*, what their relative accumulations are, and whether they exhibit organ- or developmental-specific regulation. Available data are discussed in Section 4, below.

Exceptions to the plastid-only localization rule do appear to exist. *Arabidopsis* AtSig4 and AtSig5 had more ambiguous localization patterns based on GFP fusions (Fujiwara *et al.*, 2000). Closer analysis of AtSig5 showed either localization to plastids, or a dual localization to mitochondria and chloroplasts that was organ-specific and resulted from differential splicing of the mRNA leading to different start codon usage (Yao *et al.*, 2003). ZmSig2B from maize had been

*Table 1.* Sigma factors in *Arabidopsis* and maize

| Sigma factor | Localization[a] | Expression pattern | Knockout or RNAi phenotype |
|---|---|---|---|
| AtSig1 (At1g64860) | Plastid[1] | Protein is more abundant in cotyledons than leaves. but accumulates only after imbibition in the light[5] | None[5] |
| AtSig2 (At1g08540) | Plastid[1,2] | Same as AtSig1 [5] | Pale green[5,6] |
| AtSig3 (At3g53920) | Plastid[1,2] | Protein is more abundant in leaves than cotyledons yet pre-accumulates in embryonic cotyledons[5] | None[5] |
| AtSig4 (At5g13730) | Plastid (?)[3] | NA | NA |
| AtSig5 (At5g24120) | Plastids & Mitochondria[4] | Protein accumulates to its highest level in stems and flowers[4]. | Embryo-lethal[4] |
| AtSig6 (At2g36990) | Plastid[3] | NA | NA |
| ZmSig1 (AF058708) | Plastid[7] | Transcripts detectable only in green tissue[7] | NA |
| ZmSig2 (AF099111.1) | NA | Transcripts detectable in green and etiolated tissues[7] | NA |
| ZmSig2A (AF099110) | Plastid[8,9] | Protein most abundant in green tissue with mature chloroplasts[8] | NA |
| ZmSig2B (AF099110) | Plastids & Mitochondria[8,10] | Transcripts detected in all tissues. Protein most abundant in yellow tissue [9,10] | NA |
| ZmSig3 (AY091464) | NA | NA | NA |
| ZmSig6 (AF099112) | NA | Protein most abundant in non-green tissues[8] | NA |

[1] Kanamaru *et al.*,1999
[2] Isono *et al.*, 1997
[3] Fujiwara *et al.*, 2000
[4] Yao *et al.*, 2003
[5] Privat *et al.*, 2003
[6] Shirano *et al.*, 2000
[7] Tan and Troxler, 1999
[8] Lahiri and Allison, 2000
[9] Lahiri *et al.*, 1999
[10] Beardslee *et al.*, 2002
[a] Localization is a compilation of *in vitro* import of radiolabeled precursor proteins into purified organelles, confocal microscopy-based analysis of transiently expressed fusions of putative transit peptides to GFP, and immunoblot data.

previously shown to co-localize to mitochondria and chloroplasts (Beardslee *et al.*, 2002) and may use a mechanism similar to *Arabidopsis* Sig5 (S. Chowdhury, T. J. Bollenbach, D. B. Stern and L. A. Allison, unpublished results). The function of mitochondrial localization has yet to be determined but is curious, given the absence of eubacterial RNA polymerases in mitochondria except in the single known case of a protozoan whose mtDNA still encodes Rpo proteins (Lang *et al.*, 1997).

## 2. Reverse Genetic Analysis of Sigma Factors

The ready availability of T-DNA insertion lines, and other tools such as antisense expression, allowed rapid analysis of null or near-null phenotypes for four of the six *Arabidopsis* sigma-like factors. Of these, only two exhibited recognizable phenotypes—AtSig2 and AtSig5. AtSig1 and AtSig3 (Privat *et al.*, 2003) appear to be either functionally insignificant or redundant, which should be clarified, e.g., by double mutant construction.

An AtSig2 knockout from a T-DNA insertion (Shirano *et al.*, 2000), or reduction of its expression using an antisense transgene (Privat *et al.*, 2003), produced viable plants with light green cotyledons and impaired chloroplast development. Cotyledon morphology appeared to be unaffected by the absence of AtSig2, but electron microscopy revealed small chloroplasts with poorly developed lamellar structure (Shirano *et al.*, 2000). Interestingly, the *Sig2* antisense gene appeared to be ineffective in mature leaves, where AtSig2 protein was found at wild-type levels. Reduction of transcription in AtSig2 mutants was observed only for a subset of genes, namely *psbA*, *trnE*, *trnD* and *trnV* in the antisense line (Privat *et al.*, 2003), and for the photosynthetic proteins in addition to *trnV*, *trnM*, *trnE* and *trnD* in the knockout line (Kanamaru *et al.*, 2001). A more detailed analysis of *psbD* transcription

initiation in an AtSig2 knockout line showed a failure to initiate from one of its four promoters. Additionally, *trnE* transcripts lacked an unprocessed precursor 26 bases longer than mature *trnE*, whereas *trnV* was nearly undetectable (Hanaoka *et al.*, 2003).

Two AtSig5 T-DNA insertion lines suggested that AtSig5 is essential for seed or embryo development. Self-fertilized heterozygous plants had defective siliques with obvious spaces where seeds had not developed, and no homozygous progeny were detected (Yao *et al.*, 2003). These results suggested that AtSig5 may be important in seed production, or at least for ovule development. Consistent with this hypothesis, AtSig5 protein accumulates to a higher level in reproductive tissue (flowers) than in any other part of the plant. Interestingly, flowers but not leaves contain the minor version of AtSig5 mRNA, which allows targeting of GFP to both mitochondria and chloroplasts.

## 3. Implications of Reverse Genetic Results for Sigma Factor Function

The sigma knockout experiments and transcript analysis have focused on two opposing definitions of sigma factor function. The first of these is specialization; each sigma factor directs transcription from a specific set of promoters. The second is redundancy; all of the sigma factors direct transcription from all PEP promoters. Results so far have failed to universally reject either hypothesis. But definitive results (simply by their nature) have so far supported the first scenario, which assigns specific transcription initiation roles to each sigma factor with a redundancy of promoters amongst genes. AtSig2, for instance, specifically promotes transcription from the −10 and −35 consensus sequences (discussed below). AtSig5 homozygous knockouts were not obtained because of its putative indispensable function, as discussed above. From this we can assign a putative specific transcriptional role, although determining this role will likely require weaker mutant alleles.

The lack of a strong phenotype among AtSig2 knockouts appears to result from genes with multiple functionally-redundant promoters, consistent with the hypothesis that multiple plastid sigma factors that result from ancient gene duplications have redundant roles. The lack of a phenotype in the AtSig1 and AtSig3 antisense lines may also result from redundant functionality, or at least largely overlapping specificities (Homann and Link, 2003). Still, it is equally possible that certain growth conditions and/or stages require individual family members for optimal adaptation or even survival. This hearkens back to bacterial situations where sigma factors "lie in wait", such as those required for sporulation in *Bacillus subtilis* (Kroos and Yu, 2000).

## 4. Sigma Factor Expression

An important line of evidence that gives clues to the functions of sigma factors is their own expression patterns. Generally, sigma factor expression can be described as highest in leaves, with a positive regulation by light (Allison, 2000). There have also been reports of circadian control of sigma factor expression in tobacco and wheat (Morikawa *et al.*, 1999; Oikawa *et al.*, 2000). Table 1 includes a summary of sigma factor expression patterns in *Arabidopsis* and maize.

Nonetheless, the expression of individual factors does differ between plant organs. AtSig1 and AtSig2 are more abundant in cotyledons than in leaves, and AtSig3 is more abundant in leaves than in cotyledons (Privat *et al.*, 2003). The small amount of AtSig3 that does accumulate in cotyledons pre-accumulates in seed-bound cotyledons, and the protein is detectable in dry seeds as well as dark-grown seedlings. In contrast, AtSig1 and AtSig2 accumulate only after imbibition in the light. It is perplexing that AtSig3 accumulates as though it is important for early developmental events, yet reducing its levels by antisense gave no detectable phenotype (Privat *et al.*, 2003). Another sigma factor with a putative essential role in early development, AtSig5, is induced by blue light (Tsunoyama *et al.*, 2002), which suggests that it may be responsible for transcription of the blue-light induced *psbD* transcripts. Indeed, mutagenesis of the barley chloroplast *psbD* promoter showed that the −10 box was required for blue light induction, although other upstream elements, unlikely to interact with sigma factor, also play a regulatory role (Kim *et al.*, 1999). AtSig5 protein accumulates to its highest level in stems and flowers, supporting a possible role in embryo and seed development (Yao *et al.*, 2003).

Organ-specific up-regulation was also observed for *Sinapis alba* sigma factors. SaSig1 and SaSig2 are most abundant in green tissue of light-grown seedlings, whereas SaSig3 is most abundant in etiolated tissue. Interestingly, full length SaSig3 will bind to promoters but does not promote transcription. The authors suggest that the full length SaSig3 protein may be a transcription repressor in etiolated tissues and that its proteolytic removal upon chloroplast maturation activates the promoter. They also suggest that all three

sigma factors can bind to the same promoters and may be functionally redundant but developmentally significant (Homann and Link, 2003).

Maize (and potentially other monocots) offers an analytical advantage over other systems in the form of a developmental gradient along leaves, in which cells at the base of the leaf possess immature plastids, and chloroplasts mature progressively towards the tip. Expression of maize sigma factors differs across this developmental gradient, suggesting putative developmental roles. For instance, ZmSig2B protein accumulates to higher amounts near the leaf base than the tip (Beardslee *et al.*, 2002), ZmSig2A (previously called Sig1) protein is light-responsive and accumulates to its highest level in green leaf tissue, such as the tips of leaves, but ZmSig6 (previously called Sig3) protein is most abundant in non-green tissue, such as the leaf base, etiolated leaves and even roots (Lahiri and Allison, 2000). Another interesting quality of ZmSig2B is its co-localization to both plastids and mitochondria. When reconstituted with a bacterial core, ZmSig2B can form a holoenzyme that recognizes the plastid *psbA* promoter, which strongly supports a transcriptional role in chloroplasts. However, its role in mitochondria, if any, is unknown.

### 5. Sigma Factor Interacting Proteins

Although sigma factors presumably interact with PEP, at least one study suggests that chloroplast sigma factors may interact with additional proteins. AtSig1 was used in a two-hybrid screen, which identified the AtSig1-binding protein (SibI). A SibI homolog (T3K9.5) was subsequently identified by an EST database search (Morikawa *et al.*, 2002). Both proteins exclusively interact with the R4 region (a conserved sigma factor domain that interacts with the −35 element of $\sigma^{70}$ -type promoters) of AtSig1 and have no significant similarity to other characterized proteins. By analogy to bacterial anti-sigma factors, the authors suggest that these proteins may function as negative transcriptional regulators. Considering that AtSig1 knockouts have no specific phenotype and that SibI and T3K9.5 do not resemble anti-sigma factors in primary sequence, further analysis will be required to understand the significance of these findings.

## D. PEP Promoter Recognition

PEP promoters generally resemble eubacterial $\sigma^{70}$ promoters with conserved −10 "TATA" and −35 "Pribnow Box" sequence elements (Stern *et al.*, 1997; Hess and Borner, 1999; Weihe and Borner, 1999; reviewed in Liere and Maliga, 2001). That the −10 and −35 elements function in chloroplasts as they do in *E. coli* was originally demonstrated using *in vitro* transcription systems, allowing high through-put testing of promoter variants (Gruissem and Zurawski, 1985a, b). Recent *in vivo* expression of *psbA* from its PEP promoter as tested by fusion to the GFP-coding region underscores the function of the −35/−10 elements (Hayashi *et al.*, 2003).

Along with the assumption that PEP recognizes canonical eubacterial promoters comes the inference of a sigma factor requirement. Evidence of such a requirement was recently offered through the analysis of AtSig2 knockout lines, where S1 nuclease protection was used to identify precise transcription start sites of affected genes. AtSig2 mutants failed to initiate *psbD, tRNE*-UUC and *tRNV*-UAC transcription from $\sigma^{70}$ like promoters, leading to the suggestion that AtSig2 specifically promotes transcription from promoters with −10 and −35 consensus sequences of TTgAca and TANNaT, respectively (Hanaoka *et al.*, 2003).

Although the examples above suggest that PEP can and does recognize a typical eubacterial −35/−10 promoter, there are exceptions. A recent development regarding PEP promoters involves the rRNA operon (*rrn*), which in tobacco has two promoters. PrrnP2 is recognized by NEP, and PrrnP1, which has canonical −10 and −35 elements, is recognized by PEP. Scanning mutagenesis across the PrrnP1 promoter, however, revealed a surprisingly low reduction in transcription among −10 mutants. On the other hand, mutations both within and immediately upstream of the −35 element resulted in a severe reduction of transcript abundance. These results suggest that a 12-bp element, which includes the −35 motif plus six upstream bases, is essential for transcription from PrrnP1 and that the −10 conserved region is dispensable for this promoter (Suzuki *et al.*, 2003).

There are also exceptional promoters, such as spinach *trnS*, which has no apparent upstream elements (Gruissem *et al.*, 1986), a finding that was mirrored for *Chlamydomonas trnE* (Jahn, 1992). Whether these are transcribed by PEP or another uncharacterized polymerase is still unknown, although the lack of upstream elements is more reminiscent of promoters recognized by RNA polymerase III than of those recognized by eubacterial enzymes. In *Chlamydomonas*, two intragenic elements, $A_{40}$ and $(AAAGGG)_8$, have been shown to

act as transcriptional enhancers when placed upstream of an *aadA* transgene with the *atpA* 5′-region (Lisitsky *et al.*, 2001).

## III. Nuclear-Encoded Polymerase (NEP)

### A. NEP Gene Families

Nuclear genes that encode single-subunit, phage-like RNA polymerases (RpoT's) were isolated from several plant species (summarized in Table 2) and even earlier, partial sequences were amplified from a broad range of eukaryotes (Cermakian *et al.*, 1996). When complete genes were isolated and *in vivo* targeting was investigated, two categories were described in *Arabidopsis* (Hedtke *et al.*, 1997) and maize (Chang *et al.*, 1999; Tan & Troxler, 1999). One has a leader peptide that localized GFP to plastids, while the other targeted GFP to mitochondria. For convenience, we will use the maize nomenclature of RpoTp and RpoTm, respectively (Chang *et al.*, 1999).

One of the more suprising recent developments was the discovery that a third type of transit peptide co-localizes GFP to both plastids and mitochondria (RpoTmp) not only in *Arabidopsis* (Hedtke *et al.*, 2000) but also in *Nicotiana tabacum* (Kobayashi *et al.*, 2001a; Hedtke *et al.*, 2002), *Nicotiana sylvestris* (Kobayashi *et al.*, 2001b), and *Physcomitrella patens* (Kabeya *et al.*, 2002; Richter *et al.*, 2002). In all four cases the 5′-region of the mRNAs contains two potential in-frame start codons. When both AUG's are fused to GFP, the protein co-localizes to both organelles. However, fusions that were forced to initiate from the second AUG localized exclusively to mitochondria. Alternatively, when the second start codon was changed to an isoleucine and the proximal one left intact, the fusion protein in *Arabidopsis* and *N. sylvestris* co-localized to both organelles (Hedtke *et al.*, 2000; Kobayashi *et al.*, 2001b), but in *Physcomitrella* the fusion protein was specifically imported into plastids (Richter *et al.*, 2002). This may reflect an artifact of the chimeric construction or a species-specific difference.

The dual-localizing variant is intriguing, but there are still some very basic questions that must be addressed. For example, are the enzymes active in both organelles and indeed, is the GFP result indicative of what occurs with RpoTmp itself? Also, is this gene the vestige of a transition from the presumably older mitochondrial RpoTm to the plastid RpoTp ? The latter question could be addressed by grouped alignments, once putative localization has been determined. Multiple RpoT genes most likely arose from the original eukaryotic mitochondrial-localized, phage-like RNA polymerase. Gene duplications coupled with new transit peptides then may have given rise to differently localized RpoT's.

At least two sequences of events could have conspired to produce this end result. First, RpoTm was

*Table 2.* Localization of nucleus-encoded phage-like RNA polymerases based on GFP fusions

| | Species | RpoTp Plastid | RpoTmp Mitochondria and plastids | RpoTm Mitochondrial |
|---|---|---|---|---|
| Dicot | *A. thaliana*[1] | AtRpoT;3 | AtRpoT;2 | AtRpoT;1 |
| | *N. tabacum*[2] | NtRpoT3 | NtRpoT2 | NtRpoT1 |
| | *N. sylvestris*[3] | NsRpoTp | NsRpoT-B | NsRpoT-A |
| Monocot | *C. album*[4] | | | CaRpoT |
| | *Z. mays*[5] | ZmRpoTp | | ZmRpoTm |
| | *T. aestivum*[6] | TaRpoTp | | TaRpoTm |
| | *H. vulgare*[7] | HvRpoTp | | HvRpoTm |
| | *O. sativa*[8] | OsRpoTp | | OsRpoTm |
| Moss | *P. patens*[9] | | PpRpoT1<br>PpRpoT2 | |
| Protist | *P. falciparum*[10] | | | PfRpoT |

[1] Hedtke *et al.*, 1997, 2000
[2] Hedtke *et al.*, 2002
[3] Kobayashi *et al.*, 2001a; Kobayashi *et al.*, 2001b; Kobayashi *et al.*, 2002
[4] Weihe *et al.*, 1997
[5] Chang *et al.*, 1999; Young *et al.*, 1998
[6] Ikeda and Gray, 1999
[7] Emanuel *et al.*, 2004
[8] Kusumi *et al.*, 2004
[9] Richter *et al.*, 2002
[10] Li *et al.*, 2001

duplicated and the duplicate added a plastid transit peptide that resulted in dual localization (RpoTmp), which was then followed by duplication of RpoTmp to produce a copy that lost its mitochondrial transit peptide to yield RpoTp. Or, second, two independent duplications of RpoTm occurred that resulted in RpoTmp and RpoTp. Sequence alignments using RpoT regions from all known nuclear-encoded RpoT genes as well as viral homologs predictably grouped all plant homologs together (Kabeya *et al.*, 2002; Richter *et al.*, 2002). Within this group, RpoTp genes were clearly related, but RpoTm and RpoTmp homologs tended to group together and not form separate branches. This suggests that RpoTmp homologs diverged from RpoTm more recently than RpoTp, supporting a model of two independent duplication events, the second of which added the dual-localizing plastid transit peptides. We note that in all monocots examined to date, maize, wheat (Ikeda and Gray, 1999), barley (Emanuel *et al.*, 2004) and rice (Kusumi *et al.*, 2004), no evidence was found for a third RpoT. Thus, this putative duplication may have occurred after the divergence of monocots and dicots.

There is only one suspected example of a photosynthetic organism that lacks NEP. *Chlamydomonas* plastid transcription is fully inhibited by the PEP-specific antibiotic rifampicin, suggesting that PEP is exclusively responsible for transcription (Eberhard *et al.*, 2002). One could speculate that the importance of NEP in higher plants is developmental, and single-celled photosynthetic organisms such as *Chlamydomonas* simply do not require it.

### *B. Evidence of RpoTp Activity and NEP Transcription Factors*

In spite of the almost ubiquitous distribution of RpoTp in the plant kingdom, an understanding of the composition of the active enzyme has been elusive. To date, we have learned about the NEP promoter structure principally through development of *in vitro* transcription systems from plastid protein fractions devoid of PEP (Kapoor and Sugiura, 1999; Liere and Maliga, 1999). However, successful attempts to reconstitute specifically NEP transcription *in vitro* with purified recombinant proteins have not been reported. *In vitro* RpoTp activity was reported by Hedtke *et al.* (2000), who expressed His-tagged AtRpoTp in *E. coli*, purified it, and assayed non-specific transcription using calf thymus DNA as template. They found that the non-specific activity was insensitive to tagetin, rifampicin, and actinomycin D, all of which inhibit PEP. Similar experiments with maize RpoTm revealed a robust but non-specific transcription activity (D. B. Stern, K. Stern and S. Lerbs-Mache, unpublished results).

The lack of specificity of RpoTp or RpoTm when assayed alone is unsurprising, considering that human, yeast, *Xenopus*, and presumably all other mitochondrial core RNA polymerases require at least two protein factors to initiate transcription (Antoshechkin and Bogenhagen, 1995; Shoubridge, 2002; McCulloch and Shadel, 2003). While definitive plant orthologs of these factors remain to be identified, many candidates exist. Two mitochondrial transcription factors are from the RNA methyltransferase and the High Mobility Group proteins, both of which are encoded by multigene families in plants. Sorting out the subcellular localizations and biochemical activities of these gene products will take considerable effort.

Biochemical evidence does exist, however, for a plastid RpoT transcription factor, although it is apparently specialized in transcription of the *rrn16* (rRNA) operon. CDF2 (chloroplast DNA binding factor, a ~35-kDa protein) was first identified as a factor that specifically bound to the non-canonical NEP promoter ($P_c$) upstream of *rrn16* (Baeza *et al.*, 1991; Iratni *et al.*, 1997). Spinach *rrn16* also possesses an overlapping PEP promoter. Further analysis of CDF2 revealed a dual role based on two putative forms of the protein (Bligny *et al.*, 2000). One form (CDF2A) binds to $P_c$ and represses PEP initiation, while a different form (CDF2B) binds and promotes transcription by NEP-2. The data suggest that CDF2A is produced during the late fall and plays a role in shutting down plastid activity in preparation for winter, whereas CDF2B is produced in the early spring to recruit NEP-2 to the *rrn16* promoter and jump-start plastid activity. One component of CDF2 was subsequently revealed to be ribosomal protein L4 (Trifa *et al.*, 1998). Interestingly, CDF2 and its use of $P_c$ appear to be species-specific. When $P_c$ was fused to *uidA* and introduced into tobacco plastids, no transcription of the transgene was detected, which suggested that the requisite CDF2 was not present (Sriraman *et al.*, 1998), although in any species a ribosome-bound form of L4 is highly likely to be present.

### *C. NEP-Specific mRNAs and Promoter Recognition Sequences*

That certain plastid transcripts were entirely dependent on NEP activity was first proposed after analysis of plants lacking PEP. Analysis of monocots that lack PEP because of non-functioning plastid ribosomes (Hess *et al.*, 1993; Hubschmann and Borner, 1998;

Silhavy and Maliga, 1998b; Cahoon *et al.*, 2003), and tobacco lines with directed transplastomic *rpo* gene knockouts (Allison *et al.*, 1996; Hajdukiewicz *et al.*, 1997; Serino and Maliga, 1998; Krause *et al.*, 2000; Legen *et al.*, 2002), revealed accumulation of approximately 15 transcripts in spite of the missing PEP. Of these, the levels of three transcripts (*clpP*, *accD* and *rpoB*) were much greater in the PEP-deficient plants than in their wild-type counterparts. The implications of this finding are discussed below.

Analysis of NEP promoter sequences has been extensively reviewed (Weihe and Borner, 1999; Liere and Maliga, 2001). To summarize briefly, RpoT promoters bear no resemblance to PEP promoters but rather resemble mitochondrial promoters, which in plants (Caoile and Stern, 1997; Brennicke *et al.*, 1999) and yeast (Biswas *et al.*, 1987), e.g., have been studied through the use of *in vitro* systems. The promoters in plastids can be broken down into three groups. Type Ia consists of a YRTA motif embedded in a 15-nt AT-rich region. Type Ib has the same YRTA box with a second GAA-box 18- to 20-nt upstream. This GAA sequence was shown to be essential for *atpB* transcription *in vivo* using transplastomic tobacco (Xie and Allison, 2002). Type II promoters are approximately 30-nt long and mostly reside downstream of the transcription start site. Although apparently conserved among many plant species, the importance of a Type II promoter has been demonstrated only for tobacco *clpP*. There is also at least one recognized active non-canonical promoter, spinach *rrn16* $P_c$, which was discussed above. Interestingly, the promoter types are not universally conserved among the same genes between species. For example, *clpP* in *N. tabacum* has a Type Ia promoter, whereas the same gene in barley and maize has a Type Ib promoter.

The hyper-accumulation of certain transcripts in PEP-deficient tobacco contributed to the enumeration of NEP-derived transcripts, while simultaneously raising the question of whether NEP activity increases in the absence of PEP in a compensatory manner. Examination of plastid transcription activity by run-on assays in PEP knockout tobacco lines, combined with RNA accumulation measurements, suggested that all plastid genes can be transcribed by NEP to some degree, and that the increased levels of certain mRNAs are largely a function of mRNA stability (Krause *et al.*, 2000; Legen *et al.*, 2002). Similar experiments with wild-type maize leaves also highlighted the importance of post-transcriptional processes and identified two transcript stability classes corresponding to NEP- and PEP-derived transcripts (Cahoon *et al.*, 2004). In this view, selective transcription by NEP from specific promoters is not the primary cause of transcript hyper-accumulation, and thus primary control of RNA accumulation may be post-transcriptional. The primacy of post-transcriptional control in plastids has been advocated for some 15 years (Deng and Gruissem, 1987; Mullet and Klein, 1987; Deng and Gruissem, 1988; Deng *et al.*, 1989), and these newer studies match an older picture. An ancillary issue raised by the knockout studies is how well they represent gene regulation in wild-type plants, either in terms of transcriptional or post-transcriptional control. Existing data suggest, however, that NEP does not transcribe "PEP genes" in wild-type plants, because PEP-transcript 5′-ends generally map to canonical PEP promoters.

### *D. Dynamics of PEP-NEP Activity during Plastid Development*

To date, inferences regarding regulation of NEP activity are based upon expression of the *RpoTp* genes themselves. The best estimates of RpoTp expression during plastid development came from the dissection of maize, barley, and rice leaves, which offer a developmental gradient ranging from etioplasts at the base of the leaf to fully mature chloroplasts at the tip (Leech *et al.*, 1973). *RpoTp* transcripts and protein were detected along the length of maize leaves using quantitative PCR (Chang *et al.*, 1999) and immunochemical detection with isolated plastids (Cahoon *et al.*, 2004). Steady-state transcript abundance was high in the base and increased progressively along the leaf, peaking near the mid-point of the leaf and then decreasing precipitously, such that abundance at the tip was only about 1/4 of that at the base. Protein abundance followed a similar pattern and was highest near the base and then tapered off along the length of the leaf. Very similar transcript and protein patterns were observed in rice stems and leaves (Kusumi *et al.*, 2004). In barley leaves, the highest levels of transcript occurred in the youngest part of the leaf (in the base), declined in the region just above the base, rose to a second lower peak around 1 cm from the base, and then remained constant along the remainder of the leaf. Whether these *RpoTp* transcript levels are reflective of the enzyme's activity is unknown, because it might be regulated at other levels or limited by the availability of transcription factors. Nevertheless, we can infer that it may, because the transcript accumulation for three genes with NEP promoters (*clpP*, *rpl2* and *rpoB*) closely mimics the transcription of *RpoTp* in barley leaves (Emanuel *et al.*, 2004). Among *Nicotiana* species, transcripts for the dual-targeted RpoT2 in *N. sylvestris* were found to be most abundant in mature leaves and lowest in cotyledons (Kobayashi *et al.*, 2001b). Transcripts for

the plastid-specific RpoT3 in PEP-deficient *N. tabacum* were more than double the amounts found in wild-type cells, which suggested an up-regulation of NEP-driven transcription to compensate for the lack of PEP (Hedtke *et al.*, 2002).

Although complete elimination of NEP has not been accomplished, a few recent reports tentatively confirm the *in vivo* role of NEP. T-DNA insertions of the dual-targeted RpoTmp (*rpoT2*) were recently isolated and analyzed (Baba *et al.*, 2004). Knockout lines exhibited retarded seedling development marked by short roots, round wrinkled leaves, and delayed greening. As the plant matured, however, growth and appearance began to resemble normal plants. Chloroplast transcription was analyzed via macro-arrays and was most affected during early development for all genes tested, which included both NEP- and PEP-derived transcripts. Interestingly, mitochondrial transcription was unaltered by the knockout. The authors suggest that RpoTmp is implicated in early plant development but is not essential for plant survival. The alternative approach of over-expressing NEP was also examined. A substantial increase in the levels of transcripts from genes with NEP promoters were reported, offering *in vivo* evidence that *RpoTp* does indeed encode NEP (Magee and Kavanagh, 2002).

## IV. The Big Picture: Transcriptional Regulation in Chloroplasts

### *A. Does Transcriptional Regulation Play an Important Role in Chloroplast Biogenesis?*

It is not surprising that removing PEP activity from a plastid removes photosynthetic capabilities, as described above. Unexpected, however, is the essential nature of Sig5, which when missing appears to result in non-viable embryos. Based upon its similarity to eubacterial sigma factors, a role in PEP transcription can be assumed. However, because PEP can be removed while retaining viability, a further function for Sig5 must exist. One can envision that Sig5 is an NEP transcription factor in plastids and/or in mitochondria, in which it is also imported, and it is the deficiency in NEP activity that confers the non-viable phenotype. Alternatively, Sig5 might have a non-transcriptional and/or mitochondrial function that remains to be recognized.

### *B. Revision of the Original Model*

The current model of chloroplast transcription is based upon a division of labor between the two enzymes, PEP and NEP. The model suggests that the majority of NEP promoters are recognized by NEP in tissues with undifferentiated proplastids, but that the same promoters are then unused or less recognized by NEP in mature chloroplasts (Liere and Maliga, 2001; Xie and Allison, 2002). This conclusion principally derives from analysis of PEP-deficient plants and cell lines, which revealed that only a handful of plastid genes are preferentially transcribed by NEP. Also, transcripts from these NEP-transcribed genes were more abundant in some of the PEP-deficient tissues and cells, suggesting that there may be a developmental regulation of NEP (Han *et al.*, 1993; Hess *et al.*, 1993; Hess *et al.*, 1994; Allison *et al.*, 1996; Silhavy and Maliga, 1998a, b; Kapoor and Sugiura, 1999; Zubko and Day, 2002; Cahoon *et al.*, 2003).

This model still appears to be valid in many respects, but must be updated and reinterpreted in light of new data, as we have done in Fig. 1. Fig. 1a depicts

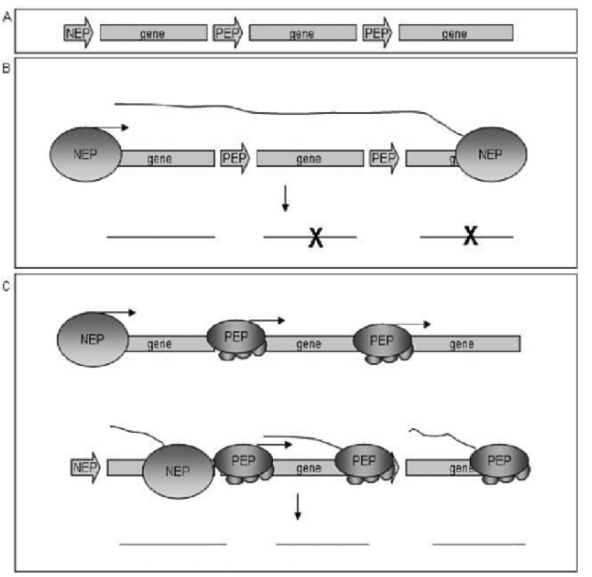


*Fig. 1.* Chloroplast transcription model. Panel A represents a portion of the chloroplast genome. The arrow labeled "NEP" represents a nuclear-encoded RNA polymerase promoter region. The arrows labeled "PEP" represent plastid-encoded RNA polymerase promoter regions. Panel B depicts transcription in non-photosynthetic proplastids, where NEP is the polymerase exhibiting the highest activity. In this diagram, NEP initiates transcription at a specific promoter, and we postulate that elongation is highly progressive, making it possible for NEP to synthesize long pre-mRNAs. The transcripts produced in the immature plastid are processed and selectively degraded. Panel C represents transcription in the mature chloroplast. PEP is now the polymerase exhibiting the highest activity, initiating exclusively at PEP promoters. Although NEP is still present and active, its function is reduced either through *trans*-acting protein factors or physical exclusion by the now abundant PEP.

a series of genes and promotors in the chloroplast genome. Here we propose that in immature plastids, NEP is the predominant polymerase activity (Fig. 1b). It recognizes NEP-type promoters and polymerizes long transcripts that may extend from the initiating promoter to the next NEP promoter or farther. Considering the degenerate nature of known NEP promoters, NEP may even prime non-specifically at sequences within and outside of genes, resulting in non-functional transcripts. Whatever their origin, these transcripts are processed and most are selectively degraded, leaving behind the transcripts that had been previously defined as NEP-derived. As the immature plastid matures into a chloroplast (Fig. 1c), PEP becomes more abundant and begins transcribing from the numerous canonical $\sigma^{70}$-type promoters. The sheer abundance of PEP, in this model, limits NEP initiation by obscuring promoters (Bligny *et al.*, 2000) or through mechanical disruption when the polymerases bump into one another along the coding DNA strands (Brewer, 1988). NEP that is not associated with a template might then be susceptible to proteolytic degradation, or regulation could occur via a transcription factor(s). While this model is consistent with currently available data, many aspects of it are untested, particularly because the biochemical components of the transcriptional apparatus remain to be defined with certainty, particularly for NEP. Breakthroughs on this front are likely to make possible a leap forward in our comprehension of this unexpectedly complex process.

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# Chapter 9

# Plastid-to-Nucleus Signaling

Åsa Strand* and Tatjana Kleine
*Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden*

Joanne Chory
*Plant Biology Laboratory and Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA, U.S.A.*

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## Summary

The function of the eukaryotic cell depends on the regulated and reciprocal interaction between its different compartments. This includes not only the exchange of energy equivalents but also information. Most information exchange flows from the nucleus to the organelles, because the large majority of genes encoding proteins with organellar function are encoded in the nucleus. Nevertheless, there is evidence that the expression of these genes is regulated by signals originating in the organelles. It is clear that several different plastid processes produce signals influencing nuclear photosynthetic gene expression, and to date the tetrapyrrole Mg-protoporphyrin IX, the redox state of the thylakoid membrane and reactive oxygen species are the best characterized plastid signals. This chapter will describe the tetrapyrrole-mediated pathway in detail, but also discuss plastid-to-nucleus communication during chloroplast development and the interaction between light and plastid signaling pathways.

*Author for correspondence, email: Asa.Strand@plantphys.umu.se

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## I. Introduction

Chloroplasts, like mitochondria, evolved from free-living prokaryotic organisms that entered the eukaryotic cell through endosymbiosis. The genome of the cyanobacterium *Synechocystis* encodes more than 3,100 genes (Kotani and Tabata, 1998) while the plastid genome codes for less than 100 of the estimated 3,000 proteins in the chloroplast (Leister, 2003). The gradual conversion from endosymbiont to organelle during the course of evolution has clearly been accompanied by a dramatic reduction in genome size as the chloroplasts lost most of their genes to the nucleus and became dependent on their eukaryotic host. The genes that remained in the chloroplast genome are photosynthesis related or encode components of the plastid gene expression machinery (rRNA, a complete set of tRNA and some ribosomal proteins) (Wakasugi *et al.*, 2001).

Nuclear-encoded plastid proteins are translated as precursors on 80S ribosomes in the cytosol and targeted post-translationally to the chloroplasts (see Chapter 3). Each precursor protein carries an $NH_2$-terminal targeting signal, the transit peptide that guides the protein to the chloroplasts (Bruce, 2001). Translocon complexes in the outer and inner envelope membrane, Toc and Tic, mediate import to the chloroplast (Jarvis and Soll, 2002). After translocation, the proteins are processed by stromal peptidases and are folded to their final configuration by molecular chaperones (Keegstra and Cline, 1999). The plastid-encoded proteins are translated in the plastid on prokaryotic-like 70S ribosomes, a relic of the endosymbiotic past of the organelle.

The photosynthetic apparatus is composed of proteins encoded by genes from both the nucleus and the chloroplast. For example, in the photosynthetic electron transport complexes of the thylakoid membrane, the core subunits are encoded by the chloroplast genome and the peripheral subunits are encoded by the nuclear genome. In the stroma, the large subunit of Rubisco is chloroplastically encoded whereas the small subunit is nuclear encoded. To ensure that all these photosynthetic complexes are assembled stoichiometrically, and to enable their rapid reorganization in response to a changing environment, the activities of the nuclear and chloroplast genomes must be closely coordinated through intracellular signaling.

The necessity of a tight coordination of expression by the different genomes has led to the evolution of mechanisms to coordinate nuclear and organellar gene expression. These include both anterograde and retrograde controls (Rodermel and Park, 2003). Anterograde mechanisms (nucleus-to-plastid) coordinate gene expression in the plastid with cellular and environmental cues that are perceived and choreographed by genes in the nucleus. This type of traffic includes nuclear-encoded proteins that regulate the transcription and translation of plastid genes. Retrograde (plastid-to-nucleus) signaling, on the other hand, coordinates the expression of nuclear genes encoding plastid proteins with the metabolic and developmental state of the plastid (Susek *et al.*, 1993).

## II. Intracellular Communication During Chloroplast Development

Plastids exhibit a very clear developmental program where all plastids are derived from proplastids present in meristematic cells. Transcriptional activity is low in the proplastids but is activated during the development to chloroplasts (Baumgartner *et al.*, 1989). The initiation of chloroplast development in the light and synthesis of the chloroplast transcription and translation apparatus is dependent on anterograde mechanisms through nuclear-encoded components (Leon *et al.*, 1998).

Chloroplast genes of higher plants are transcribed by at least two types of RNA polymerases, one is the nuclear-encoded plastid RNA polymerase (NEP), a T3-T7 bacteriophage-type that predominantly mediates the transcription of house-keeping genes such as components of the gene expression machinery during the early phase of chloroplast development. NEPs are encoded by the *RPOT* genes, and in *Arabidopsis* three *RPOT* genes have been identified, *RPOT;1* and *RPOT;3* are directed to mitochondria and chloroplasts, respectively, whereas the third *RPOT;2* has dual targeting properties (Hedtke *et al.*, 1997, 2000). The other type is a plastid-encoded RNA polymerase (PEP), which is a eubacterial-type, multi-subunit enzyme. Photosynthesis related genes such as *PSBA, PSBD* and *RBCL* are transcribed by PEP (Allison *et al.*, 1996; DeSantis-Maciossek *et al.*, 1999). Although the core subunits for PEP (rpoA, rpoB, rpoC1 and rpoC2) are encoded by the plastid genome, the promoter specificity factors, the sigma factors, of this enzyme are encoded by nuclear genes. To date, six genes encoding putative PEP

*Abbreviations:* Mg-ProtoIX – Mg-protoporphyrin IX; Rubisco – ribulose 1,5-*bis*phosphate carboxylase/oxygenase; NEP – nuclear-encoded plastid RNA polymerase; PEP – plastid-encoded RNA polymerase; LHC – light-harvesting complex; RBCS – Rubisco small subunit; PSI – photosystem I; PSII – photosystem II; PQ – plastoquinone; ROS – reactive oxygen species; gun – genome uncoupled.

sigma factors (SIG1-SIG6) have been identified and characterized in *Arabidopsis* (Hanaoka *et al.*, 2003).

Transcriptional control is the key element in regulating the expression of plastid-encoded genes but post-transcriptional and translational events also play a role during chloroplast development (Leon *et al.*, 1998). RNA processing and transcript stability seems to be important for the accumulation of several photosynthetic mRNAs during the transition from proplastid to chloroplast. Nuclear-encoded RNA binding proteins are required for correct mRNA processing (Hayes *et al.*, 1996) and nuclear-encoded proteins have been identified that interact with specific stem-loop sequences in the 5′UTR of chloroplast transcripts (Danon and Mayfield, 1991; Hauser *et al.*, 1996; Hirose and Sugiura, 1996). These RNA binding complexes seem to modulate ribosome binding, acting as translational activators or repressors (Mayfield *et al.*, 1995). Furthermore, some of the ribosomal proteins are nuclear encoded and are imported to the chloroplast to form ribosomes. Thus, chloroplast development is under strong anterograde control, where the nucleus initiates chloroplast differentiation and provides key components of the transcriptional and translational machinery that are also required for later stages of development (Mullet, 1993; Mayfield *et al.*, 1995).

Although the control of chloroplast development appears to be overwhelmingly under nuclear control, several reports demonstrated that nuclear genes encoding key components in the photosynthetic machinery are regulated by retrograde mechanisms, signals originating in the plastids. When inhibitors of plastid transcription and translation are applied to seedlings, induction of expression of genes encoding light-harvesting complex apoproteins (*LHC*) and the small subunit of Rubisco (*RBCS*) is strongly inhibited (Oelmüller *et al.*, 1986; Rapp and Mullet, 1991; Sullivan and Gray, 1999). Interestingly, these inhibitors have no effect when applied later than 48 to 72 hours after germination, suggesting that the plastid signal must involve a product of early plastid gene expression (Sullivan and Gray, 1999).

## III. Retrograde Communication Through "Plastid Signals"

### *A. Mutants With Aberrant Plastids Provided Evidence for Plastid Signals*

Some of the earliest evidence for the existence of a "plastid signal" came from studies of mutants with morphologically aberrant plastids. These mutants are either albinos, have a pale phenotype or show white-green variegations. Several mutants within these categories demonstrated reduced expression of nuclear-encoded plastid components (Table 1). These include mutants with defective plastid protein synthesis such as the plastid ribosome-deficient *albostrians* barley mutant and the *Brassica napus al* mutant (Bradbeer *et al.*, 1979; Hess *et al.*, 1994; Zubko and Day, 1998). The *Arabidopsis cla1* and *alb3* mutants and the *dag* mutant of *Antirrhinum* also demonstrate severe inhibition of chloroplast development and reduced expression of nuclear-encoded photosynthesis genes (Long *et al.*, 1993; Chatterjee *et al.*, 1996; Mandel *et al.*, 1996; Estevez *et al.*, 2000). The *CLA1* and *DAG* genes both encode plastid-localized proteins of unknown function (Chatterjee *et al.*, 1996; Mandel *et al.*, 1996) whereas *ALBINO3* encodes a protein localized to the chloroplast that shares similarity with a yeast mitochondrial protein required for the assembly of the cytochrome oxidase complex (Sundberg *et al.*, 1997). Reduced expression of nuclear-encoded photosynthesis genes is also observed in carotenoid deficient mutants of maize and the *Arabidopsis* mutant *immutans* (Mayfield and Taylor, 1984; Wetzel *et al.*, 1994; Wu *et al.*, 1999). Carotenoids are synthesized in the plastids and protect the organelle from photooxidative damage by scavenging triplet excited chlorophyll and singlet oxygen, and carotenoid deficient mutants all show a characteristic photobleached phenotype.

However, some mutations that disturb plastid morphology and leaf development do not affect nuclear gene expression, demonstrating that not all aspects of plastid physiology impact plastid-to-nucleus signaling and that the signals for chloroplast development and plastid-to-nucleus communication are not necessarily the same. For example, transgenic lines over- and under-expressing the *Arabidopsis* ankyrin repeat gene, *AKR*, the *pale cress*, *pac* mutant of *Arabidopsis*, the tobacco *ali* mutant and the tomato *dcl* mutant, all affect chloroplast development but do not show reduced expression of nuclear-encoded *LHC* genes (Table 1) (H. Zhang *et al.*, 1992, 1994; Reiter *et al.*, 1994; Keddie *et al.*, 1996; Bae *et al.*, 2001; Bellaoui *et al.*, 2003). AKR deficiency blocks proplastid to chloroplast development in *Arabidopsis* but the specific mechanism is unknown (H. Zhang *et al.*, 1992). Both the *Arabidopsis PAC* gene and the tomato *DCL* gene encode novel components that control the development of chloroplasts. The gene affected in the tobacco *ali* mutant is unknown but the mutants show chlorophyll deficiency in leaves, stem and cotyledons and do not develop organized thylakoid membranes (Bae *et al.*, 2001).

*Table 1.* Mutants with morphologically aberrant plastids divided into different categories depending on their effect on nuclear genes encoding plastid proteins

| Mutant | Species | Phenotype/Plastid morphology | Encoded protein/ function miscellaneous | Expression of Plastid-encoded photosynthesis genes | Expression of nuclear-encoded photosynthesis genes | Reference |
|---|---|---|---|---|---|---|
| | | | 1. Nuclear and plastidic photosynthesis genes influenced | | | |
| *al* | *Brassica napus* | chorophyll deficiency, poorly formed, incompletely differentiated plastids; plastid ribosome deficiency | ? | *PSBA* mRNA transcript level reduced | *LHCB* expression not investigated, but LHCII proteins not detectable | Zubko and Day, 1998 |
| *albino* | Barley | 16S, 23S rRNA mRNA undetectable or greatly reduced | ? | *RBCL*, *PSBD-PSBC* mRNA undetectable or greatly reduced | *LHCB* expression reduced | Dunford and Walden, 1991 |
| *albostrians* | Barley | proplastid-like in leaves; plastid ribosome deficiency | ? | *RBCL* and *PSBA* transcript level reduced | *LHCB* transcript level reduced | Hess *et al.*, 1993, 1994 |
| *cla1* | *Arabidopsis* | albino, number of thylakoids and the extent of stacking into grana significantly reduced | plastid DXP synthase (1-deoxyxylulose 5-phosphate synthase) | *PSAB* expression reduced | *LHCB* expression reduced | Mandel *et al.*, 1996 Estevez *et al.*, 2000 |
| *cue1* | *Arabidopsis* | white-green reticulate; interveinal regions of leaves are pale, whereas paraveinal regions are green; light-sensitive; mesophyll cells: aberrant and small plastids with few grana; bundle sheath cells: normal chloroplasts | plastid PPT (phospho-enolpyruvate/ phosphate translocator) | *PSBA* and *RBCL* transcript level reduced | *LHCB* and *RBCS* expression reduced | Li *et al.*, 1995 Lopez-Juez *et al.*, 1998 Streatfield *et al.*, 1999 |
| *zebra* | Rice | leaves with chlorotic stripes; under continuous light at 30°C no chlorotic stripes | ? | *RBCL* and *PSBA* transcript level reduced to appr. 70% of wild type level | *RBCS and LHCB* transcript level reduced to appr. 10% of wild type level | Kusumi *et al.*, 2000 |
| | | | 2. Nuclear photosynthesis gene expression influenced, plastidic gene expression not yet determined | | | |
| *alb3* | *Arabidopsis* | 5% chlorophyll content of wild type; defective plastid protein synthesis | plastid protein with homology to a bacterial membrane protein and to yeast mitochondrial OXA1; seems to be part of a complex which is needed to integrate LHCP into thylakoid membranes | ? | *LHCB* and *RBCS* expression reduced | Long *et al.*, 1993 Sundberg *et al.*, 1997 Moore *et al.*, 2000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *chm* | *Arabidopsis* | white-green-yellow variegated; white sectors: abnormal plastids with reduced granal stacking, green sectors: normal chloroplasts | a lot of aberrant mitochondria, mtDNA alterations, mitochondrial protein expression altered | ? | ? | Martinez-Zapater *et al.*, 1992<br>Sakamoto *et al.*, 1996 |
| *dag* | *Antirrhinum* | white leaves with green revertant sectors, white sectors: proplastid-like, green sectors: normal chloroplasts | plastid 26 kDa protein (unknown function); *RPOB* (plastidial RNA polymerase beta subunit) expression reduced | ? | *LHCB* and *RBCS* expression reduced | Chatterjee *et al.*, 1996, 1997 |
| *immutans* | *Arabidopsis* | white-green variegated, temperature- and light-sensitive; white sectors: normal chloroplasts and vacuolated plastids lacking lamellae; green sectors: normal chloroplasts | plastid AOX (alternative oxidase) homolog | ? | white sectors: *LHCB* expression reduced, green sectors: normal | Wetzel *et al.*, 1994<br>Carol *et al.*, 1999<br>Wu *et al.*, 1999<br>Aluru *et al.*, 2001 |
| 3. Nuclear photosynthesis gene expression not influenced | | | | | | |
| *ali* | Tobacco | chlorophyll deficiency in leaves, stems and cotelydons, no organized thylakoid membrane system; transcript level of 16S and 23S rRNA slightly reduced | ? | ? | *LHCB* expression not affected | Bae *et al.*, 2001 |
| *akr* | *Arabidopsis* | AKR deficiency blocks proplastid to chloroplast development | protein with four ankyrin repeats | *RBCL, PSBA* not influenced | *LHCB, CAB4, RBCS, GAPA* not influenced | H. Zhang *et al.*, 1992, 1994 |
| *dcl* | Tomato | white-green variegated; white sectors: proplastid-like, green sectors: normal chloroplasts; aberrant polysome assembly in the aberrant chloroplasts | plastid 25 kDa protein (unknown function)/ protein level of POR and TOC75 not influenced | *RBCL*, *PSBA*, *PETA* and ATPB mRNA steady state level not influenced. But lower protein level of RBCL, D1, CF$\alpha$ | *LHCB* and *RBCS* expression normal, but protein level of *RBCS, LHCB, PSI-D* reduced | Keddie *et al.*, 1996<br>Bellaoui *et al.*, 2003 |
| *pac-1* | *Arabidopsis* | chlorophyll and carotinoid content less than 3% of wild type; plastids show less thylakoid development and retain the irregular shape of etioplasts | plastid 36 kDa protein, role in plastid mRNA maturation, controls development of leaves and chloroplasts | *PSBA* expression decreased | *LHCB* expression not altered | Reiter *et al.*, 1994 |

Why defects in these different proteins do not influence the retrograde communication is not known but clearly these proteins function in a different pathway or at a different developmental state from the one controlling expression of nuclear-encoded photosynthetic genes. More information on the function of these proteins and detailed comparisons of the different groups of mutants could help elucidate the mechanisms behind plastid-to-nucleus communication during chloroplast development.

### B. Redox Control of Nuclear Gene Expression

There is considerable evidence that photosynthesis itself provides signals that modulate nuclear photosynthetic gene expression (Escoubas *et al.*, 1995; Pfannschmidt *et al.*, 2001; Pfannschmidt, 2003). In photosynthesis, a chain of electron carriers connects the two separate light-driven reaction centers, photosystem II (PSII) and photosystem I (PSI) (see Chapter 2). The redox state of one of the connecting electron carriers, plastoquinone (PQ), has been shown to influence the expression of photosynthetic genes encoded both in the chloroplast and in the nucleus (Escoubas *et al.*, 1995; Huner *et al.*, 1998; Pfannschmidt *et al.*, 1999; Pfannschmidt *et al.*, 2001; Pfannschmidt, 2003). The redox state of the chloroplast also affects phosphorylation of thylakoid proteins (Rintamaki *et al.*, 2000) and the phosphorylation state has been suggested to be involved in the regulation of *LHC* expression in the nucleus.

At high irradiances the production of reactive oxygen species (ROS) increases (see Chapter 27). In correlation with the increased production of ROS, there is an induction of nuclear genes encoding proteins involved in the antioxidant defense system (Karpinski *et al.*, 1997, 1999). Changes in concentrations or rates of production of ROS could be additional initiators of signaling pathways originating in the chloroplast (Mullineaux and Karpinski, 2002). Increases in foliar $H_2O_2$ concentrations have been shown to be important for the induction of the ascorbate peroxidase gene *APX2*. $H_2O_2$ is thought to diffuse as easily as water across biological membranes (Karpinski *et al.*, 1999) so chloroplast-derived $H_2O_2$ could act as a plastid signal. The potential for $H_2O_2$ to act as an intracellular signaling molecule is demonstrated by its role in the systemic response of plants exposed to excess light (Karpinski *et al.*, 1999). It has also been suggested that there is an interaction between sugar and retrograde signaling pathways controlling the expression of *LHC* genes via the plastid redox state (Oswald *et al.*, 2001).

### C. Tools to Study Plastid Signals

Plastid-to-nucleus signaling pathways can be accessed by treating plants with various inhibitors and pharmacological agents that affect chloroplast metabolism. Inhibitors of plastid translation such as lincomycin and chloramphenicol have frequently been used to study the retrograde mechanisms controlling expression of nuclear-encoded photosynthesis genes during chloroplast development (Oelmuller *et al.*, 1986; Sullivan and Gray, 1999, 2000, 2002). The redox state of the PQ pool can be manipulated by inhibiting its reduction with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, which blocks the flow of electrons from PSII to PQ, leaving the PQ pool oxidized in the light. Treatment with 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone inhibits electron transfer from PQ to the cytochrome $b_6f$ complex and consequently increases the reduction state of the PQ pool in the light. These two inhibitors were used to demonstrate the correlation between the reduction state of PQ and the repression of nuclear-encoded photosynthetic genes (Escoubas *et al.*, 1995; Durnford and Falkowski, 1997; Pfannschmidt *et al.*, 2001).

The response of the carotenoid-deficient mutants can be mimicked by using the herbicide norflurazon, which inhibits phytoene desaturase in carotenoid biosynthesis. The photooxidation caused by norflurazon treatment is limited to the plastid and results in complete destruction of the thylakoid membrane but does not affect the envelope membrane (Oelmuller and Mohr, 1986; Puente *et al.*, 1996). The resulting inhibition of chloroplast function leads to decreased transcription of nuclear-encoded photosynthetic genes, as demonstrated by nuclear run-on assays (Burgess and Taylor, 1988). Because the norflurazon treatment does not alter normal light-grown seedling morphology, it has been widely used to study the effect of chloroplast development on nuclear gene expression. Experiments with the Affymetrix *Arabidopsis* oligoarray containing ~8,200 genes revealed that, in addition to the well documented photosynthetic genes *LHCB* and *RBCS*, 322 genes changed their expression more than three-fold (182 were repressed and 140 were induced) in wild-type seedlings grown on 5μM norflurazon (Strand *et al.*, 2003). Among the genes repressed by norflurazon were those encoding proteins involved in light harvesting and electron transport reactions of photosynthesis, and enzymes involved in carbon

metabolism and tetrapyrrole biosynthesis. Genes that were induced by norflurazon treatment primarily encode proteins involved in stress responses and secondary metabolism.

### *D. Mutants That Disrupt Chloroplast-to-Nucleus Communication*

Taking advantage of the effect of norflurazon on nuclear gene expression, Joanne Chory's laboratory was able to identify components of the plastid-to-nucleus signaling pathways (Susek *et al.*, 1993). Susek *et al.* fused a *LHCB1* promoter to two different reporter genes, *HPH* and *UIDA*, which confer resistance to the antibiotic hygromycin and provide the colorimetric ß-glucuronidase activity, respectively. The chimeric construct was transformed into *Arabidopsis thaliana* and used to screen for *Arabidopsis* mutants no longer able to repress *LHCB* expression when grown on norflurazon. The mutants identified are referred to as the genome *un*coupled mutants, or *gun* mutants. Five non-allelic loci were identified as *gun* mutants (*gun1* to *gun5*) that express nuclear-encoded photosynthetic genes in the absence of proper chloroplast development (Susek *et al.*, 1993; Mochizuki *et al.*, 2001). None of the *gun* mutations affects tissue specificity, light or circadian regulation of the *LHCB* genes, suggesting the mutations are specifically affecting the plastid-to-nucleus communication (Susek *et al.*, 1993).

Genetic analysis of the five different *gun* mutants suggested there were two separate signaling pathways, the *GUN1* and *GUN2-5* pathways (Mochizuki *et al.*, 2001). This was confirmed when the expression profiles of three *gun* mutants, *gun1, gun2* and *gun5* were compared (Strand *et al.*, 2003). To date, four of the *GUN* loci have been cloned. The GUN2, GUN3, GUN4 and GUN5 proteins are all essential for tetrapyrrole biosynthesis (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003) and all mutants exhibit a pale phenotype. The *gun2* and *gun3* are alleles of *hy1* and *hy2*, respectively. *HY1*/*GUN2* encodes heme oxygenase and *HY2/GUN3* encodes phytochromobilin synthase. These enzymes are required for the synthesis of phytochromobilin, the chromophore of phytochrome. Repression of chlorophyll synthesis in these mutants is thought to be mediated through allosteric inhibition by heme accumulation of the glutamyl-tRNA reductase, which catalyzes the committed step in the conversion of glutamate to ALA (Beale, 1999; Terry and Kendrick, 1999). The *GUN5* gene encodes the H-subunit of Mg-chelatase (Mochizuki *et al.*, 2001). Mg-chelatase catalyzes the first reaction in the "chlorophyll branch" of tetrapyrrole biosynthesis by inserting $Mg^{2+}$ into the protoporphyrin ring and is composed of three subunits referred to as ChlH, ChlD and ChlI (Jensen *et al.*, 1996). *GUN4* has recently been cloned and encodes a novel chloroplast protein demonstrated to bind both protoporphyrin IX (ProtoIX) and Mg-protoporphyrin (Mg-ProtoIX) and to activate Mg-chelatase *in vitro* (Larkin *et al.*, 2003). GUN4 is predicted to be a soluble protein but has been recovered with fractions of the chloroplast stroma, thylakoids and envelopes. GUN4 could also play a role in photoprotection by binding the photooxidizing ProtoIX and Mg-ProtoIX. Consistent with this proposal is the observation that *gun4* seedlings bleach under high light (Larkin *et al.*, 2003).

GUN1 represents a component in a signaling pathway separate from the GUN2-5 pathway. In the *gun1* mutant the expression of nuclear-encoded photosynthesis genes is strongly uncoupled from the metabolic and developmental state of the plastid. Unlike the *gun2-5* mutants, the *gun1* mutant does not repress *LHCB* expression when treated with inhibitors of plastid translation (Sullivan and Gray, 2002; Å. Strand and J. Chory, unpublished results). This suggests that GUN1 encodes a component upstream of where different plastid signals converge.

## IV. Mg-ProtoIX, a Link Between the Plastids and the Nucleus

### *A. Regulation of the Tetrapyrrole Pathway*

Higher plants synthesize four major tetrapyrrole molecules–chlorophyll, heme, siroheme and phytochromobilin–via a common, partly branched pathway. Many tetrapyrroles are excited by light and, when unquenched, can lead to the formation of highly toxic radicals. Therefore, tetrapyrrole synthesis must be accomplished while preventing the accumulation of pools of intermediates that might endanger the plant cell, which requires tight regulatory control of the pathway. ALA synthesis is the primary control point, determining the flux through the pathway via allosteric regulation of glutamyl-tRNA reductase by accumulated heme (Beale, 1999). However, the chlorophyll branch also exerts control over the flux through the pathway. Evidence for this regulation comes from the *flu* mutant that over-accumulates protochlorophyllide. The FLU protein is localized to chloroplast membranes and functions as a negative regulator of chlorophyll synthesis (Meskauskiene *et al.*,

2001). Consistent with the key role of glutamyl-tRNA reductase in controlling the flux though the pathway, FLU interacts with this enzyme in a yeast two-hybrid assay (Meskauskiene and Apel, 2002). A key aspect of the control of tetrapyrrole biosynthesis is the need to coordinate the synthesis of tetrapyrroles in the plastid with synthesis of apoproteins in the plastid and the cytosol, which is achieved by plastid-to-nucleus communication.

### *B. The Gun Mutants Revealed One of the Plastid Signals*

In earlier studies with higher plants, perturbations in tetrapyrrole biosynthesis were shown to affect the expression of nuclear genes encoding photosynthetic proteins (Kittsteiner *et al.*, 1991; La Rocca *et al.*, 2001) but the source of the signal was elusive. However, the genome uncoupled mutants *gun2-5*, with restrictions in defined steps in tetrapyrrole biosynthesis, have provided conclusive evidence that Mg-ProtoIX acts as a signal molecule initiating retrograde communication between the chloroplast and the nucleus (Jarvis, 2003; Rodermel and Park, 2003; Strand *et al.*, 2003). Exposure to stress can cause a build-up of Mg-ProtoIX, and wild-type *Arabidopsis* plants were found to accumulate high amounts of Mg-ProtoIX when grown on norflurazon. This accumulation of Mg-ProtoIX was partly or completely absent in *gun2* and *gun5* mutants due to reduced flux through the pathway of tetrapyrrole biosynthesis. The accumulation of Mg-ProtoIX in norflurazon grown seedlings can be explained by the fact that the last step in chlorophyll biosynthesis is thought to be localized to the thylakoid membranes (Joyard *et al.*, 1998), and these structures are destroyed by the effects of norflurazon treatment (Oelmuller and Mohr, 1986; Puente *et al.*, 1996). Other conditions that affect membrane structure and function could also result in an imbalance in the tetrapyrrole pathway and accumulation of Mg-ProtoIX.

Reduced accumulation of Mg-ProtoIX is the reason photosynthetic genes are misregulated during norflurazon treatment in *gun2* and *gun5*, and when investigated, a large number of other mutants with different lesions in the pathway for tetrapyrrole biosynthesis upstream of Mg-ProtoIX also demonstrated a *gun* phenotype (Strand *et al.*, 2003). In contrast, a T-DNA knockout mutant in one subunit, CRD (Tottey *et al.*, 2003) of the cyclase complex down-stream of Mg-ProtoIX, did not exhibit a *gun* phenotype when grown on norflurazon (Å. Strand, unpublished data). Other evidence for the role of Mg-ProtoIX as a signaling metabolite comes from studies with green algae. Treatment with the Fe-chelator 2,2'-dipyridyl, which results in an accumulation of Mg-ProtoIX, has been demonstrated to strongly repress *LHC* expression in *Chlamydomonas reinhardtii* (Johanningmeier and Howell, 1984). Furthermore, Beck and colleagues demonstrated a direct effect of Mg-ProtoIX and Mg-ProtoIX methyl ester on nuclear expression in *Chlamydomonas*, where the light responsive gene *HSP70*, encoding a heat-shock protein, could be induced in the dark by Mg-ProtoIX (Kropat *et al.*, 1997, 2000). *Chlorella vulgaris* cells have also been shown to accumulate Mg-ProtoIX in response to low temperatures (Wilson *et al.*, 2003) but whether Mg-ProtoIX plays a role in the cold induced repression of *LHC* genes (Strand *et al.*, 1997) remains to be determined.

Is the signaling molecule Mg-ProtoIX exported from the chloroplast to transduce the signal to the nucleus? This question could be addressed by taking advantage of the photoreactive properties of Mg-ProtoIX. Accumulation of Mg-ProtoIX in ALA-fed norflurazon grown seedlings could be visualized using a multiphoton laser microscope, and the fluorescence images demonstrate the accumulation of Mg-ProtoIX in the cytosol (Å. Strand, unpublished data). How Mg-ProtoIX exits the chloroplast is unclear but related molecules such as heme, heme precursors, phytochromobilin and chlorophyll degradation products are exported from the chloroplasts (Thomas and Weinstein, 1990; Matile *et al.*, 1992; Jacobs and Jacobs, 1993; Terry *et al.*, 1993) and it is feasible that Mg-ProtoIX could use the same export mechanism(s). GUN4 could potentially be involved in promoting export of Mg-ProtoIX to the cytosol (Larkin *et al.*, 2003).

Our working model is that Mg-ProtoIX accumulates in the chloroplasts and is transported to the cytosol (Fig. 1). In the cytosol, Mg-ProtoIX is bound by a regulatory protein, possibly a transcription factor, and modifies the activity and/or the translocation of this protein, perhaps through a photoreactive action. As a consequence, expression of photosynthetic genes is inhibited. A large number of genes are regulated by the Mg-ProtoIX mediated signal. The Affymetrix *Arabidopsis* oligoarray, containing ~8,200 genes, revealed that 67 genes are not repressed in the *gun5* mutant when grown on norflurazon compared to wild-type (Table 2). A large number of nuclear genes encoding proteins closely associated with photosynthesis are regulated by Mg-ProtoIX. However, the components transducing the signal to the nucleus and the *trans*-acting factors

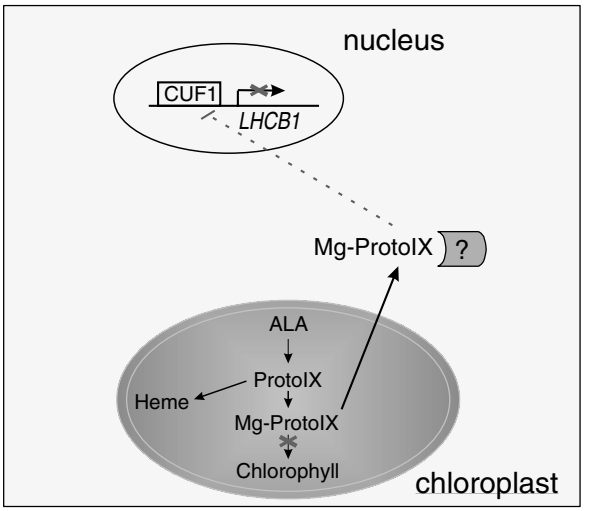


*Fig. 1.* Model of Mg-ProtoIX-mediated retrograde signaling. Under conditions where the latter steps in chlorophyll biosynthesis are inhibited, Mg-ProtoIX accumulates in the chloroplasts and eventually diffuses or is transported to the cytosol. In the cytosol, Mg-ProtoIX is bound by a regulatory protein, possibly a transcription factor, and modifies the activity and/or the translocation of this protein, perhaps through a photoreactive action. As a consequence, expression of photosynthetic genes is inhibited.

controlling the expression of photosynthetic genes remain to be discovered and are challenging tasks for the future.

### *C. Regulation of Nuclear Genes by Mitochondrial Heme*

In yeast (*Sacharomyces cerevisiae*), a similar route of communication using signaling metabolites has been demonstrated where heme synthesized in the mitochondria regulates transcription of nuclear genes encoding mitochondrial proteins (Forsburg and Guarente, 1989). Heme synthesis is directly correlated to oxygen levels in the cellular environment and when cells are grown aerobically, heme is synthesized in the mitochondria and imported to the nucleus where it activates the transcription factor HAP1. Binding of heme to HAP1 permits HAP1 to bind to upstream activation sequences, promoting transcription of many genes required for oxygen utilization and for controlling oxidative damage (L. Zhang and Hach, 1999). HAP1 also activates expression of the *ROX1* gene, encoding the aerobic repressor that represses genes encoding proteins required for anaerobic growth (L. Zhang and Hach, 1999). Because plants contain both chloroplast and mitochondria, it is necessary for the chloroplast to communicate with the nucleus via a plastid-specific molecule, such as Mg-ProtoIX. The *Arabidopsis* genome has not revealed any homologs of the yeast HAP1 protein, so it remains to be clarified whether plant mitochondria also communicate with the nucleus via heme.

## V. Plastid-Responsive Promoter Elements in Nuclear Genes

The cytoplasmic and nuclear proteins that participate in the plastid-to-nucleus signaling pathways are poorly understood. Nevertheless, progress has been made on the identification of *cis* elements in the promoter regions of nuclear genes responding to signals originating in the plastids. Nuclear genes that encode plastid components are regulated by a diverse group of *cis* regulatory elements that act in a combinatorial manner. Promoter::reporter gene fusions have been used to identify light and plastid response elements in the promoters. However, so far it has been impossible to uncouple plastid- from light-responsive *cis* elements (Kusnetsov *et al.*, 1996; Puente *et al.*, 1996; McCormac *et al.*, 2001; Strand *et al.*, 2003).

Mutations of five nucleotides in the promoter of the *ATPC* gene, encoding the $\gamma$-subunit of chloroplast ATP synthase, causes constitutive light- and plastid-independent expression (Bolle *et al.*, 1996). The five nucleotides are just upstream of a CAAT box and are believed to be part of a repressor binding site preventing recruitment of CAAT box binding factors (Bolle *et al.*, 1996; Bezhani *et al.*, 2001). In *Chlamydomonas*, analysis of *HSP70* promoter mutants also revealed that Mg-ProtoIX specifically activates a light-signaling pathway (Kropat *et al.*, 1997). Two of the best-defined binding sites involved in light-regulated transcription of *LHCB* genes, the GT-1 (G3M) and the G-box (CUF1) elements (Terzagi and Cashmore, 1995) were used to investigate what element responds to the Mg-ProtoIX-mediated signal in *Arabidopsis.* Three different *LHCB1*::Luciferase reporter constructs were used that were truncated or contained mutations of the G3M and the CUF1 elements. These three different *LHCB1*: Luciferase constructs were transformed into wild-type plants and subsequently crossed into the *gun5* mutant. The result demonstrated that the G-box motif defined by CUF1 responds to the Mg-ProtoIX-mediated signal in *Arabidopsis* (Strand *et al.*, 2003). Furthermore, a true CUF1 element (CACGTA) is present in the promoter region of 18 of the genes

*Table 2.* Genes that are regulated by Mg-ProtoIX. The Affymetrix *Arabidopsis* oligoarray, containing ~8,200 genes revealed that 67 genes are not repressed in *gun5* when grown on norflurazon. Proteins with predicted chloroplast localization are marked with (cp).

| Category | gene # | Function |
|---|---|---|
| Light harvesting/Electron transport | | |
| | At1g15820 | Lhcb6 protein |
| | At1g19150 | PSI type II chlorophyll a/b-binding protein |
| | At1g23410 | PsbS protein |
| | At1g30380 | photosystem I subunit X precursor |
| | At1g52230 | photosystem I subunit VI precursor |
| | At1g55670 | photosystem I subunit V precursor |
| | At2g05100 | Lhcb2 protein |
| | At2g30570 | photosystem II reaction center 6.1KD protein |
| | At2g34420 | photosystem II type I chlorophyll a/b binding protein |
| | At3g16140 | photosystem I subunit VI precursor |
| | At3g29670 | 33 kDa oxygen-evolving protein |
| | At3g50820 | oxygen-evolving enhancing protein |
| | At3g54890 | chlorophyll a/b-binding protein |
| | At3g61470 | Lhca2 protein |
| | At4g02770 | photosystem I reaction center subunit II |
| | At4g12800 | photosystem I subunit XI precursor |
| | At4g21280 | photosystem II oxygen-evolving complex protein 3-like |
| | At4g28750 | photosystem I subunit PSI-E-like protein |
| | At5g01530 | CP29 |
| | At5g23120 | PSII stability factor (HCF136) |
| | At5g60580 | photosystem I subunit III precursor |
| | At5g64040 | PSI-N |
| Metabolism/Transport | | |
| | At1g04340 | lesion-inducing protein |
| | At1g42970 | glyceraldehyde-3-phosphate dehydrogenase (cp) |
| | At1g68010 | hydroxypyruvate reductase |
| | At2g02130 | plant defensin-fusion protein |
| | At2g21330 | fructose bisphosphate aldolase (cp) |
| | At2g26080 | glycine dehydrogenase (P-protein) |
| | At2g28190 | copper/zinc superoxide dismutase (cp) |
| | At2g40490 | uroporphyrinogen decarboxylase (cp) |
| | At2g43560 | FKBP type peptidyl-prolyl cis-trans isomerase (cp) |
| | At3g26650 | glyceraldehyde-3-phosphate-dehydrogenase subunit (cp) |
| | At3g55800 | sedoheptulose-1,7-bisphosphatase SBPase (cp) |
| | At4g00170 | vesicle associated membrane protein (VAMP) |
| | At4g17090 | beta-amylase (cp) |
| | At4g18480 | protoporphyrin-IX Mg-chelatase, CHLI (cp) |
| | At4g33550 | lipid transfer protein (LTP) |
| | At4g38970 | fructose-bisphosphate aldolase (cp) |
| | At5g08280 | hydroxymethylbilane synthase (cp) |
| | At5g09660 | microbody NAD-dependent malate dehydrogenase |
| | At5g14740 | carbonic anhydrase (cp) |
| | At5g20630 | germin like protein |
| | At5g24150 | squalene epoxidase homologue |
| | At5g35100 | peptidyl-prolyl cis-trans isomerase (cp) |
| | At5g35790 | glucose-6-phosphate dehydrogenase (cp) |
| | At5g42650 | allene oxide synthase (cp) |
| | At5g63850 | amino acid transporter |
| Signaling/Regulation | | |
| | At1g09750 | cp nucleoid DNA binding protein (cp) |
| | At1g20700 | homeobox-leucine zipper protein |
| | At2g47940 | DegP2 protease (cp) |
| | At4g36450 | MAP kinase like protein |

*(continued)*

*Table 2.* (Continued)

| Category | gene # | Function |
|---|---|---|
| Translation of cp encoded proteins | | |
| | At1g79860 | ribosomal protein S17 |
| | At2g24090 | chloroplast ribosomal protein L35 |
| | At2g33800 | 30S ribosomal protein S5 |
| Proteins with unknown function | | |
| | At1g05340 | unknown protein (cp) |
| | At1g09340 | unknown protein |
| | At2g21280 | unknown protein (cp) |
| | At2g35260 | unknown protein (cp) |
| | At2g46820 | unknown protein (cp) |
| | At2g47910 | unknown protein |
| | At4g01150 | unknown protein (cp) |
| | At4g04330 | unknown protein |
| | At4g12970 | unknown protein |
| | At4g22890 | unknown protein (cp) |
| | At4g24430 | unknown protein |
| | At4g28080 | unknown protein |
| | At4g38160 | unknown protein |

mis-regulated in *gun5*, and an additional 24 genes have the closely related CACGTG (Strand *et al.*, 2003).

## VI. Interaction Between Light- and Plastid-Signaling Pathways

The involvement of multiple plastid signals complicates our understanding of retrograde regulation. Characterization of the *Arabidopsis gun* mutants has demonstrated the existence of two signaling pathways, one of which is a stress signal where accumulation of the tetrapyrrole Mg-ProtoIX communicates metabolic imbalance of the chloroplast to the nucleus (Strand *et al.*, 2003). Expression of nuclear-encoded photosynthetic genes requires plastid translation during the first days of seedling development (Sullivan and Gray, 1999, 2000). The reduction state of the plastoquinone pool is correlated with *LHC* expression and accumulation of reactive oxygen species affects nuclear gene expression (Mullineaux and Karpinski, 2002; Pfannschmidt, 2003). As the picture emerges, it is becoming clear that there are multiple signals produced by the plastids that control nuclear gene expression. Furthermore, it has been demonstrated that these signals are produced at different developmental stages of the plant (Sullivan and Gray, 2002).

Apart from plastid signals, the expression of nuclear-encoded photosynthetic genes is regulated by numerous stimuli such as light, sugars, hormones and circadian mechanisms. The plastid signals are probably integrated into regulatory networks that share common routes or interact with these other signaling pathways. A close connection between light- and plastid-signals was demonstrated by the *c*ab *u*nder *e*xpression, *cue* mutants in *Arabidopsi*s. The CUE1 locus has been cloned and encodes the plastid envelope phospho*enol*pyruvate/phosphate translocator (Streatfield *et al.*, 1999). The *cue1* mutant is affected in the aromatic amino acid biosynthesis in the plastids and also has defective chloroplasts. The *cue1* mutant shows reduced expression of the *LHC* genes in the light because it is unable to de-repress the *LHC* expression in response to phytochrome activation (Lopez-Juez *et al.*, 1998). Furthermore, this reduced expression is mediated through the light-responsive G-box, CUF1, of the *LHCB1* promoter (Streatfield *et al.*, 1999). Interestingly, this is the same promoter element responding to the Mg-ProtoIX-mediated signal. The *cue* mutants indicate that some plastid signal(s) is closely associated with the pathway through which phytochrome regulates the expression of nuclear genes encoding plastid proteins (Streatfield *et al.*, 1999).

Even though light and plastid signals frequently appear to use the same *cis* elements, the two signals are distinct. A number of dark grown photomorphogenic mutants, the *det, cop* and *fus* class of mutants, assemble pre-chloroplasts in the dark. Nuclear genes encoding chloroplast proteins are over-expressed in the dark in these mutants (Fankhauser and Chory, 1997). However, dark expression of those genes in the mutants is prevented by inhibitors of plastid translation, which also blocks pre-chloroplast development (Sullivan and Gray, 1999, 2000). To act on common promoter

elements, light and plastid signals must converge at some point, and because a plastid signal is required for over-expression of nuclear photosynthesis genes in the *cop1* and *lip1* mutants, the point of intersection appears to be downstream of COP1 (Sullivan and Gray, 1999, 2000). In the dark, COP1 is a nuclear protein that is required for suppression of photomorphogenesis by mediating targeted proteolysis through the 26S proteosome (Osterlund *et al.*, 2000). Determining the CUF1-binding factor responding to the Mg-ProtoIX-mediated signal could potentially reveal shared components between light and plastid signals.

## VII. Concluding Remarks

While it is true that the chloroplast is dependent on the nucleus to supply much of the genetic information necessary for its function, it is also clear that the plastids produce multiple signals at different times of their development, and in response to changes in the environment, that orchestrate major changes in nuclear gene expression, giving the chloroplast a significant role in running the cell. There also appears to be interaction between plastid signals and light signaling pathways in the plant cell, and dissecting these complex networks of signaling pathways will be challenging tasks for the future.

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# Chapter 10

# Trace Metal Utilization in Chloroplasts

Sabeeha S. Merchant*
*Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, U.S.A.*

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*Author for correspondence, email: merchant@chem.ucla.edu

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## Summary

Redox reactions, which are central to metabolism, depend on redox active functional groups on enzymes such as cysteinyl thiols, organic cofactors like pyridine or flavin nucleotides, or metal cofactors such as Fe, Cu, Mn, or Mo. Accordingly, certain metals are nutritionally essential for life. The green organs of plants display the most descriptive symptoms of metal-deficiency because of the importance of the chloroplast in various metal-dependent redox pathways. The impact of trace element deficiency on chloroplast function at the molecular level has been studied most extensively in microorganisms such as the alga *Chlamydomonas*, for whom the growth media are more readily manipulated. Studies of trace metal distribution and its regulation in cyanobacteria are also relevant to our understanding of chloroplast processes. Fe is the most limiting metal nutrient for all forms of life. In chloroplasts, Fe is found as a redox-active cofactor in FeS centers, heme, mononuclear and di-iron enzymes, and also in ferritin, which functions as an iron "store" and iron "buffer" to maintain intracellular iron homeostasis. The plastid is the key organelle for heme biosynthesis, but FeS cluster synthesis occurs in both plastids and mitochondria. The machinery for cluster synthesis is derived from bacteria, with the process in mitochondria derived from the Isc system and that in plastids containing components of both the Suf system and the Isc system. In iron-deficient chloroplasts, the abundance of iron-containing proteins and specific chlorophyll-proteins is reduced by hierarchical post-transcriptional regulatory mechanisms that may receive signals from iron-dependent enzymes in the tetrapyrrole biosynthetic pathway. The abundant copper enzymes in chloroplasts include plastocyanin and, in some plants, polyphenol oxidase in the thylakoid lumen, and CuZn-superoxide dismutase in the stroma of plants but not green algae. Distributive copper transporters and chaperones are responsible for delivery of the metal to specific sub-organellar compartments. Again, a hierarchical pattern of copper allocation is noted, with plastocyanin receiving copper with higher priority in *Arabidopsis* where plastocyanin is essential, but not in *Chlamydomonas* where a heme protein can substitute for plastocyanin function. A master regulator of copper nutrition called Crr1 regulates degradation of apoplastocyanin in *Chlamydomonas*. The mechanisms of manganese delivery and distribution have not been studied in eukaryotic photosynthetic organisms but, by analogy to metal uptake pathways required for loading Mn-enzymes in bacteria and mitochondria, could involve MntA and MntH/Nramp-like transporters. Mn-deficiency impacts the water oxidation machinery in the chloroplast and also mitochondrial superoxide dismutase.

## I. Introduction

### *A. The Transition Metals Function as Redox Catalysts*

Redox reactions are central to metabolism. Biosynthetic reactions involve the reduction of inorganic compounds—$CO_2$, nitrate, $N_2$ and sulfate—to more reduced compounds such as carbohydrates, fatty acids and functional groups such as amines, alcohols and thiols. Energy producing reactions involve the oxidation of reduced organic compounds at the expense of a terminal electron acceptor, which is oxygen in most aerobic organisms. The relevant pathways are replete with enzymes that carry redox active cofactors, commonly pyridine and flavin nucleotides or various metal centers. Several transition metals are useful biological catalysts because ionic species of different oxidation states form stable chelates with functional groups found in proteins (Merchant and Dreyfuss, 1998). The use of particular metals in biology does not reflect their abundance in the earth's crust. Those elements that are bioavailable at neutral pH, either because of the high solubility of the aqua complexes of the low oxidations states (e.g. $Cu^{2+}$ and $Fe^{2+}$) or of the corresponding hydrated oxyanions for the higher oxidation states (e.g. molybdate and vanadate), are used preferentially (Kaim and Schwederski, 1994; Raven *et al.*, 1999).

The use of these metals for catalysis in particular pathways makes organisms that use those pathways dependent on the availability of such metals, leading to the concept of essential or beneficial nutrients (Frieden, 1985; Marschner, 1995). Accordingly, organisms have evolved mechanisms for assimilating the essential metals from the environment, often accumulating them to high levels against a concentration gradient. Because the metal ions are reactive, the assimilation pathways are regulated by supply and demand, and when supply does not meet demand, adaptive mechanisms for

*Abbreviations:* ATPase – adenosine 5′-triphosphatase; EDTA – ethylenediaminetetraacetic acid; PSI – Photosystem I ; PSII – Photosystem II ; SOD – superoxide dismutase.

conserving, re-distributing and prioritizing the metal nutrient come into play. Based on its amount in an organism, the metal is classified as a micronutrient (e.g. Fe), a trace nutrient (e.g. Cu) or an ultra-trace nutrient (e.g. Se, Mo or Mn). The amount required is organism-specific. Plants carry out different metabolism than do animals, and therefore their micronutrient requirements are distinct.

### *B. Trace Metal Deficiency Impacts the Chloroplast*

The impact of metal nutrition on the chloroplast has long been recognized because the symptoms of metal nutrient deficiency, which invariably include some form of "chlorosis" or chlorophyll-deficiency, are easily visualized in the green organs. There is a substantial amount of descriptive older literature on Fe-, Cu- and Mn-deficient plants, which established the importance of these metals in various metabolic pathways (Marschner, 1995). More recent research has focused on understanding the cell biology of metal homeostasis, and for this purpose, microorganisms such as algae and cyanobacteria have been useful because of the facility with which the growth media can be metal-depleted or fortified, coupled with the possibility of monitoring a large homogeneous population of cells.

### *C. Metalloprotein Assembly—Thermodynamics vs. Kinetics*

It is useful to emphasize that the fundamental biochemical principles that are taught in the context of the so-called "central metabolic pathways" apply also to the metabolism of the metal nutrients. For instance, a requirement for catalysis of metalloprotein assembly was not appreciated historically because metal-binding to apoproteins was known to be a thermodynamically favorable reaction, based on the fact that metalloproteins are usually more stable than their corresponding apoproteins. Also, in the case of FeS centers, the uncatalyzed reaction occurred readily and produced the correct cluster as long as the appropriate reducing conditions were provided (Malkin and Rabinowitz, 1966). Nevertheless, metalloprotein assembly has been documented for many proteins to be selective *in vivo* relative to the uncatalyzed *in vitro* metal reconstitution reactions. Not surprisingly, the rate of assembly *in vivo* is much faster than for the corresponding uncatalyzed *in vitro* reaction. The use of assembly factors *in vivo*, aside from accelerating a specific reaction, also provides a means for regulation of metal cofactor utilization. This is relevant in a cell where a limiting micronutrient like iron may be required both for respiratory chain function as well as for photosynthesis. In this situation, iron delivery pathways to individual organelles allow assembly pathways to respond to metabolic demand. In a multicellular organism, there may also be differentiation of function and hence expression of particular metal-utilizing metabolic pathways in specialized organs or at particular developmental states, which suggests operation of inter-cellular signals for metal nutrient homeostasis. This area of metabolism is presently under-studied, especially in the context of chloroplast function in plants. The interested reader is referred to the work of Raven for an excellent treatment of variation in metal requirement in photosynthetic organisms in response to metabolic demand (Raven, 1988, 1990; Raven *et al.*, 1999).

Another point to consider is the use of equilibrium constants in describing intracellular metal distribution. This treatment is valid only in a system that is at equilibrium, which is distinctly not the case in a living cell. The assimilation and distribution of metal cofactors in most organisms requires an input of energy to maintain the system at a steady state away from equilibrium. This is grasped easily for metal transport (e.g. by metal-transporting P-type ATPases) but it applies also to other steps in metalloprotein assembly, including preparation of the apoprotein substrates (e.g. maintenance of ligand oxidation state) and formation of clusters (e.g. the $Mn_4Ca$ complex involved in oxygen evolution). Theoretical calculations of concentrations of "free" metals or particular metal-ligand complexes based on equilibrium constants must be interpreted with caution because they may not reflect the true dynamic in a cell, which is in a non-equilibrium situation.

### *D. Fe, Cu and Mn*

The metals that are well-studied in the context of chloroplast biogenesis and function are Fe, Cu and Mn because of their abundance in the photosynthetic apparatus (Raven *et al.*, 1999). Also, the corresponding metalloproteins are readily monitored in the holoform by spectroscopic methods, and changes in metalloprotein expression are therefore easy to visualize. Other important metals such as Zn are spectroscopically silent, and hence, much less is known about the biogenesis of Zn-enzymes in the chloroplast despite their prevalence and abundance. The discussion in this chapter is restricted to the impact of Fe, Cu and Mn nutrition

on chloroplast function, especially the photosynthetic apparatus.

## II. Fe

A variety of iron enzymes occur in chloroplasts: heme proteins like cytochromes and P450s, soluble and membrane-bound two- and four-iron-sulfur proteins, di-iron enzymes and mononuclear-iron enzymes, and iron bound to ferritin (Jäger-Vottero *et al.*, 1997; Kerfeld and Krogmann, 1998; Merchant and Dreyfuss, 1998; Briat *et al.*, 1999; Froehlich *et al.*, 2001; Berthold and Stenmark, 2003; Gray *et al.*, 2004; Tian and DellaPenna, 2004). These enzymes function in electron transfer reactions of the photosynthetic apparatus and in various redox reactions in pathways that produce secondary metabolites and natural products. Because iron is an actively acquired nutrient that limits most life forms, organisms generally do not excrete iron when it is in intracellular excess beyond what is needed for maintenance of the iron enzymes, but rather store it. The reactivity of iron in an aerobic environment requires that it be stored in a protected form, as in the protein ferritin. When iron is required for de novo synthesis of iron-containing proteins, it can be mobilized from the stored form, and when it is released as iron-containing enzymes are degraded, the store can be re-built. Ferritin is therefore a key component of iron homeostasis.

### A. Ferritin

The protein ferritin accounts for stored iron in a plant and is the source of iron for chloroplast development. The reader is referred to substantial reviews by Briat and Theil and their co-workers for details of ferritin chemistry and biology (Briat and Lobréaux, 1997; Briat *et al.*, 1999; Curie and Briat, 2003; Theil, 2003, 2004). The protein consists of 24 subunits that bind up to $4.5 \times 10^3$ atoms of iron as ferric-oxy-hydroxide within an internal core. This mineral core is built by movement of iron into the core and oxidation of ferrous to ferric ions by the ferroxidase activity of the ferritin subunits (Lawson *et al.*, 1989). Plant ferritins are distinguished from animal ferritins by being localized within an organelle, the plastid, and by their subunit composition. While animal ferritins consist of two types of related chains, H and L, plant ferritins have a single type of chain. The plant ferritin subunit, encoded by a multigene family, is more closely related to the H-chain but also has features of the L chains that facilitate iron nucleation and yield a stable core (Van Wuytswinkel *et al.*, 1995; Wardrop *et al.*, 1999). The mineral core in plant ferritin is also unique in containing a high proportion of phosphate like bacterioferritins (Waldo *et al.*, 1995). The proteins encoded by the *FER* genes in plants and algae include an N-terminal plastid-targeting sequence in the precursor, and an N-terminal region in the mature protein that is a determinant of stability and protease susceptibility *in vitro* (Ragland *et al.*, 1990; van Wuytswinkel *et al.*, 1995; Wardrop *et al.*, 1999; La Fontaine *et al.*, 2002). Interestingly, plastid ferritin is quite distinct from bacterioferritin found in cyanobacteria, which indicates that chloroplast ferritin is a function acquired from the host rather than retained from the endosymbiont (Laulhere *et al.*, 1992).

Ferritin expression is determined by multiple signals because of the nutritional importance of iron, the variation in iron demand at different stages of growth, and the potential for toxicity of iron in an aerobic environment. Ferritin abundance is regulated by changes in RNA abundance through transcriptional regulation and polypeptide abundance through post-transcriptional mechanisms in response to multiple signals, including iron supply, developmental stage, and wounding (Lescure *et al.*, 1991; Lobréaux and Briat, 1991; Ragland and Theil, 1993; Fobis-Loisy *et al.*, 1996; Tarantino *et al.*, 2003). Therefore, it is important to monitor protein abundance for a picture of ferritin action *in vivo*, while RNA abundance only presents a picture of the potential or capacity for ferritin action. Recent studies in animals and plants suggest that ferritin can also be found in mitochondria under certain conditions (Levi *et al.*, 2001; Zancani *et al.*, 2004), which adds another layer of complexity in understanding the biology of ferritin. Another relevant aspect of ferritin function is the amount of iron in the mineral core, which can change during growth and development (e.g. van der Mark *et al.*, 1981), but this has not been studied systematically.

The four ferritin-encoding genes in *Arabidopsis*, *FER1*, *FER2*, *FER3* and *FER4*, show unique patterns of expression in response to iron nutrition, environmental stress and developmental stage, which reinforces the importance of plastid Fe homeostasis for plant growth and physiology (Petit *et al.*, 2001; Tarantino *et al.*, 2003). Ferritin accumulates to high levels in non-green plastids and decreases in abundance as they green, suggesting that ferritin is the source of iron found in heme- and other iron-containing proteins in the photosynthetic apparatus, although the direct movement of labeled Fe from ferritin to an Fe-containing enzyme has not been monitored. As the leaf gets older, the ferritin

content decreases until the organ is at the stage of senescence, when the ferritin content increases again, presumably to accommodate iron that is released from enzymes as the proteins of the chloroplast are degraded. The re-appearance of ferritin is attributed to increased gene expression and de novo synthesis (Tarantino *et al.*, 2003). Ferritin accumulation is also increased under conditions of oxidative stress, which presumably exacerbate the toxicity of iron (Briat *et al.*, 1999; Petit *et al.*, 2001).

Although ferritin accumulation involves post-transcriptional regulatory mechanisms, when a soybean *FER* cDNA was over-expressed in tobacco plants via a 35S promoter-driven construct, ferritin did over-accumulate in the mature plants (Van Wuytswinkel *et al.*, 1998). The plants accumulated more iron but displayed symptoms of iron-deficiency (i.e. inter-veinal chlorosis) even when the ferritin was targeted to the plastid. These studies emphasize the role of plastid ferritin in iron homeostasis and the function of ferritin as an iron "buffer" that keeps iron available in the cell but in a non-toxic form. This work also raised the question of how iron might be mobilized from ferritin. The more abundant ferritin in the over-expressing plants catalyzes and accommodates the over-chelation, but the fact that it is not available for normal chloroplast development indicates that the iron mobilization system can not circumvent the over-chelation. Because oxidation is involved in the deposition of iron in the ferritin core, it is generally assumed that reduction is required for iron mobilization and there are some experiments that support this notion (e.g. Bienfait and van den Briel, 1980). In that *in vitro* study, a connection between copper and ascorbate- and oxygen-dependent iron mobilization from ferritin was noted, suggesting perhaps a role for an enzyme like ascorbate oxidase. Nevertheless, an *in vivo* connection has not yet been established. The models considered for iron release from ferritin in animal cells favor either lysosomal degradation of the protein and/or unfolding of the iron cores, but mobilization of iron from the mineral would still depend on reduction (reviewed by Theil, 2004). Besides serving as a buffer for iron, ferritin is also an important store of Fe for the next generation and does accumulate in seeds (Masuda *et al.*, 2001).

## B. Heme, FeS and Fe Cofactor Synthesis

During development of the chloroplast in germinating seedlings, the disappearance of ferritin is correlated with the appearance of iron-containing catalysts such as cytochromes that contain heme (or Fe-protoporphyrin IX) and iron-sulfur proteins (of either the $Fe_2S_2$ variety as in ferredoxin or the $Fe_4S_4$ variety as in PSI). It is possible that iron distribution to various enzymes is regulated based on physiological demand for, or importance of, particular metabolic pathways (see below). For an understanding of the principles underlying the regulation, it is first useful to have a description of the iron-utilizing pathways.

The biosynthesis of heme and FeS centers represent major iron utilizing pathways in the cell, and the organelles (mitochondria and plastids) contain an abundance of these redox cofactors. In *Saccharomyces cerevisiae*, inhibition of mitochondrial heme synthesis blocks transcriptional activation of the iron uptake genes, and disruption of iron-sulfur metabolism results in mitochondrial iron accumulation and hence altered cellular iron homeostasis, substantiating the importance of cofactor biosynthesis in iron homeostasis (e.g. Knight *et al.*, 1998; J. Li *et al.*, 1999; Lange *et al.*, 2000; Crisp *et al.*, 2003).

### 1. Heme

In fungi and animal cells, the heme biosynthetic pathway is distributed between the cytosol and the mitochondrion. In plants, the entire pathway, from δ-aminolevulinate to Fe-protoporphyrin IX, is localized in plastids, but the last two enzymes—protoporphyrinogen oxidase and ferrochelatase—occur also in the mitochondrion (Chow *et al.*, 1997; Lermontova *et al.*, 1997; Beale, 1999; Watanabe *et al.*, 2001). In *Arabidopsis*, two ferrochelatase-encoding genes, *FC-I* and *FC-II*, are expressed under different conditions (Singh *et al.*, 2002). Interestingly, *FC-I*, whose product is probably localized to both plastids and mitochondria, showed increased expression in leaves upon wounding or upon treatment with salicylic acid, which suggested that an increased potential for heme synthesis, presumably to provide cofactors for induced P450s (see below), is part of the defense response. It is not presently known whether heme found outside the plastid, in peroxisomes (peroxidases), cytosol (hemoglobin), endoplasmic reticulum (P450s and non-heme oxygenases), or the cell wall (peroxidases), is derived from the plastid pool or the mitochondrial pool.

Iron deficiency impacts heme levels and reduces the content of cytochromes in the photosynthetic apparatus (Duggan and Gassman, 1974; Moseley *et al.*, 2002a). Because the tetrapyrrole pathway is feedback regulated by heme, synthesis of δ-aminolevulinate is promoted

under these conditions (Cornah *et al.*, 2003; Franklin *et al.*, 2003).

For many organisms, heme is a source of iron, especially under conditions of nutritional deficiency. Heme or Fe-protoporphyrin IX is oxidized by a mixed function-type oxidase reaction, which requires molecular oxygen and a reductant, to Fe-biliverdin (usually the $\alpha$ isoform) and CO. This reaction, catalyzed by a heme oxygenase, is driven by removal of product through the action of biliverdin IX$\alpha$ reductase (Franklin *et al.*, 2003). The step also releases iron bound to the tetrapyrrole. In animals, fungi and bacteria, a heme oxygenase is a key target of iron deficiency because it releases iron from heme for use in other iron-containing enzymes (e.g. Poss and Tonegawa, 1997; Protchenko and Philpott, 2003; Frankenberg-Dinkel, 2004; Skaar *et al.*, 2004).

The role of ferrochelatase as an iron-utilizing enzyme and heme oxygenase as an iron-releasing enzyme in plastid iron homeostasis is intriguing but not yet analyzed thoroughly in plants. The major role of the plastid heme oxygenases lies in the production of bilins for light harvesting and signaling (Willows *et al.*, 2000; M. Terry *et al.*, 2002). In a red alga, the gene *pbsA* in the plastid genome, encoding a heme oxygenase, is transcriptionally activated by iron-deficiency (Richaud and Zabulon, 1997). In *Chlamydomonas* neither of the two typical heme oxygenase-encoding genes appears to be transcriptionally regulated by iron nutrition (J. Kropat and S. Merchant, unpublished results), and the question of regulation by iron has not yet been addressed for the various heme oxygenases of plants. Nor has the issue of whether plastid heme levels may signal organelle or cellular iron status, as evidently is the case for mitochondrial heme in yeast, been addressed.

## 2. FeS

### a. Discovery and Function of Prototypical Bacterial Nif, Isc and Other Components

The biosynthesis of FeS centers requires mobilization of sulfur from cysteine, mobilization of Fe, assembly of the cluster and transfer of the cluster to apoenzymes (Merchant and Dreyfuss, 1998; Lill and Kispal, 2000; Frazzon and Dean, 2003). Although not all of the molecular events are understood, the necessary components have been defined in many organisms through classical and reverse genetic approaches. The first components were identified in the context of nitrogenase function (called NifS and NifU) and, subsequently, related molecules encoded by *isc* genes in *Escherichia coli* were shown to be responsible for the assembly of clusters in various iron-sulfur proteins (Zheng *et al.*, 1993; Takahashi and Nakamura, 1999). Sulfur is mobilized from cysteine by NifS/IscS in a pyridoxal phosphate-dependent reaction catalyzed by a cysteine desulfurase, which yields alanine and sulfane sulfur. This activity is required also for the synthesis of other sulfur containing compounds in bacteria such as thiamine and thionucleosides. NifU, a three-domain protein, is responsible for building the FeS cluster (Yuvaniyama *et al.*, 2000). An N-terminal iron-binding domain related to IscU is the assembly scaffold and binds a "transient" cluster through three cysteine residues (Agar *et al.*, 2000). IscU interacts with the chaperone system, Hsc66 and Hsc20, encoded in the *isc* operon (Table 1) (Hoff *et al.*, 2000). The middle domain of NifU holds a permanent FeS cluster, presumably with redox function during cluster assembly. The C-terminal domain, called the NFU domain or CnfU, is involved in transferring the FeS cluster to apoproteins. The domain contains a CxxC motif of unknown function (Frazzon *et al.*, 2002). IscA is another cluster assembly component providing a scaffold for transient formation and binding of an FeS cluster, but how it relates to IscU function is not understood (Ollagnier-de-Choudens *et al.*, 2001; Cupp-Vickery *et al.*, 2004). A ferredoxin (itself containing an FeS cluster) is associated with the *isc* operon and is required for cluster biogenesis, presumably for reducing ferric to ferrous iron. This ferredoxin interacts physically with IscA (Ollagnier-de-Choudens *et al.*, 2001).

Interestingly, under anaerobic but not aerobic conditions, a simple system consisting solely of NifS- and NifU-homologues is sufficient for FeS cluster assembly in *E. coli* lacking both the *isc* and *suf* (see below) operons (Ali *et al.*, 2004). This suggests that the additional components of the Isc system evolved to accommodate an aerobic environment.

In a genetic screen for defects in FeS cluster metabolism in *Salmonella enterica*, two new assembly factors were identified, called ApbC and ApbE (Skovran and Downs, 2003). Phenotypic analysis suggests that these proteins are required for the maintenance (repair) or assembly of oxygen-labile FeS clusters in the enzymes ThiH (required for thiamine biosynthesis), succinate dehydrogenase (containing multiple FeS clusters) and aconitase. The specific role of the proteins is not known, but ApbC does catalyze ATP hydrolysis and the *apb* mutants (unlike *isc*

*Table 1*. Biochemical functions required for cluster biosynthesis in bacteria and organelles. A list of components and types of activities required for FeS cluster assembly in various organisms. See text for details.

| Function | Gene |
|---|---|
| Sulfur mobilization from cysteine | bacterial IscS, NifS |
| | mitochondrial Nfs1 |
| | bacterial and plastid SufS[3]+SufE |
| ATP dependent sulfur transfer? | bacterial and plastid SufC+SufB+SufD |
| ATP-dependent Repair / synthesis of (oxygen labile) $Fe_4S_4$ clusters | bacterial ApbC / Mrp |
| | cytosolic Cfd1p / Nbp35p |
| | plastid Hcf101 |
| Cluster assembly and provision of scaffold for assembly | bacterial NifU-N terminus, IscU |
| | mitochondrial Isu1, Isu2 |
| Cluster assembly | bacterial IscA |
| | mitochondrial Isa1, Isa2 |
| | plastid ISAI[3] |
| | bacterial and plastid SufA[4] |
| Cluster transfer | bacterial NifU-C terminus with conserved CxxC motif |
| | mitochondrial Nfu1 |
| | plastid NFUs |
| Reductant | bacterial NifU-permanent cluster in central portion |
| | bacterial Fdx |
| | mitochondrial Yah1 |
| Chaperones | bacterial ATP-dependent Hsp70, HscA, Hsc66 + |
| | bacterial J-type co-chaperone, HscB, Hsc20 |
| | mitochondrial Ssq1 + Jac1 |
| Iron metabolism | mitochondrial frataxin |

[3] Also called CsdB in *E. coli* or NifS-Type II.
[4] SufA is related to IscA and the nomenclature used depends on genic context (i.e. whether the gene occurs in a *suf* vs. *isc* operon).

mutants) can be suppressed by exogenous iron. These features are reminiscent of the essential P-loop ATPase Cfd1p in *S. cerevisiae*, which is involved in repair or synthesis of the aconitase cluster in the cytosol, and indeed, Cfd1p and ApbC share sequence similarity (Roy *et al.*, 2003). Cfd1p contains a conserved CxxCxxC motif, of which the first two cysteine residues are functionally important and conserved also in ApbC.

### *b. Eukaryotic Homologs of Nif and Isc Components Function in Mitochondria*

The discovery of homologs in eukaryotes of the proteins encoded in the bacterial *nif*/*isc* operon led to the description of a related machinery in mitochondria, consisting of Nfs1 (related to NifS), Nfu1 (related to the C-terminal domain of NifU), Isu1/2 (related to the N-terminal domain of NifU), Isa1/2 (related to IscA), Yah1 (a mitochondrial ferredoxin) and the molecular chaperone system, Ssq1 plus Jac1 (related to Hsc66 and Hsc20) (Table 1) (Garland *et al.*, 1999; Kispal *et al.*, 1999; J. Li *et al.*, 1999; Schilke *et al.*, 1999; L. Jensen and Culotta, 2000; Lange *et al.*, 2000; Mühlenhoff *et al.*, 2002). In *S. cerevisiae*, and probably animals as well, this machinery appears to be the source of clusters for all FeS proteins in the cell (Lill and Kispal, 2000), although additional components, like Cfd1p, Nar1p and Nbp35p, are required for the synthesis of extra-mitochondrial clusters (Roy *et al.*, 2003; Balk *et al.*, 2004).

Nevertheless, several lines of evidence suggested that plastids make their own FeS clusters. First, isolated plastids could incorporate sulfur from cysteine into acid-labile clusters in ferredoxin in a reaction requiring ATP and NADPH (Takahashi *et al.*, 1986; Takahashi *et al.*, 1991a; Takahashi *et al.*, 1991b). Second, newly imported apo-ferredoxin could be converted into the holoform *in vitro* in isolated plastids in the absence of cytosol (H.M. Li *et al.*, 1990; Pilon *et al.*, 1992). And third, most of the iron in the plant cell is plastid-localized (see above). Therefore, it was concluded that plant cells must have at least two FeS assembly machineries, one in the mitochondrion and one in the plastid.

### *c. Plastid Components That are Nif/Isc-Related*

Analysis of the *Arabidopsis* genome revealed two *nifS*-related genes. One, At*NFS1*, is suggested to

encode a mitochondrially-targeted protein, and another, At*NFS2*, a plastid-localized one with cysteine desulfurase activity (Kushnir *et al.*, 2001; Léon *et al.*, 2002). Subsequently, five NFU proteins, each containing the conserved C-terminal CxxC motif, and encoded by At*NFU1* through *NFU5* were described, as well as a plastid-localized IscA homolog, AtISA1 (Léon *et al.*, 2003; Yabe *et al.*, 2004). The NFU proteins were distinguished into two sub-types, the NFU1-3 type[1] being plant-specific and proposed to be plastid-localized based on immunodetection, GFP fusions and *in vitro* import studies, and NFU4 and NFU5 being mitochondrial. A T-DNA insert in the *NFU* gene on chromosome V, *CNFU2* or *NFU2* (encoding a plastid-type protein), resulted in decreased abundance of photosystem I, ferredoxin, sulfite reductase, and reduced stromal iron-sulfur cluster assembly activity (Touraine *et al.*, 2004; Yabe *et al.*, 2004). On the other hand, $Fe_3S_4$ glutamate synthase activity was not reduced, nor was the abundance of the $Fe_2S_2$ Rieske protein or subunits of the cytochrome $b_6f$ complex reduced, suggesting distinct pathways for plastid iron-sulfur cluster assembly (Touraine *et al.*, 2004).

### *d. The More Recently Discovered Suf System Functions in FeS Cluster Assembly in Bacteria and Plastids*

The Suf pathway was revealed through analysis of suppressors of *E. coli* strains in which the ISC machinery was deleted (Takahashi and Tokumoto, 2002). In the suppressed strain, the Suf pathway, which is important in bacteria under conditions of oxidative stress and iron limitation (Nachin *et al.*, 2003; Outten *et al.*, 2004; Wang *et al.*, 2004), is mis-expressed, allowing the SUF system to cover the loss of the ISC system. Homologs of the SUF components (called SufABCDES) are found in many bacteria, archaea and plastid-containing eukaryotes (Ellis *et al.*, 2001), and the operation of the Suf pathway in *Arabidopsis* plastids was demonstrated recently (Xu and Møller, 2004). As mentioned above, anaerobic conditions facilitate cluster assembly. The corollary is that conditions that favour oxidation reactions make cluster maintenance and assembly more difficult. The use of the Suf system in cyanobacteria and chloroplasts perhaps represents an adaptation to greater oxidative stress in this compartment relative to the mitochondrion.

SufC, a cytoplasmic ABC-type ATPase in bacteria, is a key component of the SUF system because of its high degree of conservation and the severity of phenotype associated with loss of function (Nachin *et al.*, 2003). SufC associates with SufB and SufD to form a complex that is required for "repair" of labile FeS clusters that are damaged during oxidative stress, which may mean energy-dependent insertion of $Fe^{2+}$ into $Fe_3S_4$ centers.

SufS is related to NifS and in bacteria constitutes one subunit of a cysteine desulfurase. SufS also exhibits selenocysteine lyase activity, which is required for the synthesis of selenoproteins. The second subunit, SufE, enhances the cysteine desulfurase over the selenocysteine lyase activity (Loiseau *et al.*, 2003). The plastid SufS, called CpNifS or AtNFS2, also shows both cysteine desulfurase and selenocysteine lyase activity (Pilon-Smits *et al.*, 2002). Interestingly, the recombinant protein shows only a fraction (1 to 2%) of the activity of the endogenous protein in stromal extracts in the assembly of iron sulfur clusters of ferredoxin (Ye *et al.*, 2004). One possibility is that only a fraction of the recombinant protein is active. Another possibility, raised by the function of SufE in bacteria, is that the *in vivo* reaction involves other factors, such as a plastid SufE-homolog (Xu and Møller, 2004). The latter model is supported by the observation of a high molecular weight CpNifS-containing complex (Ye *et al.*, 2004). SufS and NifS are related, but the key difference may be the dual role of SufS in both Se and S metabolism, dependent on interaction with SufE. SufA is related to IscA and by analogy probably has a role in cluster assembly.

### *e. Directly Discovered Plastid Components*

In a screen for mutants of *Arabidopsis* defective in the assembly of PSI, Meurer and co-workers identified HCF101 as a candidate FeS cluster assembly factor in the plastid stroma (Lezhneva *et al.*, 2004; Stöckel and Oelmüller, 2004). They proposed a role for HCF101 in the assembly of $Fe_4S_4$ clusters as opposed to $Fe_2S_2$ clusters based on a drastic decrease in the abundance of PSI reaction center polypeptides PsaA and PsaB, attributable to degradation of the apoproteins as a result of a post-translational block in assembly. A less dramatic increase in the peripheral subunits argued for an effect of HCF101 on cofactor biogenesis and, more specifically $Fe_4S_4$ centers, owing to a 50% decreased abundance of ferredoxin thioredoxin reductase but not of ferredoxin or the Rieske FeS protein.

[1] The nomenclature of Léon et al. (2003) is used here owing to precedence. The gene names for the mitochondrial forms are at*NFU1* and at*NFU2* and for the plastid forms at*CNFU1* through at*CNFU3* in the work of Yabe et al. (2004).

The sequence relationship between HCF101 and ApbC (Lezhneva *et al.*, 2004), and also between HCF101 and Cfd1p, solidifies its role in FeS biogenesis in the plastid, as does its iron-dependent expression (Stöckel and Oelmüller, 2004), but what specifically that role might be is unclear. HCF101 also contains a conserved CxxC motif (Lezhneva *et al.*, 2004). As appropriate for a gene encoding a PSI assembly factor, *HCF101* is expressed in green organs (Stöckel and Oelmüller, 2004). The *Arabidopsis* genome encodes three different HCF101-like proteins—HCF101, HCF101-L1 and HCF101-L2, but L1 and L2 have not been characterized functionally as yet.

HCF101 is highly conserved with homologs in all kingdoms of life. These have been classified into four groups (Lezhneva *et al.*, 2004). HCF101 itself belongs to Class 1. The Class 2 form (L1 in *Arabidopsis*) is proposed to function in mitochondria based on the presence of an apparent N-terminal pre-sequence. The Class 3 form (L2 in *Arabidopsis*) functions in the cytosol and nucleus based on the location of the yeast representative, Nbp35p (Hausmann *et al.*, 2005), and the Class 4 form is in the cytosol. It is possible also that one of the *Arabidopsis* homologs is a redundant factor in the plastid (accounting perhaps for the weak impact of loss of HCF101 on ferredoxin thioredoxin reductase).

It is likely that a continued classical genetic approach to the study of plastid FeS cluster biogenesis could reveal new components besides those related to the products of the well-studied *isc* operon and the more recently discovered *suf* operon. A recent publication suggests that *Arabidopsis* APO1 may be involved in $Fe_4S_4$ cluster biosynthesis (Amann *et al.*, 2004), but the pleiotropic impact of loss of APO1 on membrane structure and the absence of homologs in *Chlamydomonas* and cyanobacteria suggest that APO1 may be more generally involved in a development-specific aspect of thylakoid biogenesis.

### 3. Hydrogenase Fe Cluster

Besides heme and FeS centers, there are other iron-containing cofactors in enzymes, including mono- and di-iron enzymes as well as uncharacterized iron sites (Fox, 1998; Plank *et al.*, 2001; Berthold and Stenmark, 2003; Hausinger, 2004). One of these is the bi-nuclear Fe center of hydrogenase in *Chlamydomonas* (Happe *et al.*, 1994). Genetic analysis of hydrogenase-minus mutants revealed two new enzymes, HydE and HydG belonging to the "Radical SAM" family (Sofia *et al.*, 2001), which are required for production of active hydrogenase (Posewitz *et al.*, 2004). The restricted occurrence of HydE and HydG homologs in Fe-hydrogenase containing prokaryotes is consistent with the proposed function in hydrogenase assembly. By analogy to the biosynthesis of the nitrogenase cluster, the authors of this work suggest that HydE and HydG may be involved in mobilization of iron for assembly of the so-called H-cluster of the [Fe] hydrogenase, which also requires CN, CO and the di(thio-methyl)amine ligand, but they do not rule out a function in in situ generation of the iron-coordinating ligands. Heterologous expression of hydrogenase in *E. coli* requires only the HydE and HydG factors in addition to the gene for the apoprotein, indicating that these are probably the most critical assembly factors.

### C. Transport of Iron into Chloroplasts

If ferritin is assembled with iron in the plastid, then there must be a mechanism for iron transport into the chloroplast. Also, under conditions of iron deficiency, it may be necessary to re-allocate iron from one compartment to another (such as the mitochondrion where iron is required for the function of respiratory enzymes), and intracellular transporters are expected to be central to organelle metal homeostasis. However, such molecules have not yet been discovered, although the Nramp transporters are candidates. The Nramp proteins, originally identified in mammals as iron transporters that affect resistance to microbial infection, are considered to be broad specificity, divalent cation transporters (Gunshin *et al.*, 1997). They are encoded in plant and other eukaryotic genomes as multi-gene families with members displaying distinct patterns of expression in response to divalent cation nutrition, suggesting that they may have metal-specific roles *in vivo*. While they are generally considered to be assimilatory transporters, they are found also in intracellular membranes in *S. cerevisiae* (reviewed by Van Ho *et al.*, 2002), and it is possible that they function to deliver metal ions into and out of organelles in plants. For instance, NRAMP3 in *Arabidopsis* localizes to the vacuolar membrane (Thomine *et al.*, 2003).

In *S. cerevisiae*, members of the carrier family—Mrs3p and Mrs4p—appear to be involved in iron transport across the mitochondrial inner membrane (Foury and Roganti, 2002; Mühlenhoff *et al.*, 2003; Kunji, 2004; Lesuisse *et al.*, 2004). Although the *Arabidopis* genome encodes 58 members of the mitochondrial carrier family, the probability that Mrs3p and Mrs4p homologues would function in chloroplasts is low because the plastid inner envelope transporters tend to be

distinct from the mitochondrial carriers (Flügge *et al.*, 2003; Picault *et al.*, 2004).

### *D. Fe-Deficiency Impacts the Photosynthetic Apparatus*

The abundance of the photosynthetic apparatus in chloroplasts and the numerous iron-containing redox-active proteins therein, plus the loss of chlorophyll proteins as a marker for iron-deficiency, meant that studies of the impact of iron-deficiency on plastid biochemistry have focused on photosynthesis (e.g. N. Terry, 1983; N. Terry and Abadía, 1986). Nevertheless, the discovery of di-iron enzymes in desaturation and other fatty acid modification reactions, and in carotenoid synthesis (Cunningham and Gantt, 1998; Shanklin and Cahoon, 1998), and the recent molecular identification of cytochrome P450 enzymes functioning in the biosynthesis of carotenoids, oxylipins and other isoprenoid-derived compounds, indicates the importance of iron in many other physiological processes including the production of defense metabolites (Froehlich *et al.*, 2001; Helliwell *et al.*, 2001; Tian and DellaPenna, 2004).

General principles concerning the impact of iron-deficiency on photosynthesis in plants have not yet emerged because it is difficult to directly compare individual studies with different plant material at different stages of growth and under various conditions of other nutrients. Therefore, our understanding of iron-deficiency adaptation of the photosynthetic apparatus comes largely from studies of microorganisms like cyanobacteria and green algae where iron nutrition can be readily and uniformly controlled (Moseley *et al.*, 2002a; Michel and Pistorius, 2004). Some well-documented changes in response to iron-deficiency in cyanobacteria include the replacement of iron-containing ferredoxin by iron-free flavodoxin, a decrease in the ratio of PSI to PSII from about 4:1 to 1:1, and the de novo synthesis of a new antenna for photosystem I consisting of the IsiA polypeptide or CP43' (Laudenbach *et al.*, 1988; Laudenbach and Straus, 1988; La Roche *et al.*, 1996; Bibby *et al.*, 2001; Boekema *et al.*, 2001). PSI is also a prime target in iron-deficient plants, presumably because of its high iron content (three $Fe_4S_4$ clusters), but the other two adaptations are not known to occur in chloroplasts (Nishio *et al.*, 1985). The *Chlamydomonas* genome appears to encode several different chloroplast-targeted ferredoxins. Two of these genes show a reciprocal pattern of expression dependent on iron nutrition, but the physiological function of this pattern is not known (N. Fischer and J.-D. Rochaix, personal communication; A. Terauchi and S. Merchant, unpublished results). Because ferredoxin is the source of electrons for many biosynthetic pathways in the plastid, it is possible that synthesis of alternate forms of ferredoxin may determine allocation of reducing power under iron-deficient conditions.

In a recent study with *Chlamydomonas*, a distinction was made between iron deficiency vs. iron-limitation (La Fontaine *et al.*, 2002; Moseley *et al.*, 2002a). *Iron-deficient* cells are defined as those that are not chlorotic but where the assimilator iron uptake genes, *FOX1*, *FTR1* and *FEA1*, are fully induced, whereas *iron-limited* cells are defined as symptomatic (i.e. chlorotic) and the rate of cell division is reduced. In *Chlamydomonas*, a progressive modification of the photosynthetic apparatus was observed. In marginally iron-deficient cells, the LHCI antenna was found to be physically and functionally uncoupled from PSI and this was correlated with an altered association of the PSI-K polypeptide (Fig. 1), which functions to facilitate the transfer of excitation energy from the peripheral

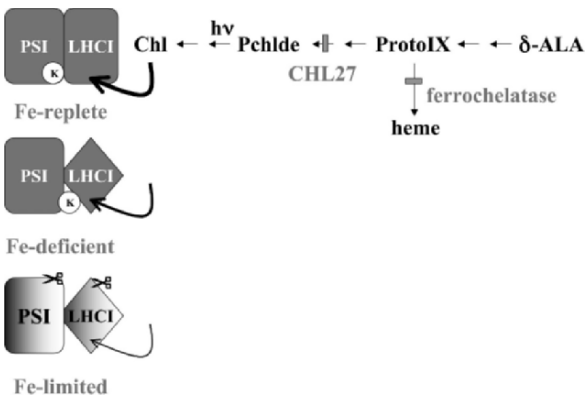


*Fig. 1.* The PSI-LHCI interaction is dependent on Fe nutrition. In Fe-replete cells, LHCI is physically and functionally associated with PSI in order to optimize energy transfer. As the cells anticipate iron-deficiency (i.e. in the situation where iron uptake is enhanced), the photosynthetic apparatus is modified to decrease excitation energy input into the PSI reaction center. This occurs via a loosening of the interaction between LHCI and PSI through a change in the association of PSI-K with PSI, and also by a change in the polypeptide composition of the LHCI complex (indicated by the change in shape of LHCI from rectangular to rhomboidal). The rationale for this modification is to avoid oxidative stress resulting from loss of iron from labile $Fe_4S_4$ clusters in PSI. When iron is limiting cell division, despite the expression of high affinity transporters, the abundances of PSI and the LHCI antenna are down-regulated by induced proteolysis to conserve iron (indicated with the scissors symbols). The plastid iron status may be sensed by flux through the tetrapyrrole pathway. For regulating chlorophyll protein abundance, the level of chlorophyll synthesized is relevant, and this is sensitive to iron nutrition because the aerobic oxidative cyclase (CHL27) is an iron-containing enzyme. For regulating heme proteins, the level of heme may be relevant, and this is clearly dependent on iron availability at the step catalyzed by ferrochelatase.

antenna to the reaction center (P. Jensen *et al.*, 2000). This response was suggested to function as a protective mechanism to avoid photo-oxidative damage resulting from damaged or improperly assembly FeS clusters in PSI. And indeed, iron-deficiency or loss of LHCI antenna can "rescue" the light sensitivity of a *Chlamydomonas* strain lacking PsaF (Hippler *et al.*, 2000; Moseley *et al.*, 2002a).

As iron-deficient *Chlamydomonas* cells progress to iron limitation, specific subunits of the LHCI antenna proteins are degraded, and the abundance of both photosystems and the cytochrome complex is reduced to about 5% of the level maintained in iron-replete cells coincident with a decreased rate of cell division. The abundance of the ATP synthase is unchanged by iron deficiency. Interestingly, the chlorophyll content on a per cell basis is maintained at about 50% of the level found in iron-replete cells, with most of the chlorophyll associated with LHCII complexes that are not associated with a reaction center. This complex may serve as a reservoir of chlorophyll for de novo synthesis of photosystems during the re-greening process initiated by iron nutrition. De novo chlorophyll synthesis does occur even in iron limited cells, but the new pigment must be selectively allocated to the LHCII in -Fe cells.

### *E. Fe-Sensing*

The impact of Fe-deficiency on the biosynthesis of chlorophyll (or chlorophyll proteins) was noted decades ago and is a classic symptom of Fe-deficiency (Bogorad *et al.*, 1958; Machold, 1971; Spiller and Terry, 1980; N. Terry, 1980). The aerobic oxidative cyclase or CHL27, now known to be a di-iron enzyme, was suggested to be a key target of iron-deficiency (Spiller *et al.*, 1982; Tottey *et al.*, 2003). The loss of chlorophyll proteins in iron-deficiency was therefore attributed to reduced flux through the chlorophyll biosynthetic pathway (Spiller and Terry, 1980). However, as mentioned above, iron-deficiency chlorosis represents a specific re-programming of chlorophyll protein synthesis rather than a general decrease in all chlorophyll proteins. Because a *Chlamydomonas* mutant with reduced cyclase activity recapitulated parts of the program initiated by marginally iron-deficient cells, it was suggested that the cyclase might represent an iron sensor in the plastid (Moseley *et al.*, 2002a). According to this model, the occupancy of the active site iron in CHL27 would be proportional to iron availability in the plastid. As the cell, and hence the organelle, becomes iron-deficient, the rate of chlorophyll synthesis is decreased, leading to restriction of chlorophyll for the de novo synthesis of chlorophyll proteins. If there is a hierarchical allocation of chlorophyll to particular apoproteins (and there is considerable evidence for this), then the program of chlorophyll-protein complex accumulation can be linked to plastid iron status. In the case of PSI, it appears that the stability of PsaK is very sensitive to iron-nutrition status as well as chlorophyll biosynthesis. The model therefore suggests that occupancy of the pigment sites in PsaK determines the association of this polypeptide with PSI, and hence the functional association of the LHCI antenna with PSI (Fig. 1). Nevertheless, a causal connection between chlorophyll binding to PsaK and its assembly with PSI has not yet been established. An attractive aspect of this model is that it distinguishes between iron sensing in the plastid vs. iron sensing in the nucleus to control the expression of iron assimilatory genes. On the other hand, the mechanism is clearly relevant only in the context of the photosynthetic apparatus. Whether other plastid types have mechanisms for signaling and responding to iron nutrition is not known. One can envision a similar mechanism for regulation of heme protein accumulation in all plastid types via the action of ferrochelatase, but so far there are no studies that test this idea.

## III. Cu

Three abundant plastid proteins that contain copper are CuZnSOD in the plastid stroma and plastocyanin and polyphenol oxidase in the thylakoid lumen (Jackson *et al.*, 1978; Kieselbach *et al.*, 1998). These proteins are not present in all chloroplasts; the chlorophyte algae (such as *Chlamydomonas*) contain only plastocyanin, *Arabidopsis* has CuZnSOD and plastocyanin, and tomato and spinach have all three proteins. The relative proportion of each protein in a given plant cell depends of course on the organ, the developmental state and environmental conditions (e.g. Last and Gray, 1989; Perl-Treves and Galun, 1991; Thygesen *et al.*, 1995; Thipyapong *et al.*, 1997). The use and distribution of copper within plastid compartments and the impact of deficiency on plastid function is therefore likely to vary. Cyanobacteria, like the green algae, do not have CuZnSOD or polyphenol oxidase, but on the other hand, they do have a respiratory copper-containing oxidase.

### *A. Cu-Protein Assembly*

#### *1. Transporters*

Once copper is taken up into the plant cell, presumably by a member of the COPT1 family of transporters

(Sancenón *et al.*, 2003; Sancenón *et al.*, 2004), it needs to be distributed to organelles for the biosynthesis of copper-enzymes like cytochrome oxidase, CuZnSOD, and plastocyanin, and to other compartments for the biosynthesis of various intra- or extra-cellular multi-copper oxidases such as ascorbate oxidase and laccase. Some members of the so-called CPx-type heavy-metal transporting ATPases are responsible for intracellular copper distribution (reviewed by Williams *et al.*, 2000). Three of these enzymes have been characterized in *Arabidopsis*. RAN1 is responsible for loading copper in the secretory pathway for the biosynthesis of the ethylene receptor, while PAA1 and PAA2 function to deliver copper to the chloroplast for CuZnSOD and plastocyanin (Hirayama *et al.*, 1999; Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005).

PAA1 and PAA2 carry N-terminal MxCxxC metal-binding domains, CPC ion-transduction motifs, classical P-type ATPase phosphorylation and phosphatase domains, and ATP-binding sites. PAA1 is proposed to localize to the envelope membranes and PAA2 to the thylakoid membrane based on *in vitro* import experiments and localization of GFP fusion proteins (Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005). This distribution is analogous to the localization of two copper transporting P-type ATPases in cyanobacteria, CtaA and PacS. The former is located in the cell membrane and functions in copper acquisition, whereas the latter is located in the thylakoid membrane and functions to deliver copper to the lumen (Kanamaru *et al.*, 1994; Phung *et al.*, 1994; Tottey *et al.*, 2001). The phenotypes of *paa1* and *paa2* mutants are entirely consistent with this model. Plants carrying mutations in *PAA1* show reduced abundance of both plastocyanin and CuZnSOD whereas *paa2* plants have less plastocyanin (Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005). Although copper transporting activity has not been shown for either PAA1 or PAA2, the impact of copper nutrition on the phenotype is consistent with their function in copper transport. Specifically, the phenotype of *paa2* alleles was exacerbated in medium containing low copper and suppressed in medium containing extra copper. Also, while both *paa1* and *paa2* mutants had normal leaf copper content, when metal content was analyzed after sub-cellular fractionation, *paa1* chloroplasts were found to have less copper than those from wild-type, and *paa2* showed less copper in the thylakoid fractions but essentially normal copper content in intact chloroplasts. PAA1 is expressed in all organ types, reflecting a need for copper in all plastid types, while PAA2 expression was detected only in green organs, consistent with a function in photosynthesis (Abdel-Ghany *et al.*, 2005).

Interestingly, the genome of *Cyanidioschizon merolae* lacks a PAA1 or PAA2 homolog, but this is not incompatible with the fact that the organism also lacks a gene for plastocyanin and must use only a *c*-type cytochrome for photosynthesis (see below) (Hanikenne *et al.*, 2005).

### 2. Chaperones

The concept of a copper chaperone for delivery of copper between proteins developed through genetic analysis of copper homeostasis in *S. cerevisiae* and subsequent analysis of the function of homologous proteins in other organisms (O'Halloran and Culotta, 2000). Yeast Atx1p (homologs known as HAH1, Atox1 and CCH) is a small protein containing a metal-binding site that interacts specifically with the metal-binding site on Ccc2p (a P-type ATPase). By analogy, a stromal chaperone for copper delivery from the envelope to the thylakoid, and perhaps another in the lumenal compartment for delivery from PAA2 to apoplastocyanin, is predicted. In cyanobacteria, an Atx1-related molecule, identified through a two-hybrid interaction with the metal-binding domains of PacS and CtaA, was shown to function as a copper chaperone for plastocyanin and cytochrome oxidase assembly (Tottey *et al.*, 2002). Proteins carrying candidate copper chaperone motifs can be identified in the *Arabidopsis* genome, but these have not yet been analyzed functionally. It is also possible that because of the small size of Atx1-like copper chaperones, some candidate molecules have not been predicted accurately or they have escaped detection because of the less significant BLAST (similarity) scores.

A small copper-binding protein, related to a copper homeostasis factor in bacteria called CutA, was shown to be chloroplast-localized (Burkhead *et al.*, 2003). Its physiological function has not been deduced, but it may well function in copper trafficking and recycling (see below).

## B. Cu Deficiency and Regulation by Cu

### 1. Chlamydomonas

Because plastocyanin is the most abundant copper protein in a photosynthetic cell, it is a prime target in the face of copper-deficiency. In cyanobacteria and green algae, there is a well-regulated "back up" system, in which a heme protein, called cytochrome $c_6$,

is induced in copper-deficiency to compensate for the loss of plastocyanin in the electron transfer chain (Wood, 1978; Sandmann *et al.*, 1983; Merchant, 1998). Accordingly, copper is not essential for photosynthesis in these organisms. The regulatory events have been studied most thoroughly in the *Chlamydomonas* model (Merchant, 1998). The *CYC6* gene for cytochrome $c_6$ is associated with copper-response elements that serve as binding sites for a transcriptional activator, Crr1, in copper-deficient cells (Quinn and Merchant, 1995; Quinn *et al.*, 2000; Eriksson *et al.*, 2004). In this situation, plastocyanin is rapidly degraded because of the reduced thermodynamic stability and increased protease-susceptibility of the apo-protein vs. the holoprotein, and the Crr1-dependent expression of a degrading activity (Merchant and Bogorad, 1986; H.H. Li and Merchant, 1995; Eriksson *et al.*, 2004). In *crr1* mutants, apo- and some holo-plastocyanin accumulate even in copper-deficient cells, owing presumably to the lack of the protease. The *crr1* mutation, therefore, by affecting the "salvage" of copper from plastocyanin, has an impact on respiratory growth in addition to its impact on photosynthesis owing to loss of *CYC6* expression (S. Tottey, S. Nakamoto, J. Kropat and S. Merchant, unpublished results).

Besides regulating plastocyanin and cytochrome $c_6$ abundance, Crr1 also controls the expression of *CPX1* and *CHL27A*/*CHL27B*, encoding oxygen-dependent enzymes (coproporphyrinogen oxidase and aerobic oxidative cyclase) in the tetrapyrrole biosynthetic pathway (Eriksson *et al.*, 2004). *CPX1* encodes a plastid-targeted isoform that is about 10- to 20-fold up-regulated in copper-deficiency, while the expression of *CPX2*, encoding possibly a mitochondrial isoform, is unaffected by copper (Hill and Merchant, 1995; J. Kropat and S. Merchant, unpublished results). Copper nutrition and Crr1 reciprocally regulate the expression of *CHL27A* and *CHL27B* (Moseley *et al.*, 2000; Moseley *et al.*, 2002b). Both isozymes are plastid-localized but they may be differently distributed between the envelope and thylakoid membranes within the plastid (M. Allen and S. Merchant, unpublished results). The rationale for *CPX1* and *CHL27A*/*CHL27B* regulation by copper is not known, but it does point to a previously unrecognized connection between copper and the tetrapyrrole pathway.

### 2. Arabidopsis

Copper-deficiency has not been studied systematically in *Arabidopsis* but the work on PAA1 and PAA2 function revealed that the standard medium for *Arabidopsis* growth in the laboratory is probably slightly copper-deficient (Abdel-Ghany *et al.*, 2005). The addition of copper to that medium stimulates the accumulation of both plastocyanin and CuZnSOD with a more noticeable effect for SOD. The authors concluded that under conditions of copper limitation there is preferential allocation of copper to plastocyanin (for which there is no substitute in *Arabidopsis*) vs. CuZnSOD (for which there is a substitute). In fact, the expression of FeSOD is increased to compensate for loss of CuZnSOD.

### 3. Redistribution of Copper

Several lines of evidence indicate that metals can be redistributed from the chloroplast to other organelles or even secreted from the cells. When copper-replete *Chlamydomonas* cells become deficient, copper is reallocated from plastocyanin in the chloroplast to cytochrome oxidase in the mitochondrion (S. Tottey, S. Nakamoto, J. Kropat and S. Merchant, unpublished results). In vascular plants, the copper content of senescent tissue decreases. This process is correlated with an increase in the content of a copper chaperone, CCH, in the vascular tissue (Mira *et al.*, 2001). These processes probably require the action of transporters, chaperones or copper-binding proteins to move copper from a stable intracellular site in a protein, but the relevant molecules have not yet been identified and the process has not been subject to genetic analysis.

## IV. Mn

Mn is nutritionally essential for all living organisms (Frieden, 1985; Marschner, 1995). It functions as a redox catalyst because it can occur stably in a cell in many different oxidation states, and this is its role in Mn-containing SOD and in PSII. $Mn^{2+}$ can also activate water to generate a strong nucleophile for hydrolytic reactions (as in the enzyme arginase) or it can stabilize a leaving group (as in the nucleotide products of a glycosyl transferase reaction), but its role in these types of reactions in the plastid are not specifically described in the literature.

### A. Manganese Transport

The bulk of the manganese in a photosynthetic cell is found in PSII in the chloroplast lumen. The mechanism of assembly of this cluster is not well understood even though PSII biogenesis has been subject to considerable genetic dissection in both cyanobacteria

and *Chlamydomonas* (Pakrasi, 1995). Pakrasi and coworkers approached this problem in the *Synechocystis* model and discovered the MntABC system for manganese ion uptake into bacterial cells. In more recent work, they show that cyanobacteria contain two pools of manganese, a storage pool that is released upon treatment with EDTA but whose maintenance is energy-dependent, and a second pool in PSII that is derived from the storage pool (Keren *et al.*, 2002). By analogy, there must be mechanisms for $Mn^{2+}$ transport into the chloroplast across the inner envelope membrane plus a mechanism for transport across the thylakoid membrane. The identity of the transporters in the chloroplast is unknown. The MntABC system appears to be strictly bacterial, indicating the operation of another system for chloroplasts.

The Nramp proteins (reviewed by Williams *et al.*, 2000; Forbes and Gros, 2001) are excellent candidates for a manganese delivery system to plastids. These molecules are proton-coupled divalent cation transporters that show broad substrate specificity in many *in vitro* experiments but it is likely that some members of the gene family are $Mn^{2+}$-specific *in vivo*. The bacterial homologs of the Nramps, called MntH, indeed appear to be $Mn^{2+}$ selective (Kehres and Maguire, 2003) and Nramp homologs in *Chlamydomonas* do show increased expression in response to manganese-deficiency (M. Allen, S. Tottey, J. Kropat, J. del Campo and S. Merchant, unpublished results). Plant genomes contain multiple Nramp homologs with functionally distinct roles based on sub-cellular location, organ-specific pattern of expression, metal specificity, and pH sensitivity (Belouchi *et al.*, 1997; Curie *et al.*, 2000; Thomine *et al.*, 2000; Thomine *et al.*, 2003). While some members of the family are likely involved in iron homeostasis, others could function in manganese metabolism. But the role of plant *NRAMP* expression and function in manganese nutrition has received less attention.

A possibility for manganese acquisition by the plastid, hinted at by the intracellular organelle localization of Nramp homologs Smf1p and Smf2p in *S. cerevisiae* (reviewed by Van Ho *et al.*, 2002), is that one or more Nramps may be involved. In this context, it is worth noting that mitochondria also have a significant manganese requirement (e.g. for MnSOD) and the question of allocation of manganese to plastids vs. mitochondria in plants has not been addressed. In *S. cerevisiae*, a member of the carrier family has been proposed as a facilitator for mitochondrial manganese acquisition for MnSOD biogenesis (Luk *et al.*, 2003). It is not known whether Mtm1p is actually a $Mn^{2+}$ transporter. The mitochondrial carriers are evolutionarily distinct from most of the known plastid inner envelope translocators, and so it does not necessarily follow that a homolog of *S. cerevisiae MTM1* would function in plastid Mn-protein assembly. The diversity of $Mn^{2+}$ transporters known in nature—MntA, MntH and perhaps the Mtm1p carrier—leaves open the possibility that a completely novel molecule operates in the plastid.

A recent comparative analysis of algal genomes revealed members of the cation diffusion facilitator family (called MTP proteins in plants) that may be manganese transporters, and it is suggested that one or more of these molecules could be plastid-localized (Hanikenne *et al.*, 2005).

### *B. Manganese Deficiency*

The importance of manganese in the photochemical reactions of photosynthesis was recognized half a century ago because of the impact of Mn deficiency on phototrophic growth and oxygen evolution in algae (Pirson, 1955). The symptoms of Mn-deficiency in plants are noted as leaf discoloration, which implies an impact at the level of the chloroplast, but the biochemical consequences of deficiency have not been investigated. Mn-deficient *Chlamydomonas* cells show loss of PSII and MnSOD activity and a sensitivity to peroxides but not paraquat or Rose Bengal (M. Allen, S. Tottey, J. Kropat, J. del Campo and S. Merchant, unpublished results). Whether the oxidative stress occurs at the level of plastid or mitochondrion redox metabolism is not known.

## V. Questions for Future Investigation

The metabolism of the transition elements is intimately inter-related. For instance, in many organisms, including algae, fungi and mammals (although not plants), a copper-containing enzyme is required for high affinity iron uptake (Askwith and Kaplan, 1998; La Fontaine *et al.*, 2002). Therefore, copper-deficiency generates secondarily an iron-deficiency. A connection between copper and zinc metabolism is known in humans, where excess zinc in the diet blocks copper intake (Kumar *et al.*, 2003). Recently we noted a role for manganese in iron assimilation in *Chlamydomonas* (M. Allen, S. Tottey, J. Kropat, J. del Campo and S. Merchant, unpublished results). The use of microarray and proteomic approaches to study metal homeostasis at a whole genome level is ideal for the discovery of such inter-relationships.

In a recent microarray study on iron-deficient yeast, the concept of metabolic re-modeling was noted where certain iron-utilizing pathways are down-regulated in favor of parallel pathways that are less iron-dependent (Shakoury-Elizeh *et al.*, 2004). This phenomenon is well known in the context of the photosynthetic apparatus where flavodoxin can substitute for ferredoxin or cytochrome $c_6$ for plastocyanin, and for the SODs where Mn-SOD is up-regulated to compensate for the loss of CuZnSOD in the copper-deficient rat (Hutber *et al.*, 1977; Wood, 1978; Merchant and Bogorad, 1987; Bottin and Lagoutte, 1992; Lai *et al.*, 1994). It is possible that there are back-up systems for other metalloenzymes in nature and these may also be discovered through whole genome analyses.

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# Section III

## Photosynthetic Metabolism in Plastids

# Chapter 11

# Light/Dark Regulation of Chloroplast Metabolism

Shaodong Dai
*Howard Hughes Medical Institute, Integrated Department of Immunology, National Jewish Medical and Research Center & University of Colorado Health Sciences Center, 1400 Jackson Street, Goodman Building K404, Denver, CO 80206, U.S.A.*

Kenth Hallberg
*AstraZeneca R&D Mölndal, Structural Chemistry Laboratory, S-431 83 Mölndal, Sweden*

Hans Eklund*
*Department of Molecular Biology, Swedish University of Agricultural Sciences, Box 590, Biomedical Center, S-751 24 Uppsala, Sweden*

Peter Schürmann
*Laboratoire de Biochimie Végétale, Université de Neuchâtel, CH-2007 Neuchâtel, Switzerland*

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## Summary

Light not only provides the energy for carbon assimilation in the chloroplast, it is also an important regulatory factor of carbon metabolism. The activities of several of its key enzymes are linked to light. This enables the chloroplasts to switch between biosynthetic pathways in the light and catabolic processes in the dark. The regulatory system,

*Author for correspondence, email: hasse@xray.bmc.uu.se

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which transmits the light signal to the enzymes is the ferredoxin/thioredoxin system. It consists of several proteins constituting a redox cascade. The light signal, perceived by chlorophyll, is transferred by ferredoxin in form of electrons to ferredoxin:thioredoxin reductase. This photosynthetic enzyme, the central component of the regulatory system, has a unique molecular structure containing a Fe-S cluster and a redox active disulfide bridge in very close contact. This particular arrangement enables the enzyme to reduce its disulfide with the help of the iron-sulfur cluster transforming the electron signal into a sulfhydryl (SH) signal. Thioredoxins relay the signal by disulfide/dithiol interchange reactions to target enzymes possessing redox-active disulfides. The structures of two such enzymes have been solved providing insights into the mechanism by which their activities are controlled. NADP-dependent malate dehydrogenase possesses two regulatory disulfides per subunit, one near the N-terminus and the other near the C-terminus in the primary structure, each one having a specific effect on the activity. Whereas reduction of the C-terminal disulfide releases the intrasteric inhibition caused by the C-terminus dipping into the active site, reduction of the N-terminal disulfide relaxes its rigid structure and frees the catalytic domain to adopt its active conformation. Fructose-1,6-*bis*phosphatase has a single regulatory disulfide per subunit located on a structural loop linked via a ß-sheet with the active site. Reduction of the disulfide brings about a rearrangement of the active-site, which increases the affinity for $Mg^{2+}$, the metal ion essential for catalytic activity.

## I. Introduction

The ferredoxin/thioredoxin system involves several proteins of rather small size: ferredoxin, ferredoxin:thioredoxin reductase (FTR) and thioredoxins. The electrons needed for the reductions are provided by the photosynthetic light reactions and transmitted by ferredoxin from the thylakoids to FTR (see Fig. 1). This enzyme, unique to oxygenic photosynthetic cells, transforms the light signal, received in the form of electrons, into a thiol signal, which is then transmitted through disulfide-dithiol interchanges involving thioredoxins to the target proteins. The best-studied target proteins are enzymes involved in carbon metabolism, i.e., fructose-1,6-*bis*phosphatase and phosphoribulokinase of the Calvin-Benson cycle, and NADP-dependent malate dehydrogenase involved in the export of reducing equivalents from the chloroplasts. Activation by the ferredoxin/thioredoxin system has also been demonstrated for a number of other enzymes involved in carbon, nitrogen, and fatty acid metabolism and in energy transduction (Schürmann and Buchanan, 2001). Recently, the number of putative target enzymes has been extended through the analysis of the interaction of proteins with chloroplast thioredoxins (Motohashi *et al.*, 2001; Balmer *et al.*, 2003, 2004). In recent years the structures of the components of the ferredoxin/thioredoxin system and of two well studied target enzymes have been solved. In addition, spectroscopic analyses of FTR have provided major insights into the mechanism by which this enzyme transforms

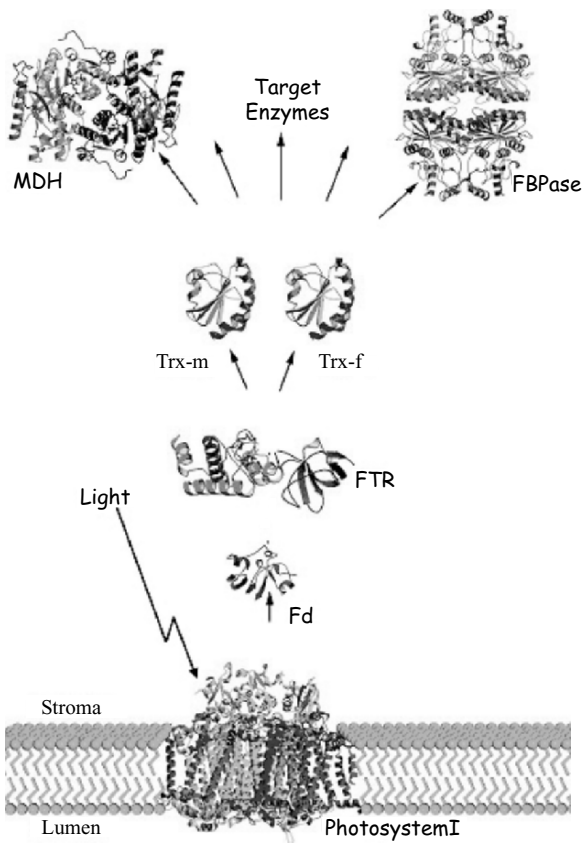


*Fig. 1.* (See also Color Plate 4, p. xxxvii.) Light activation of chloroplast enzymes by the FTR-system. Photosystem produces reduced ferredoxin upon illumination (bottom). With reduced ferredoxin, ferredoxin:thioredoxin reductase catalyzes the reduction of thioredoxins, which then reduce disulfides in target enzymes, exemplified by MDH and FBPase.

*Abbreviations*: FBPase – fructose-1,6-*bis*phosphatase; FTR – ferredoxin:thioredoxin reductase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; G6PDH – glucose-6-phosphate dehydrogenase; MDH – malate dehydrogenase; PRK – phosphoribulokinase; Rubisco – ribulose-1,5-*bis*phosphate carboxylase/oxygenase; SBPase – sedoheptulose-1,7-*bis*phosphatase; Trx – thioredoxin.

an electron signal into a thiol signal. These results taken together bring significant progress to the understanding of the functioning of the light/dark regulation at the molecular level. Different aspects of the regulatory system have been treated in a number of recent reviews (Jacquot *et al.*, 1997; Ruelland and Miginiac-Maslow, 1999; Meyer *et al.*, 1999, 2001; Dai *et al.*, 2000a; Schürmann and Jacquot, 2000; Schürmann and Buchanan, 2001; Schürmann, 2003a,b). In this chapter we will mainly focus on the structural aspects of the ferredoxin/thioredoxin system.

Light is the primary energy source for chloroplast metabolism. During daytime chloroplasts trap the energy from sunlight with their photosynthetic machinery to produce reducing equivalents, NADPH, and ATP, needed for the reduction of carbon dioxide to carbohydrates, which are then further used in the cellular metabolism as building blocks and as energy source. During nighttime, however, chloroplast metabolism depends on stored energy. By catabolic processes storage products, like starch, accumulated in the chloroplasts during daylight are broken down to provide the necessary metabolic energy. The assimilatory processes are catalyzed by enzymes of the Calvin-Benson cycle, the catabolic processes by enzymes of glycolysis and of the pentose phosphate cycle, all present in the chloroplast stroma. Some of the enzymes are even involved in both processes. To avoid futile cycling, i.e., the simultaneous functioning of assimilatory and dissimilatory pathways, the two processes are strictly controlled by a light-dependent mechanism, which might not only switch between light and dark, but also regulate the carbon flux depending on the light intensity (Buchanan 1980, 1991; Scheibe, 1994; Buchanan *et al.*, 1994, 2002; Jacquot *et al.*, 1997; Schürmann and Buchanan, 2001).

The signal for this light/dark control is light. Light activates a number of biosynthetic enzymes and inhibits a key enzyme of carbohydrate degradation, the glucose-6-phosphate dehydrogenase. Light, sensed by the thylakoid pigments, acts through a redox signaling cascade known as the ferredoxin/thioredoxin system to target the activity of key enzymes. Depending on their function in metabolism, these target enzymes are activated or deactivated by reduction of regulatory disulfides to sulfhydryl groups causing some structural change that modifies their catalytic capacity. In the dark this process is fully reversed by oxygen. Since photosynthetic electron transport produces oxygen in the light, the redox equilibrium state of the target enzymes, and hence their activity, will depend on "electron pressure" generated by the photosystems. Subsequent fine tuning of the thioredoxin-activated enzymes depends on other cellular factors such as pH and metabolite concentrations, both of which are linked to light (Buchanan, 1980; Scheibe *et al.*, 1991; Faske *et al.*, 1995).

## II. Ferredoxins

Ferredoxin is a 12 kDa, stromal-soluble, electron-carrier protein that is reduced by photosystem I (Fig. 1). Upon illumination, an electron emitted from the excited P700* near the lumenal side of the thylakoid membrane is transferred across the membrane to the [4Fe-4S] clusters of the PSI PsaC subunit on the stromal side. The [4Fe-4S] clusters in turn reduce the [2Fe-2S] cluster of ferredoxin (shown in Fig. 2). The crystal structure of cyanobacterial photosystem I has been improved to 2.5 Å resolution (Jordan *et al.*, 2001). It provides a detailed structure of the stromal ridge subunits PsaC, PsaD and PsaE (Antonkine *et al.*, 2003). Recently, the structure of a higher plant photosystem I was determined to 4.4 Å resolution (Ben-Shem *et al.*, 2003).

Several three-dimensional structures of ferredoxins from photosynthetic organisms are known (Fig. 2, see also the review by Dai *et al.* (2000a) for references). Plant-type ferredoxins possess single [2Fe-2S] clusters, which operate at low redox potentials, typically −400 mV. Several three-dimensional structures of plant-type ferredoxins share the same fold with a five-stranded β-sheet, 2-3 α-helices and a long loop. The

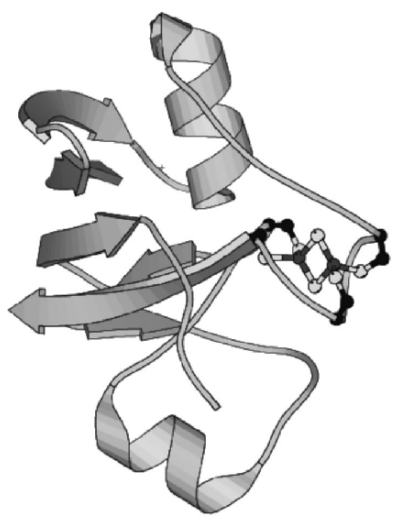

*Fig. 2.* (See also Color Plate 4, p. xxxvii.) Ferredoxin structure. Plant type ferredoxins contain a [2Fe-2S] center. The *Synechocystis* ferredoxin structure is shown, with the iron-sulfur center to the right.

iron/sulfur center is attached towards the outer edge of the molecule by four cysteine ligands in the loop with the sequence motif $CX_4CX_2CX_{22\text{-}33}C$. The two irons are tetrahedrally coordinated by inorganic sulfide ions and cysteine residues.

## III. Chloroplast Thioredoxins: *f* and *m* Type Thioredoxins

Thioredoxins are small (~12 kDa), ubiquitous redox proteins with a number of important functions (for reviews see Holmgren, 1985; Åslund and Beckwith 1999a,b; Powis and Montfort, 2001; Gromer *et al.*, 2004). Thioredoxins are α/β-proteins which have a central five-stranded β-sheet surrounded by four α-helices with a redox-active disulfide at the N-terminus of α2 (Fig. 3).

The ferredoxin/thioredoxin system involves two types of chloroplast thioredoxins: Trx-*f* and Trx-*m* (for recent reviews see Baumann and Juttner (2002); Jacquot *et al.*, (2002)). They were originally distinguished by their target enzyme specificity. Trx-*f* displays a high specificity towards chloroplast fructose-1,6-bisphosphatase (FBPase) and several other Calvin-Benson cycle enzymes whereas Trx-*m* interacts very efficiently with malate dehydrogenase (MDH), thereof their names. When tested under conditions approaching the *in vivo* situation, the Calvin-Benson enzymes, Rubisco activase, the ATP synthase CF1 complex, and MDH are exclusively or most efficiently activated by Trx-*f* (Jacquot *et al.*, 1997; Meyer *et al.*, 1999; Schürmann and Jacquot, 2000; Schürmann and Buchanan, 2001). On the other hand, glucose 6-phosphate dehydrogenase interacts specifically with Trx-*m*, which suggested that for carbohydrate metabolism, reduced Trx-*f* functions mainly in regulating carbon fixation whereas Trx-*m* acts in carbon catabolism (Schürmann and Jacquot, 2000).

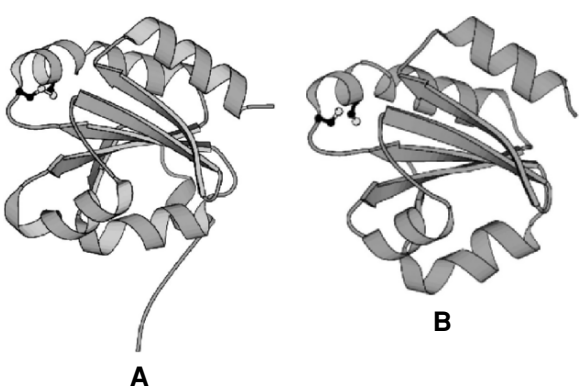


*Fig. 3.* (See also Color Plate 4, p. xxxvii.) Thioredoxin structure. Thioredoxin *f* and thioredoxin *m* structures. The active site disulfide in its oxidized form (Trx-*f*, left panel) and its reduced form (Trx-*m*, right panel) is shown in ball and stick representation.

Crystal structures have been determined for oxidized, recombinant Trx-*f,* an N-terminal truncated form of Trx-*f*, and Trx-*m* in oxidized and reduced forms (Capitani *et al.*, 2000). The structures show the typical thioredoxin fold with a central, twisted, five-stranded β-sheet surrounded by four α-helices (Fig. 3). Trx-*f* contains an additional N-terminal α-helix. The overall three-dimensional structures of the two chloroplast thioredoxins are quite similar but they have a significantly different surface topology and charge distribution around the active site.

The primary structures of the two chloroplast thioredoxins differ significantly and have only 30% identities. A difference between Trx-*f* and Trx-*m* is the presence of a third cysteine, which is conserved in all Trx-*f*s described so far. In the crystal structure, this Cys73 is exposed on the surface, 9.7 Å away from the accessible active-site Cys46 (Capitani *et al.*, 2000). Mutagenesis studies show that this residue may participate in the interactions with the target enzyme (del Val *et al.*, 1999). In addition to the third cysteine, Trx-*f* has a different distribution of polar, charged and hydrophobic residues around the active site with respect to *E. coli* thioredoxin or Trx-*m*. The active site of Trx-*f* is surrounded by a number of positive charges, which may be involved in orienting Trx-*f* correctly when it interacts with its target proteins (Wangensteen *et al.*, 2001).

Genome sequencing has revealed new chloroplast Trxs (Meyer *et al.*, 2002; Lemaire *et al.*, 2003). Some of them were shown to be unable to activate NADP-MDH while being active donors of reducing equivalents to peroxiredoxins, or thioredoxin peroxidases, involved in detoxification of $H_2O_2$ (Collin *et al.*, 2003; Lemaire *et al.*, 2003). The sequencing of the genome of *Arabidopsis thaliana* revealed that this plant contains several previously unknown isoforms of thioredoxin. On the basis of sequence comparison, seven putative chloroplastic Trxs were identified, four belonging to the *m*-type, two belonging to the *f*-type, and one belonging to a new x-type. A green fluorescent protein approach confirms the plastidal localization of these Trxs (Collin *et al.*, 2003). The study confirms the strict specificity of fructose-*bis*phosphatase for Trx-*f*, reveals that some Trxs are unable to activate NADP-malate dehydrogenase, and shows that the new *x*-type is the most efficient substrate for peroxiredoxin while being inactive toward the two other targets. This suggests that

the Trx-*x* isoform might be specifically involved in resistance against oxidative stress. Three-dimensional modeling shows that one of the *m*-type Trxs, Trx-*m3*, which has no activity with any of the three targets, exhibits a negatively charged surface surrounding the active site.

## IV. Ferredoxin: Thioredoxin Reductase

### *A. Structure: Subunits, Active Site*

FTR transduces the general redox signal from the one-electron donor ferredoxin to thioredoxins, which are two-electron donors. In contrast to bacterial, plant and mammalian thioredoxin reductases, which are flavoproteins reduced by NADPH, FTR is an iron-sulfur protein. FTR utilizes a [4Fe-4S] cluster and a disulfide bridge close to this center to mediate electron transfer in two steps from ferredoxin to cleave disulfide bridges of thioredoxins. Reduced thioredoxin then transfers the electrons to target enzymes via thiol/disulfide interchange reactions.

Most biochemical investigations have been carried out on the spinach enzyme, for which a careful analysis of the iron-sulfur center has been performed (Staples *et al.*, 1996, 1998). The only three-dimensional structure has so far been determined (at 1.6 Å resolution) for the cyanobacterial FTR from *Synechocystis* sp. PCC6803 (Dai *et al.*, 2000b). This FTR shows no functional difference from the spinach enzyme but it is significantly more stable and can be obtained in larger amounts and was thus more suitable for crystallographic studies (Schwendtmayer *et al.*, 1998).

FTR is an αβ-heterodimer composed of a 13-kDa catalytic subunit with conserved sequence between species and a variable subunit of similar or smaller size. The catalytic subunit contains the redox active disulfide and the [4Fe-4S]-center and is essentially an α-helical structure containing five helices (Fig. 4). The N-terminal half of the subunit, together with the C-terminal helix, forms an α-helical structure that covers the iron-sulfur center while the intervening 40 residues contain all the iron ligands and redox active cysteines. The iron center is surrounded exclusively by hydrophobic residues, all coming from the catalytic subunit. The FTR variable subunit is a heart-shaped, open β-barrel structure which contains five antiparallel strands with two loops forming the upper, outer parts of the heart.

The irons of the iron-sulfur center are coordinated by cysteines 55, 74, 76 and 85 (Fig. 5). All ligands are located in short sequence motifs CXC, which

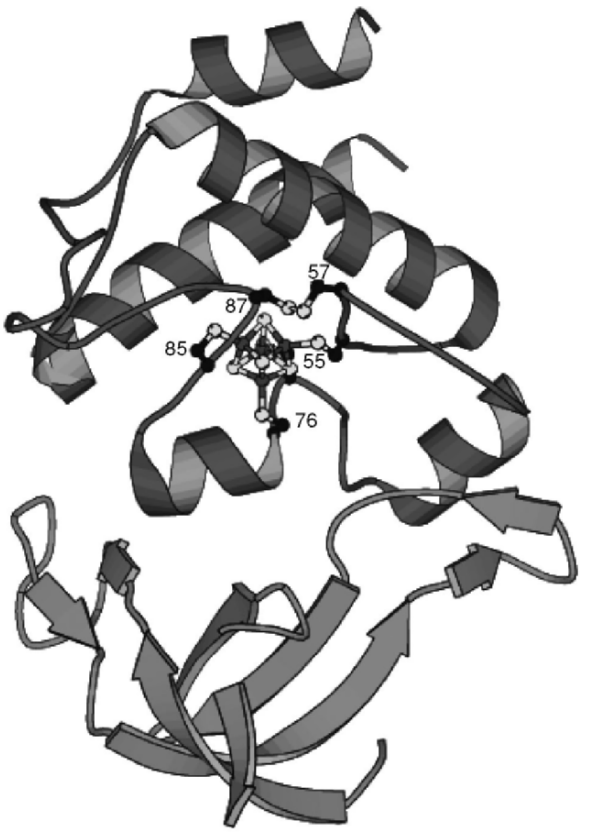


*Fig. 4.* (See also Color Plate 4, p. xxxvii.) FTR structure. The catalytic subunit at the top contains the iron-sulfur center, with iron atoms coordinated by cysteines 55, 74, 76 and 85, and the redox active disulfide bridge, formed between cysteines 57 and 87. The variable subunit is located at the lower part of the molecule.

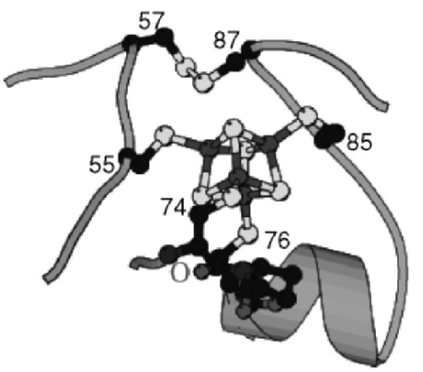


*Fig. 5.* (See also Color Plate 4, p. xxxvii.) Iron center—disulfide structure. The active site cysteines and Pro75 are shown in ball and stick models. Incoming electrons from ferredoxin can pass through proline and the iron center to the disulfide bridge.

constitutes a unique arrangement with the fingerprint $CPCX_{16}CPCX_8CHC$. Both cysteines in the central CPC motif are ligands to the iron-sulfur center while in the other two motifs the liganding cysteines are connected to the redox active cysteines in one CPC and one CHC motif where the second cysteines in these motifs form the disulfide bridge between Cys57 and Cys87. The active site disulfide bridge is in van der Waals contact with the iron center: the sulfur atom of Cys87 is in van der Waals contact to one iron atom, the coordinating sulfur atom of Cys55 and to a sulfide ion of the cluster.

### B. Spectroscopic Investigations of FTR

Spectroscopic investigations of FTR have been important to delineate the properties of the iron-sulfur center and its role in the reaction mechanism. The UV, visible and CD spectra and the absence of an EPR signal are consistent with the presence of an $S = 0$ $[4Fe\text{-}4S]^{2+}$ cluster (Droux *et al.*, 1987; Hirasawa *et al.*, 1988; Schürmann and Gardet-Salvi, 1993). This cluster exhibits unusual redox properties and is redox-inactive over the potential range of $-650$ mV to at least $+300$ mV. Reduction trials with dithionite, dithionite/DTT, dithionite/methyl viologen, and deazaflavin-mediated photoreduction produced no evidence of a paramagnetic ($S = 1/2$ or $3/2$) $[4Fe\text{-}4S]^{+}$ cluster. It was thus concluded that the midpoint potential for the $[4Fe\text{-}4S]^{2+/+}$ couple must be $<-650$ mV (Staples *et al.*, 1996).

It is also very difficult to oxidize the iron-sulfur center to the $[4Fe\text{-}4S]^{3+}$ state. Oxidation to an $S = 1/2$ $[4Fe\text{-}4S]^{3+}$ state by direct electrochemical measurements attributed a potential of +340 mV to the $[4Fe\text{-}4S]^{3+/2+}$ couple (Salamon *et al.*, 1995). Since the redox potentials of the disulfides in FTR and thioredoxins are about $-300$ mV (Hirasawa *et al.*, 1999), it was considered unlikely that this cluster is involved in mediating electron transfer from ferredoxin to the active site disulfide.

A stabilized form of a one-electron-reduced FTR reaction intermediate could be obtained by alkylation of one of the active-site cysteines with N-ethylmaleimide (NEM) with spectroscopic signals of a $S = 1/2$ $[4Fe\text{-}4S]^{3+}$ cluster (Staples *et al.*, 1996, 1998). In this intermediate, the cluster was found to be covalently attached to a cysteine formed by reduction of the disulfide bridge.

Mössbauer spectroscopy revealed weak interaction of the active-site disulfide with one Fe site of the $[4Fe\text{-}4S]^{2+}$ cluster in the resting enzyme and cleavage of the active-site disulfide with concomitant coordination of one of the cysteines to yield a $[4Fe\text{-}4S]^{3+}$ cluster with a five-coordinate Fe site ligated by two cysteine residues in the NEM-modified enzyme (Jameson *et al.*, 2003).

Recent mutational studies on the *Synechocystis* FTR corroborate results obtained with the NEM-modified protein and underline the importance of Cys87 for the stability of the Fe-S cluster (Glauser *et al.*, 2004).

The spectroscopic studies indicate that the function of the Fe-S cluster is more complex than simply transferring an electron from ferredoxin to the disulfide bridge. It can fulfill its task of reducing the disulfide because of the unique structural organization with a very close contact between Fe-S cluster and disulfide bridge.

### C. Mechanism of FTR: Structure-Function

FTR has the task to reduce the disulfide of thioredoxin, which needs two electrons, with an electron donor, ferredoxin, capable of delivering only one electron at a time. It therefore has to either store one electron until a second electron is delivered or it has to provide the second electron by taking it from the Fe-S cluster. Because of the unusual redox-potentials of FTR, the one electron reduction of the protein can not result in a reduction of the Fe-S cluster. It actually leads to an oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster to an $[4Fe\text{-}4S]^{3+}$ cluster and a full reduction of the disulfide (Staples *et al.*, 1996, 1998) (see Fig. 6). In this first step, one electron from the ferredoxin and one electron from the iron-sulfur cluster of FTR are used to cleave the disulfide bridge. In this intermediary state, labeling has shown that only one of the cysteines, Cys57, is a reactive thiol while the second cysteine is protected by the iron-sulfur cluster (Staples *et al.*, 1996).

Two possibilities for this intermediate were originally proposed: (i) a disulfide bridge formed between the cysteine and a sulfide ion in the cluster or (ii) a five-coordinated iron where the fifth ligand should be Cys87. Staples *et al.*, (1998) favored the first possibility, the intermediate formed as a disulfide with a sulfide ion. The main argument for this was that in a chemical reaction, the sulfide ion should be a good nucleophile for the reaction. However, in the structure of FTR, Cys87 of the bridge has a tight interaction to one of the iron atoms of the cluster, which led to the suggestion that Cys87 instead coordinates the iron in the intermediate stage forming a penta-coordinated iron (Dai *et al.*, 2000b). The Mössbauer studies appear to support such a structure (Jameson *et al.*, 2003).

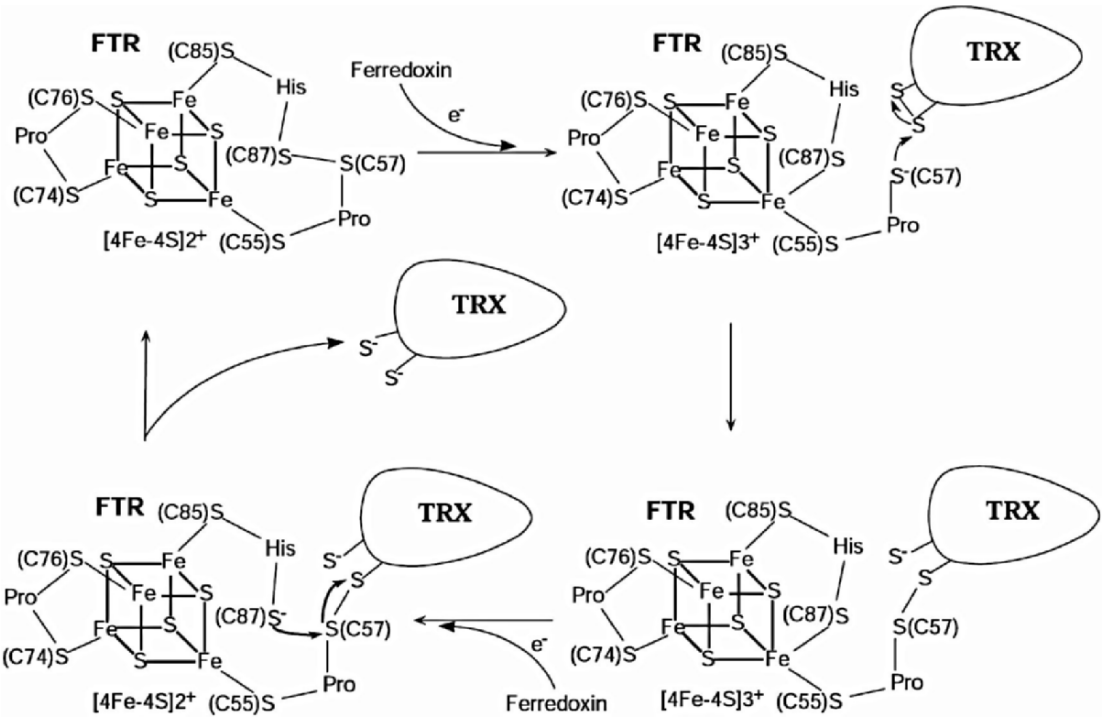


*Fig. 6.* Mechanism of action of FTR. Schematic representation of thioredoxin (TRX) reduction by FTR. The reduction of thioredoxin proceeds via a covalent intermediate between FTR and thioredoxin (lower right) where Cys87 of FTR is coordinating one of the iron atoms of the center. See text for details.

Analogous modified redox potentials have been reported for synthesized penta-coordinated iron sites of [4Fe-4S] clusters (Ciurli *et al.*, 1990; Staples *et al.*, 1996, 1998). A lowering of redox potential by about 350–700 mV is observed for penta-coordinated iron clusters compared to tetra-coordinated iron clusters (Ciurli *et al.*, 1990). Similarly, for the FTR intermediate, the redox potential of the $[4Fe\text{-}4S]^{3+/2+}$ couple is lowered from +420 mV to −210 mV, which is in the same region as the redox potential for the active-site disulfide (Salamon *et al.*, 1995; Staples *et al.*, 1996, 1998; Hirasawa *et al.*, 1999).

In the second step, a transient heterodisulfide is formed between the FTR and thioredoxin by the nucleophilic attack of Cys57 on the disulfide of Trx. The transient reaction intermediate that had been mimicked by the NEM-modified FTR has recently been stabilized using mutant thioredoxins (Glauser *et al.*, 2004).

In the third step, the second electron delivered by a second ferredoxin molecule to the iron-sulfur center reduces it back to its original 2+ oxidation state while Cys87 becomes a nucleophilic thiol that can attack the heterodisulfide bridge between FTR and thioredoxin and thereby release the fully reduced thioredoxin in the forth step. His86 might increase the nucleophilicity of the attacking Cys, a proposal supported by the mutational studies, which show that the H86Y mutant FTR is significantly less active (Glauser *et al.*, 2004). The unique property of the FTR iron-sulfur center to be able to cleave a disulfide appears to rely on the close proximity to the active site disulfide bridge. Cys87 is so close to the iron sulfur center that it does not appear to exist as a free thiol until the final reduction step and interacts during previous steps with the iron-sulfur center. Cys57, on the other hand, at the molecular surface of FTR should be the nucleophile attacking the thioredoxin disulfide in the first step of the reaction.

### *D. FTR Interactions: Complex Formation with Ferredoxin and Thioredoxin*

The FTR heterodimer is a thin, concave, disk-shaped molecule only 10Å across the center of the molecule where the iron-sulfur center is located (Fig. 4). The surfaces on both sides of the molecule, which interact with ferredoxin and thioredoxin, are highly conserved.

The reduction of thioredoxin proceeds via a mixed disulfide between thioredoxin and FTR (Staples *et al.*, 1996, 1998) (Fig. 6). Such mixed disulfides have been stabilized and purified using mutant thioredoxins and

they have been shown to be able to form noncovalent complexes with ferredoxin (Glauser *et al.*, 2004).

Three-dimensional structures of the FTR/Trx-*f* and FTR/Trx-*m* complexes demonstrate that all interactions in the complexes are with the catalytic subunit of FTR. FTR interacts mainly with the Trx's main chain and there are very few interactions between these two proteins (S. Dai, unpublished results). The FTR/Trx *f* complex with a hetero-disulfide bond between Cys57 of FTR and Cys46 of Trx-*f*, solved at high resolution, unambiguously demonstrated a five-coordinated iron-sulfur cluster where the Cys87 of FTR is ligated to an iron atom of the cluster (S. Dai, unpublished data) (Fig. 6). The thioredoxin interaction area (Fig. 7) contains mainly hydrophobic residues and the absence of many charged groups makes the thioredoxin interaction area less specific, which might be important since FTR reduces different thioredoxins present in the cell, as is the case in the spinach chloroplast. Even *Synechocystis* FTR is perfectly capable of reducing spinach Trx-*f*.

The shape of the opposite side is complementary to ferredoxin and ferredoxin can bind non-covalently to FTR (Fig. 7). Together with hydrophobic residues around the Fe-S cluster, positive residues form a docking area for negatively-charged ferredoxin. Due to the presence of these charged residues, the ferredoxin interaction area is specific. A lower affinity between spinach ferredoxin and *Synechocystis* FTR was observed than that of the homologous spinach ferredoxin and FTR couple (Hirasawa *et al.*, 1988; Schwendtmayer *et al.*, 1998).

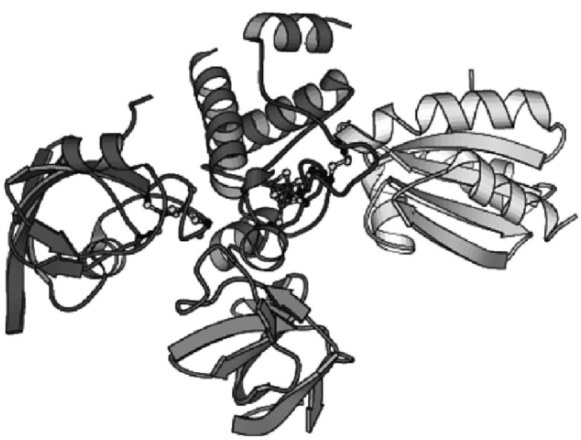

*Fig. 7.* (See also Color Plate 4, p. xxxvii.) FTR interactions with ferredoxin and thioredoxin. FTR (center) is shown interacting with ferredoxin (to the left) and thioredoxin (to the right). The figure is based on preliminary data on binary complexes with ferredoxin and thioredoxin respectively.

Recently, a crystal structure of the complex of *Synechocystis* FTR and ferredoxin has been determined (S. Dai, unpublished results). In this complex, there are no interactions between ferredoxin and the variable subunits of FTR and all intermolecular interactions occur with the catalytic subunit. This is in contrast to the NADP/ferredoxin reductase-ferredoxin complex, where ferredoxin interacts extensively with both the $NADP^+$ domain and the FAD domain (Morales *et al.*, 2000; Kurisu *et al.*, 2001). The interaction surface of the ferredoxin complex with FTR is smaller than the one of the NADP/ferredoxin reductase complex. The iron-sulfur ligands on the ferredoxin side of the disk-shaped FTR molecule (Fig. 7) are connected by Pro75 in a CPC motif (Fig. 5). The main-chain of this motif is exposed towards the ferredoxin side and docking of a ferredoxin to this site should give a short through-bond electron transfer route from the bound ferredoxin to the iron-sulfur center.

An intermediate Trx-complex would cover one of the sides of the flat FTR molecule and the second electron for the reduction should be delivered by the next incoming ferredoxin, which has to dock on the opposite side of the flat disk-like heterodimer (Fig. 7). The short distance through the enzyme from the ferredoxin docking site through the iron-sulfur center to the closely associated Cys87 of the active site disulfide seems ideally suited for its purpose.

## V. Target Enzymes

A number of chloroplast enzymes have been shown to be light-regulated and many of these are regulated by the thioredoxin pathway. Redox-active regulatory cysteines have been located in the primary structure for several target enzymes involved in photosynthetic carbon assimilation. In these cases, the principal activator thioredoxin has been determined (Table 1) (Schürmann and Jacquot, 2000; Schürmann and Buchanan, 2001).

Recent proteomic approaches have revealed new putative targets and confirmed enzymes known to interact with thioredoxins (Motohashi *et al.*, 2001; Balmer *et al.*, 2003). The relatively small number of known thioredoxin-linked proteins (about 16) raised the possibility that others remain to be identified (Balmer *et al.*, 2003). To pursue this opportunity, thioredoxins *f* and *m* were mutated such that the buried cysteine of the active disulfide has been replaced by serine or alanine, and bound to affinity columns to trap target proteins of the chloroplast stroma. The covalently-linked proteins were eluted with DTT, separated on gels, and

*Table 1.* Target enzymes.

| Target Enzyme | Organism | Conserved regulatory cysteines | Means of activation | Structural information | Reference |
|---|---|---|---|---|---|
| NADP-MDH | *Flaveria bidentis*<br>*Sorghum vulgare* | -QKPE<u>C</u>FGVF<u>C</u>LTYD-<br>-TRKD<u>C</u>FGVF<u>C</u>TTYD-<br>24 29<br><br>-AEKK<u>C</u>VAHLTGEGIAV<u>C</u>DLPEDTMLPGEM<br>-AEKK<u>C</u>VAHLTGEGNAY<u>C</u>DVPEDTMLPGEV<br>365 377 | Activated only by reduction of <u>both</u> disulfides by either Trx *f* or Trx *m* | Crystal structures of *F. bidentis* and *S. vulgare* enzymes in oxidized form | Carr *et al.*, 1999<br>Johansson *et al.*, 1999 |
| FBPase | *Pisum sativum* | -E<u>C</u>LPDFGDDSDDNTLGTEEQR<u>C</u>IVNVCQP-<br>153 173 | Activated both by disulfide reduction (Trx *f*) and/or changes in pH and [$Mg^{2+}$] | Crystal structures of the pea enzyme in oxidized form | Chiadmi *et al.*, 1999 |
| PRK | *Spinacia oleracea* | -SG<u>C</u>GKSTFMRRLTSVFGX$_{17}$SDTTTVI<u>C</u>LD<br>16 55 | Activated by Trx *f* | Crystal structures of a homologue without disulfides | Porter *et al.*, 1988<br>Brandes *et al.*, 1996 |
| $CF_1$-ATPase $\gamma$-subunit | *Pisum sativum* | -EI<u>C</u>DINGN<u>C</u>VD-<br>198 204 | Activated by Trx *f* | | Schwarz *et al.*, 1997 |
| SBPase | *Triticum aestivum* | -AS<u>C</u>GGTA<u>C</u>VN-<br>52 57 | Activated by Trx *f* | | Dunford *et al.*, 1998 |
| G6PDH | *Solanum tuberosum* | -<u>C</u>RIDKRED<u>C</u>-<br>149 157 | Inactivated by Trx *m* | | Wenderoth *et al.*, 1997 |
| GAPDH | Spinach | -<u>C</u>KDNPADEE<u>C</u>-<br>349 358 | Activated by Trx *f* | Crystal structures of the non-regulatory A-form | Sparla *et al.*, 2002<br>Fermani *et al.*, 2001;<br>Falini *et al.*, 2003 |
| Rubisco activase | *Arabidopsis thaliana* | -EG<u>C</u>TDPVAENFDPTARSDDGT<u>C</u>VYNF<br>392 411 | Activated by Trx *f* | | Zhang and Portis, 1999 |

identified by mass spectrometry. This approach led to the identification of 15 potential targets that function in ten chloroplast processes not known previously to be thioredoxin-linked. Included are proteins that seem to function in plastid-to-nucleus signaling and in a previously unrecognized type of oxidative regulation. Approximately two-thirds of these targets contained conserved cysteines. Eleven previously unknown and nine confirmed target proteins were identified that are members of pathways known to be regulated by thioredoxin. In contrast to results with individual enzyme assays, specificity for thioredoxin *f* or *m* was not observed on affinity chromatography.

Information on three-dimensional structures that explain the activation process was not available until recently when the three-dimensional structures of the two most studied target enzymes NADP-MDH and pea FBPase were determined (Carr *et al.*, 1999; Chiadmi *et al.*, 1999; Johansson *et al.*, 1999). For the first time, the structural basis for light activation during the final activating steps of the redox cascade was revealed.

### *A. NADP-Dependent Malate Dehydrogenase*

NADP-dependent malate dehydrogenase (NADP-MDH) is found in the chloroplasts of C3 and C4 plants but it is only in certain C4 plants that the enzyme is directly involved in carbon fixation. In C3 plants, this enzyme functions in a shuttle mechanism exporting reducing equivalents in the form of malate from the chloroplast. In C4 plants like maize or sorghum, the chloroplast NADP-MDH is an essential enzyme in a carbon trapping and transport mechanism. $CO_2$ is first assimilated into oxaloacetate by phospho*enol*pyruvate carboxylase and the oxaloacetate is then reduced by MDH to malate. Malate is then transported via plasmodesmata from the mesophyll into the bundle-sheath cells where it is decarboxylated by malic enzyme generating NADPH and $CO_2$. The liberated $CO_2$ is fixed by the enzymes of the Calvin-Benson cycle (Hatch, 1987). This $CO_2$ trapping is strictly controlled since it is highly energy-dependent. For NADP-MDH, this control is accomplished by reversible covalent modification of two redox active disulfide bridges through the ferredoxin/thioredoxin system.

The light-regulated, NADP-dependent, chloroplastic MDHs are totally inactive in the dark and activated by light through the ferredoxin/thioredoxin system (Jacquot *et al.*, 1997; Miginiac-Maslow and Lancelin, 2002). The regulatory disulfide bridges are located in two terminal sequence extensions not present in the extra-chloroplastic, constitutively active NAD-dependent MDHs (Miginiac-Maslow *et al.*, 1997). Both disulfides need to be reduced to fully activate the enzyme. A 40-residue-long, N-terminal extension contains two cysteines that form one of the disulfides (Fig. 8). The C-terminal extension is about 13 residues long and contains one cysteine that forms a disulfide bridge with a cysteine in the last helix in the common MDH core. Both disulfides are on the surface of the protein and are easily accessible for thioredoxin reduction (Carr *et al.*, 1999; Johansson *et al.*, 1999). The mechanism of inactivation is distinctly different for the two extensions. The tip of the C-terminus reaches into the active site and acts like an internal inhibitor of the enzyme whereas the N-terminal extension sits like a wedge between domains in the enzyme, thereby probably inhibiting necessary flexibility for catalysis to occur.

The N-terminal extension is highly flexible and a region of only about 10 residues around the disulfide, which is clearly defined, forms a short $3_{10}$ helix (Fig. 8). The two cysteines in the N-terminal disulfide are separated by four residues and are positioned approximately in the middle of the extension. This extension

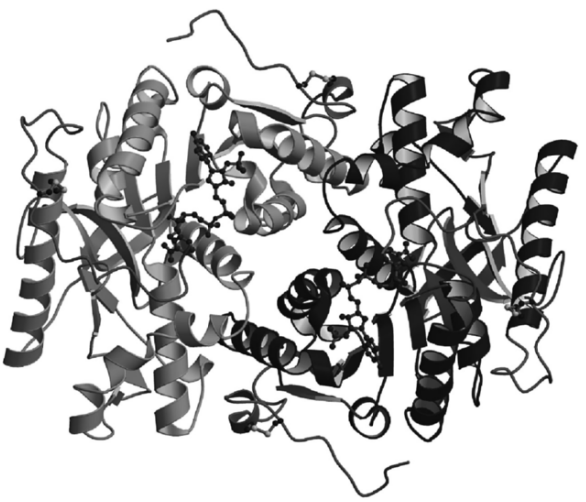

*Fig. 8.* (See also Color Plate 4, p. xxxvii.) MDH structure. The dimeric NADP-dependent MDH from *Flaveria bidentis* (Carr *et al.*, 1999). The two subunits are in lilac and yellow. In red are the N- and C-terminal extensions, specific for all chloroplastic NADP-MDHs. One regulatory disulfide bridge is present in each of the extensions. The N-terminal extensions (top and bottom) sit like wedges between the subunits, thereby locking the domains relative each other. The C-terminal extensions (at the left and right side of the molecule) fold back into the active sites and the disulfides stabilize this conformation. The very C-termini interact with residues of the active site and with the $NADP^+$ (shown in ball and stick models).

is located at the interface between subunits where it makes a number of mainly hydrophobic interactions to both the catalytic domain in one subunit and the coenzyme-binding domain in the other (Fig. 8). The breaking of the N-terminal disulfide bridge would further increase the flexibility of the whole extension and possibly remove it from its wedged position between the domains. The reduction of the disulfide is thought to relax this rigid structure and free the catalytic domain to adopt its active conformation. Mutagenesis of the N-terminal cysteines results in an acceleration of the activation rate suggesting that a rate-limiting slow conformational change follows the breaking of the N-terminal disulfide (Issakidis *et al.*, 1992).

Eleven residues separate the two cysteines of the C-terminal disulfide which is located at the edge of the molecule. When the disulfide is oxidized, access to the active site is prevented due to the C-terminus shielding the entrance and occupying the position of the natural substrate oxaloacetate (Fig. 8). Mutational studies have shown that the negative charges of the last residues are important for this mode of action. When mutating the penultimate glutamate into a glutamine or deleting the last two residues, in combination with elimination of the N-terminal disulfide, a fully active enzyme is obtained (Ruelland *et al.*, 1998). NMR experiments showed that by reduction of the C-terminal disulfide, the C-terminal extension becomes mobile (Krimm *et al.*, 1999).

It has been observed that activation of the enzyme is slowed down by the presence of oxidized $NADP^+$. A molecule of $NADP^+$ is bound in the active site of the *Flaveria* MDH structure. Its positively charged nicotinamide ring interacts with a C-terminal Glu and anchors it more tightly to the active site (Carr *et al.*, 1999), thereby slowing down the reductive activation of the enzyme. When cofactor specificity was changed from NADPH to NADH (Schepens *et al.*, 2000), the inhibitory effect was shifted from $NADP^+$ to $NAD^+$.

The redox potential of the N-terminal disulfide is isopotential with Trx, while the C-terminal disulfide is more electronegative, and would require an excess of reduced Trx to be reduced (Hirasawa *et al.*, 2000). Investigations of the transient formation of mixed disulfides between the target and the reductant demonstrated that the nucleophilic attack of thioredoxin on the C-terminal bridge proceeds through the formation of a disulfide with the most external cysteine (Goyer *et al.*, 2001). Both Trx-*f* and Trx-*m* can reduce the disulfides of MDH *in vitro*. Under certain conditions, Trx-*f* is even more efficient in reducing MDH than Trx-*m*. It has therefore been proposed that Trx-*f* is the prime activator of all light-activated enzymes involved in carbon assimilation and regulated by the ferredoxin/thioredoxin system (Schürmann and Jacquot, 2000; Schürmann and Buchanan, 2001).

### *B. Fructose-1,6-bisphosphatase*

Chloroplast FBPase, which catalyzes the conversion of fructose-1,6-bisphosphate (F-1,6-P) to fructose-6-phosphate (F-6-P) is one of the four light-regulated enzymes of the Calvin-Benson cycle. The structure of spinach chloroplast FBPase was solved in 1995 (Villeret *et al.*, 1995), but no regulatory disulfide was visible in the structure which therefore provided little information on how the oxidized enzyme is inactivated. In the light of the later solved pea chloroplast enzyme structure (Chiadmi *et al.*, 1999) and of its conformational similarity to gluconeogenic FBPases, it was plausible to propose that the spinach enzyme structure might correspond to the reduced, active form of the enzyme.

The structure of the oxidized form of pea FBPase (Fig. 9a) shows the regulatory disulfide bridge and forms the basis for the understanding how the enzyme is reductively regulated (Chiadmi *et al.*, 1999). FBPase contains an amino acid insertion varying from 14–19 residues between species. This insertion contains three conserved cysteines, two of which are involved in the regulatory disulfide. Mutations of these three cysteines result in a partial or total loss of activation (Jacquot *et al.*, 1997; Rodriguez-Suarez *et al.*, 1997; Balmer *et al.*, 2001). From the structure, it is clear that the regulatory disulfide is formed between Cys153 and Cys173 and that the third, Cys178 is located on the buried side of the following helix. Regulation is achieved by switching from an inactive conformation of the active site, stabilized through the regulatory disulfide bridge, to a catalytically competent conformation through reduction of the disulfide bridge. The activation of FBPase is in addition dependent on other light-induced changes in the chloroplasts such as pH and $Mg^{2+}$levels (Zimmermann *et al.*, 1976).

The function of the insertion in activation can be deduced quite well. When the disulfide is oxidized, the insertion is stable, packing directly against two β-strands. This stabilizes the position of the β-strands in the active site. Because of the conformation of two strands the position of a critical Glu ligand for the catalytic divalent Mg prevents the coordination of the divalent ion (Schürmann and Wolosiuk, 1978; Balmer *et al.*, 2001) and occupies part of the substrate site. Upon reduction of the disulfide by thioredoxin, the insertion

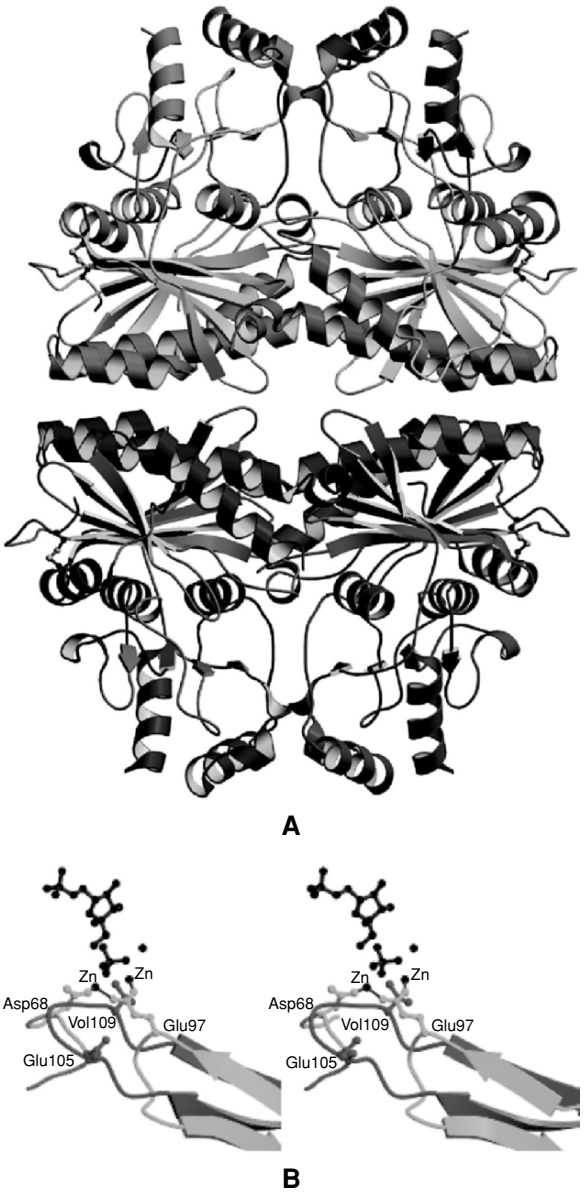


*Fig. 9.* (See also Color Plate 4, p. xxxvii.) FBPase structure. A) Structure of the oxidized tetrameric pea chloroplastic FBPase (Chiadmi *et al*., 1999). The accessible, regulatory disulfides between Cys153 and Cys173 are shown in ball and stick at the outside of the beta-sheet of each subunit.

B) Comparison of the active site area of oxidized pea chloroplastic FBPase and non-redox regulated pig enzyme. The catalytic Glu97 of the pig enzyme is together with Asp68 coordinating two zinc ions. In oxidized pea FBPase the movement of ß-strands has pushed Glu105, corresponding to the zinc coordinating Glu97 in the pig FBPase, out of the active site and positioned Val109 near the location of the cation binding site. Through reduction of the pea enzyme the catalytically important Glu105 is positioned in the active site. Fructose-6-phosphate, Pi and $Zn^{2+}$ are modeled according to the positions in the pig enzyme.

is destabilized, as seen in the spinach structure, and the strands are released. This would allow the strands to move back upon substrate binding, enabling the active site residues to adopt conformations favorable for catalysis, thus yielding a fully active enzyme.

The redox regulation of FBPase is distinctly different from the regulation of NADP-MDH in that the regulatory insertion does not interact directly with the active site, but rather it stabilizes the inactive conformation. This could explain why, under certain *in vitro* conditions, there is still a low residual activity in the oxidized FBPase, compared to oxidized NADP-MDH which is totally inactive. Interestingly, in both enzymes the redox active disulfides are at the surface of the enzyme, remote from the active site, making them accessible to reduced thioredoxin. The signal for activation is conveyed in different ways in the different enzymes to the active sites, which can then adopt their catalytic conformations.

### C. Phosphoribulokinase

The chloroplast enzyme phosphoribulokinase (PRK) is a dimeric molecule that is reversibly inactivated by formation of an intrasubunit disulfide between Cys16 and Cys55 (Brandes *et al.*, 1996). Oxidation leads to a total loss of kinase activity that has been suggested to depend on a combination of masking the sulfhydryl of Cys55 and introducing a conformational constraint as imposed by the disulfide. Comparative kinetics of activation of PRK showed Trx-*m* to be more efficient than Trx-*f* (Geck and Hartman, 2000).

No chloroplast PRK structure has yet been determined so functional considerations have to depend on the structure of *Rhodobacter sphaeroides* PRK, an enzyme that is not regulated by thioredoxin (Harrison *et al.*, 1998). The mechanism by which thioredoxin regulates the eukaryotic enzyme was deduced from the structure of the P-loop. Although the P-loop is disordered in the structure, it was estimated that the α-carbons of two specific residues are within 15 Å of each other. To achieve a disulfide bridge in the eukaryotic enzyme, the two cysteine residues at these positions would need to move at least 5 Å, distorting the P-loop and making it incapable of binding ATP.

### D. Glyceraldehyde-3-Phosphate Dehydrogenase

There are two chloroplastic forms of tetrameric glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and both are composed of two types of subunits: A- and

B-subunits and the forms have either $A_4$ or $A_nB_n$ stoichiometry. The minor non-regulatory $A_4$ variant is constitutively active (Scagliarini *et al.*, 1998) whereas the major regulatory ($A_nB_n$) isoform is redox-regulated by Trx-*f*. A catalytic difference with respect to glycolytic GAPDH, is that photosynthetic GAPDH exhibits dual cofactor specificity toward pyridine nucleotides with a preference for NADP(H) (Falini *et al.*, 2003). The enzyme is thus able to use either NADH or NADPH as a cofactor, but only the NADPH-dependent activity is redox-regulated. The sequence identity between A and B-subunits is 80%, but the B subunits have a 28 residues C-terminal extension. Two conserved cysteines that have been shown to be implicated in the redox regulation are present in the extension which acts as an autoinhibitory domain regulated by thioredoxin and NAD (Qi *et al.*, 2001; Sparla *et al.*, 2002).

The three-dimensional structures of the NADP and NAD complexes of the constitutively active $A_4$ tetramer have been determined (Falini *et al.*, 2003; Fermani *et al.*, 2001). The overall structures are very similar to that of other known glycolytic GAPDH structures. When a regulatory disulfide between Cys-349 and Cys-358 of the C-terminal extension is formed, there is a NAD-dependent association into higher oligomers and inhibition of the NADPH activity leading to GAPDH autoinhibition.

The regulatory mechanism of photosynthetic GAPDH could be correlated to the physiology of the chloroplast under varying photosynthetic conditions (Sparla *et al.*, 2002). In light, the C-terminal disulfide of GAPDH is reduced by Trx-*f* resulting in both increase of the $k_{cat}$ of the NADPH-dependent reaction and the reduction of the activation constant for the substrate 1,3-bisphosphoglycerate (Baalmann *et al.*, 1995). This induces a GAPDH-active state and 1,3-bisphosphoglycerate-dependent dissociation of oligomeric GAPDH into active tetramers. It was suggested that NAD-dependent inactivation of GAPDH may be a major mechanism of dark regulation, in addition to the redox state of the thioredoxin pool (Sparla *et al.*, 2002). In the dark, the enzyme is inactivated and associates when NAD substitutes NADP at the coenzyme-binding site. The stromal NADP(H)/NAD(H) ratio in spinach leaves is known to shift from a value of 4–5 in the light to 0.6–0.7 in the dark (Heineke *et al.*, 1991).

Regulation of GAPDH activity by the CP12 as well as by NAD and 1,3-bisphosphoglycerate has been reported (Wedel and Soll, 1998; Lebreton and Gontero, 1999; Lebreton *et al.*, 2003). These factors have an effect on the oligomerization state of the enzyme and on its association with phosphoribulokinase. CP12 is an 8.5-kDa nuclear-encoded chloroplast protein, isolated from higher plants. It forms part of a core complex of two dimers of PRK, two tetramers of GAPDH and CP12. It has been shown that oxidized, but not reduced, CP12 acts as a linker in the assembly of the complex (Graciet *et al.*, 2003).

## VI. Conclusions and Perspectives

Light does not only provide NADPH and ATP for the photosynthetic carbon assimilation, but it also regulates the activity of key photosynthetic enzymes through reduction-oxidation of protein disulfides. Reduced ferredoxin produced during light by photosystem I reduces chloroplast thioredoxins in a reaction catalyzed by the enzyme ferredoxin:thioredoxin reductase (FTR). Thereby, the redox signal from the photosystem is transmitted to target enzymes. The key electron/thiol transducer enzyme FTR in this pathway is unique in that it can reduce its disulfide bridge directly using an iron-sulfur cluster. The structure of FTR is an unusually thin, concave disk-like molecule that is easily accessible from one side by a ferredoxin, which can transfer an electron to the disulfide via the iron-sulfur center to a thioredoxin on the opposite side. Since complete reduction of thioredoxin needs two electrons, a one-electron reduced intermediate, with a unique five coordinated iron-sulfur center and a FTR-thioredoxin mixed disulfide is formed.

The three-dimensional structures of the target enzymes NADP-dependent malate dehydrogenase and fructose-1,6-bisphosphatase demonstrate that reduction of the regulatory disulfides, which are not part of the active sites, triggers conformational changes having direct consequences on the active-site structure and its catalytic capacity. The molecular mechanism for thioredoxin activation of its targets seems to be different in each case, adapted to the reaction catalyzed by the enzyme. More structures of thioredoxin-regulated enzymes are therefore needed to fully understand the various mechanisms by which light modulates the catalytic capacity of the different target enzymes.

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# Chapter 12

# Chlororespiratory Pathways and Their Physiological Significance

Peter J. Nixon*
*Wolfson Biochemistry Building, Division of Biology, Imperial College London, S. Kensington campus, London SW7 2AY, UK*

Peter R. Rich
*Glynn Laboratory of Bioenergetics, Department of Biology, University College London, Gower Street, London WC1E 6BT, UK*

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## Summary

There is now overwhelming evidence that the thylakoid membrane of green plants contains, in addition to the photosynthetic electron-transfer complexes, a set of respiratory complexes that are capable of reducing and oxidising the plastoquinone pool. These "chlororespiratory" enzymes include the Ndh complex, which is related to complex I found in mitochondria and eubacteria, and the plastid terminal oxidase (PTOX), which is a distant member of the family of alternative oxidases found in mitochondria. In addition the molecular basis of other chlororespiratory activities, including the long sought after ferredoxin:plastoquinone reductase (FQR), implicated in cyclic electron flow around photosystem one, are now being uncovered using a combination of genetics, biochemistry

*Author for correspondence, email: p.nixon@imperial.ac.uk

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and bioinformatics. Here we assess the possible components of the various chlororespiratory pathways and discuss their potential physiological importance. The emerging picture suggests that the main role of chlororespiratory enzymes, at least in mature chloroplasts, is not actually to participate in a classical respiratory chain to drive ATP synthesis. Instead, chlororespiratory activities play important auxiliary roles in various aspects of photosynthesis including cyclic electron flow around photosystem one, carotenoid biosynthesis and photoprotection. The detection of chlororespiratory enzymes in non-photosynthetic plastids suggests that their activities are not restricted to the chloroplast.

## I. Introduction

The first suggestions that respiratory enzymes might co-exist with photosynthetic electron transfer chains in thylakoid membranes arose from the recognition that the algal thylakoid plastoquinone (PQ) pool could still undergo redox changes in the dark in response to changes of physiological conditions (Goedheer, 1963). This led to the notion that thylakoid membranes might possess reductase and oxidase pathways that are distinct from related mitochondrial activities. This idea gained support with the isolation of an NADH:plastoquinone oxidoreductase that was thought to have been present in the thylakoid fraction of *Chlamydomonas* (Godde and Trebst, 1980; Godde, 1982). Bennoun (1982) was the first to postulate that distinct reductases and oxidases might interact directly with the PQ pool in *Chlamydomonas* thylakoids and coined the term chlororespiration to distinguish algal thylakoid dehydrogenases and oxidases from their mitochondrial counterparts. The notion of respiratory-like activities in thylakoids was strengthened later, in particular by the identification of 11 open reading frames (*ndhA-ndhK*) in the higher plant chloroplast genome coding for homologues of protein components of mitochondrial complex I (Ohyama *et al.*, 1988) (Table 2) and by the identification of a thylakoid homologue of the mitochondrial alternative quinol oxidase (Carol and Kuntz, 2001) (Table 1).

Analysis of chlororespiratory activities is complicated by the fact that respiratory and photosynthetic electron transfer chains interact through cytosolic redox and phosphate potentials. For example, increased glycolysis (which occurs primarily in the chloroplast stroma in *Chlamydomonas*) leads to stromal NADPH (Kow *et al.*, 1982; Rebeille and Gans, 1988), which can reduce plastoquinone *via* ferredoxin, FNR and, possibly, other pathways (Bendall and Manasse, 1995). Such interactions complicate the determination of the number, nature and capacities of other possible chlororespiratory pathways and, as a result, their numbers and biochemical natures have remained controversial.

New suggestions for the presence of various dehydrogenase and oxidase activities in both higher plant and algal thylakoids continue, increasing the numbers and types of proposed components. Nevertheless, a consistent view has yet to emerge on principle electron transfer components and their relative contributions to the observed rates of dark reduction and oxidation of plastoquinone. Figure 1 summarises the major proposed pathways by which PQ might be oxidised and reduced in the dark. The purpose of this article is to review such pathways and assess their possible physiological significance.

## II. Analyses of *Arabidopsis* and *Chlamydomonas* Genomes for Viable Candidate Components

The full and partial genome sequences, respectively, of *Arabidopsis thaliana* (http://www.arabidopsis.org/) and *Chlamydomonas reinhardti* (http://genome.jgi-psf.org/chlre2/chlre2.home.html) have provided a tremendous resource for assessing the feasibility of thylakoid-located respiratory homologues. An initial survey of the emerging *A. thaliana* genome for such information was carried out by Dr. N. Fisher (University College, London) and was summarized by Rich *et al.* (2001). The present analyses extend this initial survey.

Table 1 summarises the results of searches for possible thylakoid homologues of well-known respiratory reductases and oxidases in *A. thaliana*. In all cases, sequences were obtained in FASTA format from the

*Abbreviations:* AOX – alternative oxidase; FNR – ferredoxin:NADP$^+$ reductase; Fp – flavoprotein; FQR – ferredoxin:plastoquinone reductase; ISP – iron-sulphur protein; Ndh – complex composed of *ndh* gene products found in plastids; NDH-1 – complex composed of *ndh* gene products found in cyanobacteria; NDH-2 – single subunit type 2 NAD(P)H dehydrogenases; ORF – open reading frame; PET – photosynthetic electron transport; PQ – plastoquinone; PS I – photosystem I; PSII – photosystem II; PTOX – plastid terminal oxidase; qE – energy-dependent component of non-photochemical quenching.

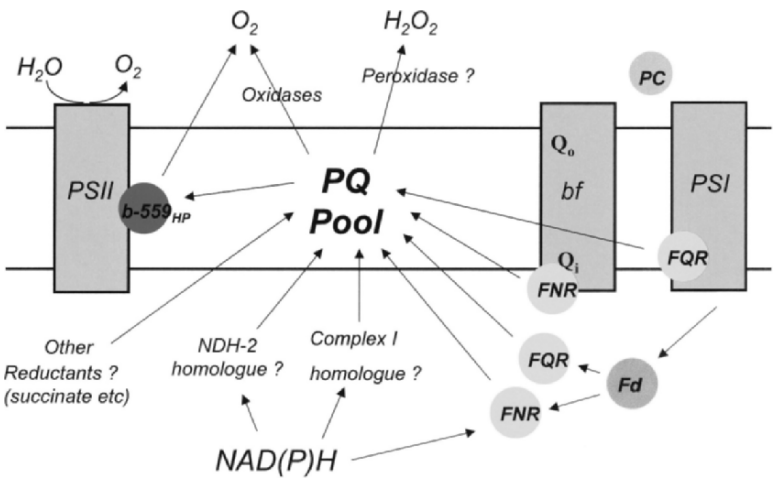


*Fig. 1.* Possible pathways for dark reduction and oxidation of plastoquinone. The figure summarises the diverse pathways that have been proposed to play a role in the dark pathways of reduction and oxidation of the plastoquinone pool in higher plant and algal thylakoids. Dark pathways of reduction include pathways *via* stromal or *bf*-bound FNR, stromal FQR, a direct site on PSI for plastoquinone reduction and various dehydrogenases including homologues of complex I, NDH-2 and succinate dehydrogenase. Routes of plastoquinol oxidation include various oxidase homologues, especially the immutans homologue of alternative oxidases, a plastoquinol peroxidase and the PSII-associated high-potential cytochrome *b*-559 (b-559 $_{HP}$). Details of these components are given in the text.

SWISS-PROT database at http://us.expasy.org/srs5/ and TBLASTN was used to interrogate the *A. thaliana* genome and EST databases. If not already specified in the database, signal sequence prediction and target analysis were performed using TargetP (www.cbs.dtu.dk/services/TargetP/). The table shows the seven homologues of NDH-2 that were originally identified (Rich *et al.*, 2001), together with a less well conserved homologue (At5g08740 gene product) that was first noted by Peltier and Cournac (2002) as a possible chloroplast component.

Two genes for each of the three subunits of succinate dehydrogenase were identified, although both flavoprotein subunits appear to be mitochondrially-targeted. The table also clearly rules out the possibility that there is a thylakoid version of cytochrome *c* oxidase, or equivalents of bacterial cytochromes *bo* and *bd*. However, there is a chloroplast-targeted homologue of the well-documented "IMMUTANS" quinol oxidase protein, in addition to five homologues of mitochondrial alternative oxidases.

Table 2 provides an analysis of the "core" mitochondrial complex I subunits in *A. thaliana*. These are the 14 subunits that are common to all analysed forms of complex I (Fearnley and Walker, 1992), but do not include the many additional subunits, mostly of unknown function, that are associated with eukaryotic forms of the enzyme (Hirst *et al.*, 2003). These are divided into three groups: the seven proteins that comprise the hydrophobic, membrane-spanning arm (ND-1 through ND-6 in bovine terminology), the three subunits (24, 51 and 75 kDa subunits) that provide the NADH dehydrogenase module and four hydrophilic subunits (PSST, TYKY and 49 and 30 kDa subunits) that provide a linkage between them. In this case the *A. thaliana* genome was interrogated with the sequences from the *P. denitrificans* genes (NQO1-14) and this allowed the majority of mitochondrial complex I core subunits to be identified. All, except TYKY, had only a single nuclear/mitochondrial DNA copy. In addition, as already known, homologues of all 11 hydrophobic and linker proteins were also present in the chloroplast genome (Ohyama *et al.*, 1988), but with the notable absence of genes for the three proteins that comprise the NADH dehydrogenase module.

Table 3 provides a survey of the available *Chlamydomonas* genome for equivalent components. In this case, the situation is roughly comparable to that in higher plants, but with several notable exceptions. Firstly, no complex I subunit homologues are encoded at all in the chloroplast DNA and it seems clear that there are sufficient nuclear genes only for components of the mitochondrial complex I. Interestingly, a homologue of the *A. thaliana* NDH-2 homologue that may be targeted to chloroplasts is present, as are two homologues of the "IMMUTANS" protein.

These findings are discussed below in the context of specific possible chlororespiratory pathways.

*Table 1.* Bioinformatic analysis of possible chlororespiratory components in the *Arabidopsis thaliana* genome. The genome sequence of *Arabidopsis thaliana* at http://www.arabidopsis.org/ was interrogated using TBLASTN with sequences obtained from the SWISS-PROT database at http://us.expasy.org/srs5/. Types of possible respiratory components searched were: NDH-2, the mitochondrial single subunit flavoprotein NADH-ubiquinone oxidoreductases that face the matrix (int) or intermembrane space (ext); Succinate DH, mitochondrial succinate dehydrogenase; Cyt *c* oxidase, mitochondrial cytochrome *c* oxidase (complex IV); Cyt *bo* and *bd* oxidases, two principal bacterial terminal respiratory ubiquinol oxidases; alternative oxidase, alternative ubiquinol oxidase found in higher plant, algal and yeast mitochondria. Targeting analyses refer to C, chloroplast, M, mitochondrial, S, secretory vesicle. EF hand motif indicates a calcium-binding module. Percent (%) values refer to sequence identity to search sequence. ESTs, number of expressed sequence tags; n.d, none detected

| Protein | SWISS-PROT search sequence | *A. thaliana* genes | Locus | Target | # ESTs | Notes |
|---|---|---|---|---|---|---|
| NDH-2 | Q9ST63_SOLTU | At5g08740 | Chromosome 5 | C ? | | 23/25% identity with potato int/ext NDH-2 |
| | Q9ST62_SOLTU | At3g44190 | Chromosome 3 | S ? | 2 | 36/27% identity with potato int/ext NDH-2 |
| | | At4g28220 | Chromosome 4 | M | 1 | 38/78% identity with potato int/ext NDH-2;EF hand motif |
| | | At2g20800 | Chromosome 2 | M | 1 | 37/59% identity with potato int/ext NDH-2;EF hand motif |
| | | At4g21490 | Chromosome 4 | S ? | 2 | 36/62% identity with potato int/ext NDH-2 |
| | | At2g29990 | Chromosome 2 | M | 2 | 72/39% identity with potato int/ext NDH-2 |
| | | At1g07180 | Chromosome 1 | M | 1 | 71/38% identity with potato int/ext NDH-2 |
| | | At4g05020 | Chromosome 4 | n.d. | 1 | 38/61% identity with potato int/ext NDH-2;EF hand motif |
| Succinate DH | | | | | | |
| Cyt b subunit | C560_MARPO | At5g09600 | Chromosome 5 | M | 29 | 42% identity with liverwort *b*-560 |
| | | At4g32210 | Chromosome 4 | — | — | 42% identity with liverwort *b*-560 |
| Flavoprotein subunit | DHSA_ARATH | At5g66760 | Chromosome 5 | M | 11 | |
| | | At2g18450 | Chromosome 2 | M | 1 | 87% identity with AT5g66760 |
| Iron-sulphur subunit | DHSB_ECOLI | At5g40650 | Chromosome 5 | M | 7 | 54% identity with *E. coli* iron-sulphur subunit |
| | | At3g27380 | Chromosome 3 | — | 12 | 53% identity with *E. coli* iron-sulphur subunit |
| Cyt *c* oxidase | | | | | | |
| Subunit I | COX1_ARATH | | mtDNA cox1 | M | | Single mtDNA copy only |
| Subunit II | | | mtDNA cox2 | M | | Single mtDNA copy+103bp N-term fragment on mt ORF291 |
| Subunit III | | | mtDNA cox3 | M | | 98% identity with mt cox3, unknown function |
| | | At2g07687 | Chromosome 2 | | | |
| Cyt bo oxidase | | | | | | |
| Subunit I | CYOB_PSUPU, | — | | — | — | Using *E. coli* or *P. putida* sequences:- |
| | CYOB_ECOLI | — | | — | — | Strong similarity with mt coxI |
| Subunit II | CYOA_PSUPU | — | | — | — | Weak similarity with mt coxII |
| | CYOA_ECOLI | — | | — | — | |
| Cyt bd oxidase | | — | | — | — | |
| Subunit I | CYDA_ECOLI | — | | — | — | No matches |
| Subunit II | CYDB_ECOLI | — | | — | — | No matches |
| Alternative oxidase | AOX1A_ARATH | At3g22370 | Chromosome 3 | M | 7 | alternative oxidase 1a precursor |
| | | At3g22360 | Chromosome 3 | M | n.d. | alternative oxidase 1b precursor (78% identity with 1a) |
| | | At3g27620 | Chromosome 3 | M | n.d. | alternative oxidase 1c precursor (76% identity with 1a) |
| | | At5g64210 | Chromosome 5 | M | n.d. | alternative oxidase 2 (71% identity with 1a) |
| | | At1g32350 | Chromosome 1 | M | n.d. | oxidase like protein (75% identity with 1a) |
| | | At4g22260 | Chromosome 4 | C | 13 | IMMUTANS protein (29% identity with 1a) |

*Table 2*. Bioinformatic analysis of homologues of core subunits of complex I in the *Arabidopsis thaliana* genome. The genome sequence of *Arabidopsis thaliana* at http://www.arabidopsis.org/ was interrogated using TBLASTN with protein sequences of *P. denitrificans* mitochondrial complex I subunits obtained from the SWISS-PROT database at http://us.expasy.org/srs5/. Percent (%) values refer to sequence identity with search sequence. UQ site, possible locus of ubiquinone binding site; N1a,1b,2-6, possible loci of binding motifs for specific iron-sulphur centres; FMN, binding site for flavin mononucleotide

| Bovine subunits | Possible location of redox binding sites | *E.coli* equivalents | *P. denitrificans* equivalents | *Arabidopsis* nuclear/mitochondrial encoded subunits | *Arabidopsis* nuclear/chloroplast encoded Ndh subunits |
|---|---|---|---|---|---|
| Hydrophobic arm | | | | | |
| ND-1 | UQ site? | NuoH | NQO8 | — | NdhA (36%) |
| ND-2 | — | NuoN | NQO14 | — | NdhB (37%)<br>ndhB-01 (37%) |
| ND-3 | — | NuoA | NQO7 | nad3 (48%) | NdhC (39%) |
| ND-4 | UQ site? | NuoM | NQO13 | nad4 (47%) | NdhD (32%) |
| ND-4L | | NuoK | NQO11 | nad4L (54%) | NdhE (44%) |
| ND-5 | UQ site? | NuoL | NQO12 | nad5 (48%) | NdhF (44%) |
| ND-6 | — | NuoJ | NQO10 | nad6 (40%) | NdhG (27%)<br>NdhL (At1g70760)[a] |
| NADH dehydrogenase | | | | | |
| 24 kDa | N1a | NuoE | NQO2 | At4g02580 (36%) | — |
| 51 kDa | FMN, N3 | NuoF | NQO1 | At5g08530 (69%) | — |
| 75 kDa | N1b, N4, N5 | NuoG | NQO3 | At5g37510 (48%) | — |
| Hydrophilic arm | | | | | |
| PSST | N2;UQ site? | NuoB | NQO6 | At5g11770 (81%) | NdhK (48%) |
| TYKY | N2;UQ site? | NuoI | NQO9 | At1g16700 (75%)<br>At1g79010 (75%) | NdhI (33%) |
| 49 kDa | N2;UQ site? | NuoCD | NQO4 | nad7 (58%) | NdhH (40%) |
| 30 kDa | | NuoCD | NQO5 | nad9 (53%) | NdhJ (35%)<br>NdhM (At4g37925)<br>NdhN (At5g58260)<br>NdhO (At1g74880) |

[a]Predicted from analysis of the NDH-1 complex of *Synechocystis* 6803 (Battchikova *et al*., 2005).

## III. Overview of Proposed Pathways

### *A. Assessment of Possible Pathways of Plastoquinone Reduction*

#### *1. Ferredoxin-Plastoquinone Reductase (FQR) and FNR*

In intact chloroplasts of both higher plants and algae, dark reduction by stromal reductants of intersystem components after photo-oxidation can be quite rapid; an oxidized PQ pool can be reduced within seconds and P700 re-reduction is even faster. These rates, and the steady-state dark reduction level of PQ, are dependent upon the metabolic history of the cells. In addition, the rate becomes much faster when respiratory oxidases are inhibited and leads to substantial steady state PQ pool reduction in the dark (Rich *et al.*, 1998). Inhibition of mitochondrial metabolism will in general elevate the mitochondrial NADH level and this will cause increased stromal NADPH, mediated by, e.g., the malate/oxaloacetate couple which equilibrates with both pools *via* NAD(P)H-dependent malate dehydrogenases (Peltier and Cournac, 2002). It is this elevated stromal NADPH that presumably accounts for the increased level and rate of reduction of PQ and other intersystem components that can occur in dark-adapted leaves and cells when mitochondrial electron transfer is inhibited (Rich *et al.*, 1998).

The number of pathways by which NAD(P)H reduces intersystem components remains unresolved. However, direct measurements of rates of reduction by added NAD(P)H in isolated thylakoids show that the fastest pathway occurs with NADPH *plus* ferredoxin (Rich *et al.*, 1998). The NADH-supported rate is slow and independent of ferredoxin. Significantly, neither process is drastically inhibited by inhibitors of mitochondrial complex I, suggesting that neither involves primarily a PQ binding site structurally related to that of complex I. Hence, a major pathway for PQ pool reduction in the dark is *via* NADPH and ferredoxin.

This pathway is presumably common to the pathway of cyclic electron transfer around photosystem I

*Table 3.* Bioinformatic analysis of possible chlororespiratory components in the *Chlamydomonas reinhardtii* genome. The partial genome sequences of *Chlamydomonas reinhardti* at http://genome.jgi-psf.org/chlre2/chlre2.home.html was interrogated with TBLASTN using protein sequences obtained from the SWISS-PROT database at http://us.expasy.org/srs5/. Because of partial sequences and interruptions with introns, percent (%) identity can not be given precisely and, instead, the sequence similarity is summarised as weak (<25% identity), medium or strong (>70% identity)

| Protein | *A. thaliana* gene products used for search | *C. reinhardtii* genes loci | Notes |
|---|---|---|---|
| Mitochondrial complex I | *nad3* | Scaffold 130 | Weak similarity with ND3 subunit |
| | *nad4,4L,5,6* | None | No nuclear or chloroplast homologues |
| | At5g11770 (PSST) | Scaffold 74 | Strong similarity to PSST |
| | At1g16700/At1g79010 (TYKY) | Scaffold 101 | Strong similarity to TYKY |
| | At5g08530 (51kDa) | Scaffold 26 | Strong similarity to 51kDa |
| | At4g02580 (24kDa) | Scaffold 32 | Strong similarity to 24kDa |
| | At5g37510 (75kDa) | Scaffold 91 | Strong similarity to 51kDa |
| Plastid "complex I" | *ndhA,B,C,D,E,F,G,H,I,K* *ndhJ* | Scaffold 45 | No homologues except mitochondrial complex I subunits. Weak similarity to NdhJ (30-kDa subunit) |
| NDH-2 | At5g08740 (possible plastid NDH-2) | Scaffold 82 | Medium identity |
| | At4g28220 (mito ext NDH-2) | Scaffold 117 | Strong similarity to int/ext NDH-2 |
| | | Scaffold 31 | Strong similarity to int/ext NDH-2 |
| | At2g29990 (mito int NDH-2) | Scaffold 595 | Strong similarity to int/ext NDH-2 |
| | | Scaffold 189 | Strong similarity to int/ext NDH-2 |
| Succinate DH (SDH) Cyt b subunit | At5g09600 | None | No close relatives found |
| | At4g32210 | None | No close relatives found |
| SDH Flavoprotein Subunit | At5g66760 | Scaffold 24 | Strong similarity to SDH Fp subunit |
| | At2g18450 | Scaffold 38 | Medium similarity to SDH Fp subunit |
| SDH Iron-sulphur protein | At5g40650 | Scaffold 20 | Strong similarity to SDH ISP subunit |
| | At3g27380 | | |
| Cyt *c* oxidase Subunit I | Cox1 | None | No nuclear homologues |
| Alternative oxidase 1a precursor | At3g22370 | Scaffold 33 | Strong similarity to AOX |
| | | Scaffold 34 | Strong similarity to AOX |
| IMMUTANS | At4g22260 | Scaffold 10 | IMMUTANS homologue |
| | | Scaffold 46 | IMMUTANS homologue |

(PSI) and is mediated by one or more ferredoxin:PQ oxidoreductases (FQRs). However, the details of the linkage between NADPH, ferredoxin and the plastoquinone pool have remained enigmatic (Bendall and Manasse, 1995). Because cyclic electron transfer is sensitive to antimycin A (Tagawa *et al.*, 1963), and antimycin A is known to tightly inhibit one of the ubiquinone binding sites (the $Q_i$ site of respiratory *bc* complexes—Slater, 1973), it was naturally assumed initially that reduced ferredoxin would reduce PQ *via* the equivalent $Q_i$ site of the homologous thylakoid cytochrome *bf* complex. However, it became clear later that the $Q_i$ site of the cytochrome *bf* complex is insensitive to antimycin A (Moss and Bendall, 1984; Rich, 1984) and that the antimycin-sensitive site must be elsewhere, probably associated with a loosely bound component in a PSI fraction (Bendall and Manasse, 1995). More recently, a motif that resembles part of the antimycin-sensitive, quinone-binding site of *bc* complexes has been identified on the photosystem I core PsaA/B subunits (Fisher and Rich, 2000), raising a further possibility for the location of this enigmatic site. Nevertheless, at present the exact nature and location of this FQR pathway remains unresolved, as do questions as to whether multiple pathways may exist (Binder and Selman, 1980). A significant step forward has come with the isolation of the *pgr5* mutant of *A. thaliana*, which lacks the antimycin-sensitive FQR activity (Munekage *et al.*, 2002). PGR5, which is a 10-kDa protein attached to the thylakoid membrane, does not appear to bind redox-active cofactors, so its role in electron transfer appears indirect.

In providing a dark pathway for reduction of plastoquinone, clearly FNR must provide the connection from NADPH to ferredoxin. However, it is known that a fraction of the FNR is membrane-bound and it has been suggested that this membrane-bound form could provide the FQR activity. Evidence for and against such a role in cyclic electron transfer has been reviewed by Bendall and Manasse (1995). More recently

however, a role for membrane-bound FNR has received support with the co-purification of a stoichiometric FNR-cytochrome *bf* complex from spinach thylakoids (Zhang *et al.*, 2001). It was demonstrated that NADPH could reduce the cytochrome *b* component in the presence of added ferredoxin and, although the rate of this process was extremely low, the authors concluded that this provided evidence for a role of FNR in the ferredoxin-dependent cyclic pathway. The idea that the cytochrome *bf* complex does indeed provide the site for plastoquinone reduction by the cyclic route has gained further support from the intriguing finding from crystallographic studies that the $Q_i$ sites of algal and cyanobacterial cytochrome *bf* complexes contain an additional haem group close to the high potential haem $b_H$ site that is absent from the homologous cytochrome *bc* complexes (Kurisu *et al.*, 2003; Stroebel *et al.*, 2003) and that might provide the elusive FQR site. How PGR5 might be involved in such a scenario is uncertain.

## 2. Mitochondrial Complex I Homologue

The first clear indication that chloroplasts might contain a homologue of the mitochondrial type I NADH:quinone oxidoreductase (also known as complex I) came with the identification of 11 plastid-encoded open reading frames (annotated *ndhA-K*) with significant sequence similarities to subunits of mitochondrial and eubacterial complex I (Ohyama *et al.*, 1988) (Table 2). Although widespread, the plastid *ndh* genes are absent in a number of species (Peltier and Cournac, 2002), including somewhat ironically the green alga, *Chlamydomonas reinhardtii*, which provided much of the early evidence for a thylakoid type I NADH dehydrogenase (Godde and Trebst, 1980; Godde, 1982).

Sazanov and colleagues showed subsequently that several of the chloroplast *ndh* gene products were components of a 550-kDa complex (so-called Ndh complex), located in the non-appressed region of the thylakoid membrane, which co-purified with an NADH-specific dehydrogenase activity (Sazanov *et al.*, 1998). Ferricyanide and a range of quinones were able to act as electron acceptors from the isolated complex; both NADH and deamino-NADH could be utilised as donor substrates (Sazanov *et al.*, 1998). It is unclear whether traditional complex I inhibitors such as rotenone also inhibit the Ndh complex, despite their wide use as inhibitors in studies on chloroplasts. Although there has been and continues to be uncertainty about its substrate specificity (Nixon, 2000), recent Ndh preparations isolated either by immunopurification (Casano *et al.*, 2000) or His-tagging methods (Rumeau *et al.*, 2005) all display a preference for NADH over NADPH.

In an interesting development, three nuclear-encoded subunits, designated NdhM, NdhN and NdhO, have been identified (Prommeenate *et al.*, 2004; Rumeau *et al.*, 2005) (Table 2). Orthologues are found in the closely related cyanobacterial NDH-1 complex, but are absent in the mitochondrial and eubacterial complex I (Prommeenate *et al.*, 2004; Battchikova *et al.*, 2005). There are, however, some limited sequence similarities between cyanobacterial NdhM and subunit B13, which is a supernumerary subunit of mitochondrial complex I (Prommeenate *et al.*, 2004). The NdhL subunit which is also found in the cyanobacterial complex, is predicted to have a homologue in the plastid Ndh complex (Battchikova *et al.*, 2005) (Table 2).

Complex I in eubacteria and mitochondria is composed of three different structural modules: an electron-input device involved in NADH-binding and oxidation, an interconnecting hydrophilic arm involved in electron transfer and a hydrophobic sub-complex possibly involved in proton translocation and ubiquinone binding (Friedrich *et al.*, 1995). By analogy, the plastid NdhA-G subunits would form the hydrophobic module and the NdhH-K subunits would form the interconnecting fragment (Table 2). As yet the identity of the subunit(s) involved in NADH oxidation is unknown, despite intensive investigations.

Immunoblotting experiments have raised the possibility that at least in oat (Quiles *et al.*, 2003) and potato (Rasmusson *et al.*, 1998) some of the "missing" Ndh plastid proteins are structurally related to the plant mitochondrial NADH-binding proteins of complex I. In the case of *A. thaliana*, however, the published genome sequence rules out the presence of a separate set of chloroplast-targeted homologues (Table 2). Dual targeting of a single gene product to both the chloroplast and mitochondrion remains possible, but attempts to show this have so far been unsuccessful (Grohmann *et al.*, 1996; Rasmusson *et al.*, 1998). The newly discovered NdhM, N, and O subunits lack obvious NADH- and other cofactor-binding sites and so are unlikely to be the "missing" subunits. Instead these proteins appear to be part of the hydrophilic arm and might play a role in binding the so-far uncharacterised electron-input module (Prommeenate *et al.*, 2004; Battchikova *et al.*, 2005; Rumeau *et al.*, 2005).

One interesting possibility is that the NDH complexes found in cyanobacteria and chloroplasts are, from an evolutionary standpoint, more closely related to the complex I homologues found in archaebacteria

than in eubacteria and mitochondria. Like the thylakoid NDH complexes, the archaebacterial complexes possess homologous subunits to the membrane and interconnecting fragments, but instead of the classical module involved in NADH-binding and oxidation, they contain a single subunit that oxidises coenzyme $F_{420}H_2$, a 5-deazaflavin derivative (Bäumer *et al.*, 2000; Brüggemann *et al.*, 2000). Intriguingly, there are homologues of this subunit predicted from analysis of the genome sequences of cyanobacteria and plants (Prommeenate *et al.*, 2004), but their involvement in Ndh function has not yet been assessed.

Despite recent advances, the chloroplast Ndh complex has still not yet been purified to homogeneity, in large part because of its low abundance and instability (Sazanov *et al.*, 1998). Thus the presence of a contaminating NADH dehydrogenase cannot be totally dismissed. If so, it is still feasible that the chloroplast and cyanobacterial NDH-1 complexes lack an attached electron-input module and perhaps function as FQRs (Friedrich *et al.*, 1995; Prommeenate *et al.*, 2004). Indeed, all the *Synechocystis* 6803 NDH-1 complexes studied so far are composed solely of the membrane and hydrophilic fragments (Prommeenate *et al.*, 2004; Battchikova *et al.*, 2005). Also, *in vitro* measurements using various tobacco *ndh* mutants support a role for the Ndh complex in ferredoxin-mediated reduction of the plastoquinone pool (Endo *et al.*, 1998; Munekage *et al.*, 2004). However, in both cases it could be argued that the electron-input module is lost upon biochemical manipulation. The use of a His-tagging approach to purify the intact Ndh complex is a promising route to clarify this point (Rumeau *et al.*, 2005).

Evidence that the Ndh complex has a role in plastoquinone reduction *in vivo* has come from the analysis of tobacco plastid (Burrows *et al.*, 1998; Kofer *et al.*, 1998; Shikanai *et al.*, 1998; Horváth *et al.*, 2000) and nuclear (Rumeau *et al.*, 2005) *ndh* mutants. Chlorophyll fluorescence measurements indicated that the *ndh* mutants were perturbed in their ability to transiently reduce the PQ pool following a light to dark transition. This phenotype is consistent with a direct role for Ndh in the reduction of the plastoquinone pool in the dark by stromal reductant (probably NADH, and not NADPH) generated during the light period (Burrows *et al.*, 1998). An additional role in cyclic electron flow around PSI in the light has also been suggested based on assays involving the oxidation and reduction of $P700^+$ (Burrows *et al.*, 1998; Shikanai *et al.*, 1998; Barth and Krause, 2002) and photoacoustic measurements (Joët *et al.*, 2002a). Immunochemical experiments indicate that the Ndh complex is only a minor component of the thylakoid membrane (approximately 1.5% of PSII levels) (Burrows *et al.*, 1998). Consequently it is only likely to make a modest contribution to the total flux of cyclic electron flow measured *in vivo* (Joliot *et al.*, 2004).

## 3. Single Subunit NAD(P)H Dehydrogenases

Mitochondria from yeasts, fungi and higher plants can contain additional NAD(P)H-ubiquinone oxidoreductases enzymes (NDH-2) that are quite different from complex I (Soole and Menz, 1995; Luttik *et al.*, 1998). Although they catalyse the same NADH:ubiquinone oxidoreductase reaction, they are single subunit enzymes that are not coupled to proton translocation across the membrane and so represent a simpler but less efficient mechanism for NAD(P)H oxidation. In the respiratory chains of plants, yeasts and algae, there are usually several distinct isoforms that face either the matrix or intermembrane space, and with different calcium requirements and specificities for NADH and/or NADPH (Soole and Menz, 1995). These NDH-2 dehydrogenases, which have homologues in many bacterial electron transfer chains (Yagi, 1993), contain a single FAD cofactor and are insensitive to complex I inhibitors. Several inhibitors have been reported (Rasmusson and Moller, 1991), in particular dicoumarol (Day and Wiskich, 1976), flavone and flavone derivatives (Ravanel *et al.*, 1990).

Corneille *et al.* (1998) studied PQ reduction by NADH and NADPH in potato thylakoids and concluded, on the basis of inhibitor sensitivities, that the activity was distinct from the FNR, FQR and complex I-homologue pathways. On the basis of its sensitivity to dicoumarol, they suggested that the NAD(P)H:PQ oxidoreductase activity that they observe might arise from a NDH-2 homologue that is targeted to the thylakoid membrane. A bioinformatic survey of the *Arabidopsis* nuclear genome does indicate the presence of eight NDH-2 homologues (Rich *et al.*, 2001; Peltier and Cournac, 2002) (Table 1), so it is possible that some of these could provide such a function, particularly the gene product of At5g08740 that may be targeted to the chloroplast and for which there is a homologue in *Chlamydomonas* (Table 3). However, dicoumarol is not a particularly specific inhibitor and its cross-reactivity with other oxidoreductases has not been well characterised. In addition, it has been found (P.R. Rich and J.T. Wiskich, unpublished) that dicoumarol interferes with the calcium-dependence, rather than the

electron-transfer pathway, of NDH-2 enzymes. Furthermore, the concentrations required for inhibition (Cornielle *et al.*, 1998) were substantial in relation to the required concentrations for inhibition of known mitochondrial NDH-2 reactions (Day and Wiskich, 1976). Hence, more direct proteomic data (Peltier *et al.*, 2000) or more specific inhibitor data are required if a role for an NDH-2 homologue in chlororespiration is to be substantiated.

### *4. Other Dehydrogenases*

It is conceivable that various other types of dehydrogenase, with electrons from diverse reductants, could provide a means of PQ pool reduction. For example, it has been suggested that a thylakoid succinate dehydrogenase could provide reductant to photosystem I in *Chlamydomonas* chloroplasts (Meyer *et al.*, 1993). However, analyses of both the *A. thaliana* and *Chlamydomonas* genomes (Tables 1 and 3) indicate that genes for the succinate dehydrogenase subunits are targeted only to mitochondria, and it seems therefore that a role for a chloroplast succinate dehydrogenase in higher plants or algae can be ruled out.

## *B. Assessment of Possible Pathways of Plastoquinol Oxidation*

In higher plant thylakoids, the PQ pool oxidation rate in the dark is rather slow, occurring over several hundred seconds (Graan and Ort, 1984; McCauley *et al.*, 1987; Rich *et al.*, 1998). The reoxidation rate tends to be multiphasic, relatively insensitive to ambient pH and neither first order in plastoquinol nor oxygen concentration (J. Jassal, P.J. Nixon and P.R. Rich, unpublished data). None of these characteristics are consistent with simple chemical autoxidation by molecular oxygen, although the rates are so slow that autoxidation must contribute to some extent to what appears to be a heterogeneous process. The oxidation rates are also insensitive to cyanide, an inhibitor of cytochrome oxidases, and to SHAM, an inhibitor of mitochondrial plant "alternative oxidases" (Rich *et al.*, 1998). Analyses of the *A. thaliana* and *Chlamydomonas* genomes (Tables 1 and 3) limit the types of plastoquinol oxidase that can be present in thylakoids. As expected, no homologues of the bacterial quinol oxidases, cytochrome *bo* and cytochrome *bd*, exist. A search for homologues of the core subunits I, II and III of cytochrome oxidase revealed only the mitochondrially-encoded versions. Hence, a chloroplast form of cytochrome oxidase can also be ruled out and casts doubt on the presence of a cyanide-sensitive thylakoid oxidase (Garab *et al.*, 1989). It seems likely in intact cellular systems, where oxidation rates can be faster, that at least some plastoquinol oxidation occurs simply by reversal of the FNR/FQR/ferredoxin pathway(s) that are involved in dark reduction and cyclic pathways. This energetically uphill process could be aided by energy-dependent reversed electron transfer through the complex I homologue subunits (Bennoun, 1983; Peltier and Cournac, 2002). However, a number of other lines of investigation (discussed below) have highlighted additional possible pathways for dark plastoquinol oxidation.

### *1. The Plastid Terminal Oxidase (PTOX)*

Studies on the variegated *immutans* mutant of *A. thaliana* (Carol *et al.*, 1999; Wu *et al.*, 1999) and the *ghost* mutant of tomato (Josse *et al.*, 2000) led to the identification of the so-called plastid terminal oxidase (PTOX), a new member of the alternative oxidase (AOX) family of quinol oxidases. Although the PTOX is only distantly related to the AOX found in plants, fungi and protists, the likely amino-acid ligands to the di-iron centre in the active site have been retained (Berthold *et al.*, 2000). The cysteine residues that are required for activation of AOX by pyruvate are, however, not conserved (Berthold *et al.*, 2000).

PTOX is a minor component of the thylakoid membrane (approximately 1% of PSII levels in *A. thaliana*) and is located in the non-appressed membranes, probably as a stroma-exposed interfacial protein (Andersson and Nordlund, 1999; Lennon *et al.*, 2003). Heterologous overexpression of IMMUTANS in *E. coli* (Josse *et al.*, 2003) and tobacco (Joët *et al.*, 2002b) has confirmed its role as a plastoquinol:oxygen oxidoreductase and also that it is sensitive to pyrogallol inhibitors, especially octyl gallate (Josse *et al.*, 2003). Based on its sensitivity to propylgallate, PTOX activity could be detected in a PSI-deficient strain of *C. reinhardtii in vivo* (Cournac *et al.*, 2000). However, no discernible PTOX activity was detected using a similar method in WT tobacco leaves, most likely because of low levels of expression (Joët *et al.*, 2002b). In an alternative approach, avoiding the use of inhibitors, we have used a chlorophyll fluorescence assay to compare the rates of plastoquinol oxidation in thylakoids isolated from WT *A. thaliana* and the *immutans* mutant lacking PTOX (Fig. 2). In agreement with the inhibitor studies, we found little evidence for a major role for IMMUTANS in oxidising the PQ pool.

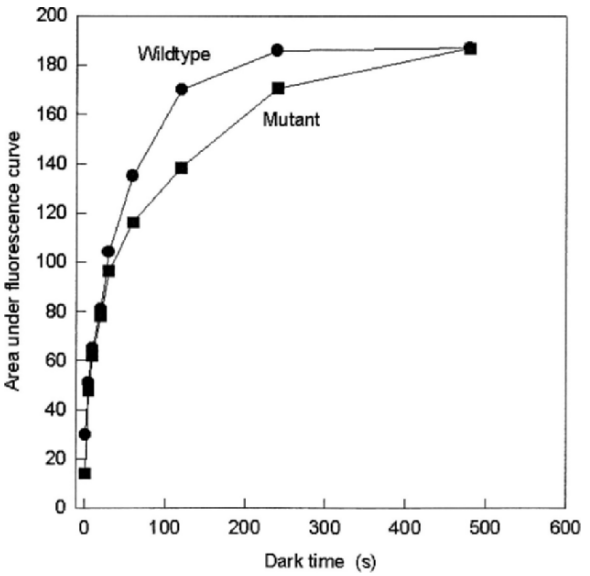


*Fig. 2.* Rate of oxidation of the PQ pool in thylakoids isolated from Wildtype and an *immutans* null mutant of *Arabidopsis thaliana.* Leaf disks of *A. thaliana* wildtype and a mutant strain lacking the IMMUTANS protein were placed at 45° to an actinic blue-light source and red fluorescence was detected with a photomultiplier at 90° to the actinic light. The PQ pool was firstly reduced by blue illumination until $F_{max}$ had been attained. The leaves were then dark-adapted for various time periods, after which the fluorescence induction curve on switching on the blue actinic light was recorded. PQ pool reduction level was assessed as the area under the fluorescence induction curve from the initial $F_o$ to $F_{max}$. The data of mutant and wildtype have been normalised to the same $F_{max}$ value. The increase in area is proportional to the extent of oxidation of the plastoquinone pool.

## 2. Plastoquinone Peroxidase

Casano *et al.* (2000) have described a membrane-associated hydroquinone peroxidase in barley thylakoids that in part co-purifies with the Ndh complex and also whose expression level follows that of the Ndh complex during development or photooxidative stress. By reconstitution of this peroxidase with purified Ndh, they showed that sustained NADH oxidation occurred, provided that PQ and hydrogen peroxide were present. From this they concluded that the peroxidase might act as a plastoquinol peroxidase, catalysing the oxidation of plastoquinol by hydrogen peroxide. In their proposal, the hydrogen peroxide would be produced by oxidation by molecular oxygen of reduced iron sulphur centres of *bf* and/or the Ndh complex to produce superoxide anions, which in turn would dismutate with superoxide dismutase to form hydrogen peroxide and oxygen.

Whilst this is a novel proposal, details remain questionable. For example, whilst Casano *et al.* (2000) quote cyanide and CO-sensitivity of plastoquinol oxidation as evidence for a role for peroxidase, plastoquinol oxidation in isolated thylakoids is insensitive to these inhibitors and, in intact systems, the secondary effects of these compounds on mitochondrial processes were not adequately excluded. Furthermore, the nature of the thylakoid hydroquinone peroxidase has not yet been established and its ability to oxidise membrane-bound plastoquinol remains to be demonstrated directly, particularly since the fact that it can oxidise hydroquinone rather suggests that its substrate site is too hydrophilic for plastoquinol. Finally, the rate of superoxide, and therefore hydrogen peroxide, generation in the dark that would be required for this process remains to be established. Hence, at present, there is little evidence to suggest that such a pathway could contribute in any major way to dark plastoquinol oxidation, although a role for such a peroxidase in control of damaging hydrogen peroxide levels does seem reasonable.

## 3. Cytochrome b-559

It is feasible that other redox centres that can equilibrate with plastoquinol could themselves be autoxidizable and, therefore, provide a dark pathway by which plastoquinol could be reoxidised, hence circumventing the observation that the kinetics of plastoquinol oxidation do not match those expected for simple, direct autoxidation. Kruk and Strzalka (1999, 2001) have indeed made such a suggestion and proposed that photosystem II-associated Cyt *b*-559 could provide such a pathway. Their proposal was based on the conclusion that direct plastoquinol autoxidation rates are likely to be too slow, which is reasonable, and the report that this *b*-559 is reducible by plastoquinone in photosystem II preparations (Gounaris *et al.*, 1988). In addition, when in its low potential form (Bendall, 1982), Cyt *b*-559 is autoxidisable at an observable rate. Again, however, there are difficulties with this model in terms of the fact that the majority of this *b*-559 in intact thylakoids is in a high potential, very poorly autoxidisable form and direct measurements of its oxidation rate in intact systems are lacking. Nevertheless, the idea of additional autoxidizable components that can themselves oxidise plastoquinol remains a possibility, though one that is again unlikely to provide a major contribution to the overall plastoquinol oxidation pathways *in vivo*.

Evidence that Cyt *b*-559 might actually be a physiologically relevant pathway has come from analysis of a tobacco mutant in which a phenylalanine residue found in a hydrophobic region of the β subunit of Cyt *b*-559

was substituted by serine (Bock *et al.*, 1994). The PQ pool was more reduced in the mutant and so it was concluded that Cyt *b*-559 functioned as a plastoquinol oxidase (Bondarava *et al.*, 2003). However, the possibility that the PQ pool became more reduced in the mutant, because of enhanced rates of non-photochemical reduction, was not examined. In contrast, a mutant of *C. reinhardti*, in which binding of the haem is destabilised by mutation of the His ligand on the alpha subunit, shows normal rates of oxidation of the plastoquinone pool in the dark in isolated thylakoids (M. Hamilton, P.R. Rich and P.J. Nixon, unpublished data). This would argue against an important role for Cyt-b559 in plastoquinol oxidation, at least in isolated thylakoids.

## IV. Physiological Role of Plastid Respiratory Enzymes

### *A. ATP Synthesis Driven by Respiration*

The original model proposed by Bennoun suggested that chlororespiration produced an electrochemical proton gradient across the thylakoid membrane in the dark, which could be used to drive ATP synthesis (Bennoun, 1982). Of the respiratory complexes so far detected in the thylakoid only the Ndh complex appears to have the potential capacity to pump protons. Thus for organisms lacking Ndh, such as *C. reinhardtii*, chlororespiration will not contribute to ATP synthesis. Even for chloroplasts containing the Ndh complex, the low levels would suggest only a minor role in ATP production in the light. Chlororespiration as a means of energising the membrane is therefore expected to be more significant in the dark or in immature chloroplasts and non-photosynthetic plastids. The detection of Ndh and PTOX in etioplasts (Guéra *et al.*, 2000; Lennon *et al.*, 2003) and chromoplasts (Aluru *et al.*, 2001; Guéra and Sabater, 2002) supports this latter possibility, but as yet there is no direct experimental evidence for a general role in plastid ATP synthesis.

### *B. Cyclic Electron Flow and Photoprotection*

The current evidence suggests that the thylakoid respiratory enzymes might have been subverted from their original function in respiration to act as auxiliary enzymes in photosynthetic processes. Analysis of the *pgr5* mutant of *A. thaliana* suggests that PGR5 has two major physiological roles: (i) it is required to protect PSI from photodamage by preventing the over-reduction of the stroma, and (ii) it is involved upon the onset of illumination in the formation of a thylakoid proton gradient, which is needed to activate the energy-dependent (qE) pathway of thermal dissipation of excess light energy (Munekage *et al.*, 2002). Given that the antimycin-sensitive FQR activity was absent in the *pgr5* mutant, Shikanai and colleagues (Munekage *et al.*, 2002) have proposed that PGR5 defines a major pathway for cyclic electron flow around PSI and that in its absence ATP levels are depleted, fixation of carbon dioxide is inhibited and NADPH is consequently accumulated. The absence of $NADP^+$ in the stroma to act as an electron acceptor ultimately causes irreversible photodamage to PSI.

Cyclic electron flow is only moderately affected in *ndh* mutants, probably because the PGR5 pathway is able to compensate (Munekage *et al.*, 2004). Consistent with this, single mutants of *A. thaliana* lacking either PGR5 or containing vastly depleted levels of Ndh are able to grow as well as WT under normal conditions. In contrast, the double mutant affected in both pathways contains less chlorophyll and grows much slower, presumably because of a more drastic effect on cyclic electron flow (Munekage *et al.*, 2004). Overall, analysis of the double mutant suggests that the Ndh and PGR5 pathways are the dominant routes of cyclic electron transfer (Munekage *et al.*, 2004).

In the case of *ndh* null mutants, growth defects have been observed under moderate water stress conditions (Horváth *et al.*, 2000) and high light exposure (Endo *et al.*, 1999), possibly due to insufficient rates of cyclic electron transport. Levels of Ndh complex also increase under circumstances demanding enhanced rates of cyclic photophosphorylation, such as in the bundle sheath cells of $C_4$ plants (Kubicki *et al.*, 1996) and when plants are exposed to various environmental stresses (Casano *et al.*, 2001; Martín *et al.*, 2004; Guéra *et al.*, 2005). The tissue specificity of Ndh expression has still not been assessed in $C_3$ plants, so it remains possible that activity is naturally up-regulated in specific cell types, such as those surrounding the vascular bundles of stems, which perform $C_4$-like photosynthesis (Hibberd and Quick, 2002).

By analogy to the mitochondrial AOX (Maxwell *et al.*, 1999), PTOX might have a role in preventing over-reduction of the PQ pool, which would otherwise produce reactive oxygen species that can cause oxidative damage (Aluru and Rodermel, 2004). Consistent with this, expression of PTOX is up-regulated in mutants that have reduced levels of the detoxification enzymes, catalase and ascorbate peroxidase (Rizhsky *et al.*, 2002).

### *C. Regulation of the Redox State of the PQ Pool*

The redox state of PQ pool plays a key signalling role in acclimation processes ranging from gene transcription in the chloroplast and nucleus (Pfannschmidt, 2003) to the redistribution of energy transfer between PSI and PSII during state transitions (Allen, 2002). For green algae, state transitions also regulate the degree of cyclic electron flow around PSI, and hence the ratio of NADPH/ATP produced by photosynthetic electron transport (Finazzi, 2005). Appropriate poising of the PQ pool is also needed for optimal rates of cyclic electron flow (Allen, 2003). Non-photochemical reduction and oxidation of the PQ pool by respiratory complexes therefore have the potential to coordinate the outputs of the light reactions to the metabolic needs of the organism such as that seen during nitrogen assimilation (Turpin and Bruce, 1990). Acclimation responses in chlororespiratory mutants have not been extensively studied as yet.

Analysis of *immutans* suggests that a key physiological role for PTOX is to keep the PQ pool sufficiently oxidised in developing chloroplasts, and possibly other plastids, to allow phytoene desaturation to proceed during carotenoid biosynthesis (Kuntz, 2004). In the absence of PTOX, leaf variegation is thought to occur because of an increase in light-induce bleaching of chlorophyll caused by carotenoid deficiency (Aluru and Rodermel, 2004; Kuntz, 2004).

## V. Conclusions and Prospects

It should be clear from the discussion above that much remains to be established in defining the non-photochemical pathways by which the redox state of the plastoquinone pool can be altered and controlled. In intact, mature plant cells, it seems likely that the pathways shared with the cyclic electron transfer route around photosystem I, involving ferredoxin, FNR and NADPH provide a major reduction, and possibly re-oxidation, mechanism that is intricately interlinked with metabolic activities of the mitochondria. However, more subtle pathways appear likely to operate that may fine-tune the responses, possibly even dominating in special types of cells or during specific developmental stages. Of these, the best substantiated at present are the alternative plastoquinol oxidase IMMUTANS protein and the complex I homologue whose major electron donor remains to be confirmed. However, it seems clear that much is left to be learned of these and other possible specialised pathways and, with the availability of bioinformatics and proteomic methods in combination with modern analytic techniques, their numbers, mechanisms and functions look likely to become much clearer.

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# Chapter 13

# $CO_2$ Concentrating Mechanisms

Sue G. Bartlett*, Mautusi Mitra and James V. Moroney
*Biochemistry and Molecular Biology Section, Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, U.S.A.*

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## Summary

Many photosynthetic organisms, from bacteria to algae to higher plants, have mechanisms for accumulating inorganic carbon in the vicinity of ribulose-1,5-*bis*phosphate carboxylase/oxygenase at the site of photosynthesis and thus minimizing photorespiration and increasing net carbon fixation. In this review components of carbon dioxide concentrating mechanisms (CCMs) are described. The best characterized of these systems, the CCM found in

*Author for correspondence, email: sbartle@lsu.edu

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some cyanobacteria, is used as a paradigm. The CCM found in microscopic green algae is then described, with emphasis on *Chlamydomonas reinhardtii*. The CCMs utilized by higher plants that carry out C4 or Crassulacean acid metabolism are then reviewed. Finally, efforts to introduce a CCM into C3 plants using genetic engineering, the effects of the CCM on the global carbon cycle, and important research directions in this field are presented.

## I. Introduction

The ability of photosynthetic organisms to use $CO_2$ for photosynthesis depends in part on the properties of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco). Rubisco can utilize either $O_2$ or $CO_2$ as a substrate. It seems that the oxygenase activity is intrinsic to Rubisco and reflects the evolution of ancestral Rubisco in an anaerobic atmosphere. The relative rates of oxygenation and carboxylation by Rubisco are major factors in determining the efficiency of photosynthesis. The chloroplast stroma of mesophyll cells of higher plants contains about 9 μM $CO_2$ and about 250 μM $O_2$ at 25°C (Keys, 1986). Rubisco from higher plants has a surprisingly low affinity for $CO_2$ with measured $K_m$ values for $CO_2$ and $O_2$ of approximately 8 to 25 μM and 360 to 650 μM respectively (Jordan and Ogren, 1983; Keys, 1986). Cyanobacterial Rubisco has an even lower affinity for $CO_2$ and the $K_m(CO_2)$ can be greater than 150 μM (Jordan and Ogren, 1981; Badger *et al.*, 1998). As the concentration of $CO_2$ in the stroma of mesophyll cells is approximately equal to the $K_m(CO_2)$, Rubisco normally functions at only half of its already low maximum velocity. The oxygenation reaction competes with the carboxylation reaction, which slows this carboxylation rate by another 30% under the current atmospheric conditions. Thus, to sustain reasonable rates of photosynthetic $CO_2$ fixation, plants must make an enormous amount of Rubisco. The enzyme may constitute up to 25% of the total nitrogen in a plant and up to 50% of the protein in the chloroplast stroma. Considering the quantity of photosynthetic tissue, Rubisco is probably the most abundant enzyme in the biosphere.

A number of photosynthetic organisms have developed mechanisms to increase the concentration of $CO_2$ at the location of Rubisco, minimize the deleterious oxygenation reaction, and reduce the nitrogen allocation cost in the form of Rubisco. These mechanisms include $C_4$ photosynthesis (Hatch, 1987) and Crassulacean Acid Metabolism (CAM), found in a number of higher plant families, as well as the $CO_2$ concentrating mechanism (CCM) found in microalgae (Osterlind, 1950; Berry *et al.*, 1976) and cyanobacteria (Turpin *et al.*, 1984; Price *et al.*, 1992). All of these photosynthetic organisms package Rubisco in a very specific location, have novel means to concentrate $CO_2$ and/or $HCO_3^-$, and have a means of rapidly converting the accumulated $HCO_3^-$ to $CO_2$ for use by Rubisco. Many of these organisms employ a mechanism of concentrating $CO_2$ that is induced only under relatively low $CO_2$ conditions (0.036% $CO_2$ in air) and their CCM does not operate when high $CO_2$ conditions are present (Osterlind, 1950; Berry *et al.*, 1976). The inducible nature of the CCM may be because many of the environments that are habituated by photosynthetic microorganisms show great fluctuations in concentrations of the gases $CO_2$ and $O_2$. Microalgae and cyanobacteria possessing an inducible CCM may be better equipped to thrive under these variable gaseous conditions. The CCM provides these organisms with an additional ecological advantage as it allows both $CO_2$ and $HCO_3^-$ species to be efficiently exploited. The utilization of $HCO_3^-$ is an advantage since in neutral and alkaline aqueous environments $HCO_3^-$ is the most abundant form of Ci.

In general, $CO_2$ concentrating mechanisms consist of the following components (Badger, 1987).

(a) A means to transport inorganic carbon species (Ci) against a concentration gradient across either the plasma membrane or the chloroplast envelope or both.
(b) A source of energy to drive the uphill inorganic carbon transport.
(c) A compartment where Rubisco is separated from the reactions that accumulate the intermediate inorganic carbon (Ci) pool. For $C_4$ plants this is the bundle sheath, for cyanobacteria it is the carboxysome and for algae it appears to be the pyrenoid.

*Abbreviations*: Ci – inorganic carbon; CA – carbonic anhydrase; CAM – Crassulacean acid metabolism; CCM – $CO_2$ concentrating mechanism; HCR – high $CO_2$-requiring; $K_{0.5}(Ci)$ – amount of inorganic carbon required for 50% of the maximal rate of photosynthesis; NDH – NADPH dehydrogenase complex; PEP – phospho*enol*pyruvate; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase.

(d) A means for releasing $CO_2$ from the captured Ci pool in the special compartment where Rubisco is localized. This might be decarboxylating enzymes such as $NAD^+/NADP^+$ malic enzyme in $C_4$ plants or carbonic anhydrases in cyanobacteria and algae.
(e) A mechanism to prevent $CO_2$ efflux away from the site of Rubisco to ensure efficient $CO_2$ fixation. This can be the thick suberized walls of the bundle sheath cells in $C_4$ plants, the protein shell of the carboxysome in case of cyanobacteria, or the starch sheath of the pyrenoid in the case of microalgae. This review will focus on the $CO_2$ concentrating mechanisms in plastids and the significance of the CCMs in photosynthetic organisms.

## II. Carbonic Anhydrases

Many photosynthetic organisms, from cyanobacteria to higher plants, have developed methods for accumulation of Ci to aid Rubisco in efficient $CO_2$ capture. Carbonic anhydrases (CAs; carbonate dehydratase; EC 4.2.1.1) play important roles in this process. CAs are zinc-metalloenzymes that catalyze the reversible interconversion of $CO_2$ and $HCO_3^-$ in two steps (Meldrum and Roughton, 1933; Lindskog, 1997), and many have turnover numbers in excess of $10^6$ $s^{-1}$ (Khalifah, 1971). The overall relationship between the three forms of dissolved Ci is shown below:

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \rightarrow$$
$$H_2CO_3 \rightarrow CO_3^{2-} + 2H^+$$

The uncatalyzed hydration and dehydration reactions are slow while the dissociation reactions are considered instantaneous. CA accelerates the hydration of $CO_2$ in solution and thus increases the rate at which the different forms of Ci interconvert. Protons abstracted from water are conducted along a proton wire to the solution (Silverman, 2000). The proportion of each Ci in solution is a function of pH. At physiological ionic strengths, $CO_2$ predominates at a pH less than about 6.4 ($pK_1$), $HCO_3^-$ predominates between pH 6.4 and 10.3 ($pK_2$), and $CO_3^{2-}$ predominates at a pH above 10.3.

The known CAs can be grouped into five types designated the $\alpha$-, $\beta$-, $\gamma$-, $\delta$-, and $\varepsilon$-CA families. (Hewett-Emmett and Tashian, 1996; So *et al.*, 2004). Although all families utilize zinc at the active site, they have no significant sequence homology and appear to be examples of convergent evolution of catalytic function. A brief description of each family is given below.

### A. α-Carbonic Anhydrases

The α-CAs are widely distributed. Although α-CAs have long been known to occur in vertebrates (Meldrum and Roughton, 1933), they have recently been identified in algae (Fukuzawa *et al.*, 1990; Fujiwara *et al.*, 1990), in the higher plant *Arabidopsis thaliana* (Moroney *et al.*, 2001), in eubacteria (Soltes-Rak *et al.*, 1997; Elleby *et al.*, 2001; Chirica *et al.*, 2001), and in viruses (Niles *et al.*, 1986; Strayer and Jerng, 1992). Most α-CAs are active as monomers of about 30 kDa with three histidines coordinating the zinc atom (Moroney *et al.*, 2001). The α-CA structure is dominated by an antiparallel β-sheet forming a spherical molecule with two halves. The active site is a funnel-shaped crater with the zinc atom located near the bottom. All known α-CAs are highly susceptible to inhibition by sulfonamide compounds (Moroney *et al.*, 1985).

### B. β-Carbonic Anhydrases

The β-CAs do not appear to be as broadly distributed as the α-CAs. β-CAs were first identified as carbonic anhydrases in higher plants (Burnell *et al.*, 1990; Fawcett *et al.*, 1990). Subsequently, β-CAs have been found in micro-algae, eubacteria (Hewett-Emmett and Tashian, 1996), archaebacteria (Smith and Ferry, 1999), and fungi (Götz *et al.*, 1999). The *Caenorhabditis elegans* and *Drosophila melanogaster* genomes also contain β-CA homologues, but this CA family appears not to be represented in any vertebrate genome.

All β-CAs have a histidine and two cysteine residues that act as zinc ligands (Bracey *et al.*, 1994; Rowlett *et al.*, 1994), and are composed of monomers of about 25kDa. The *Pisum sativum* CA is an octamer in which dimers form tetramers that are held together by their C-termini to form octamers (Kimber and Pai, 2000). An active site is at the interface of each subunit. The β-CAs from monocots lack the C-terminal residues found in α-CAs from dicots, and so they likely are tetramers.

The β-CA from the red alga *Porphyridium purpureum* is double the size of the pea CA and has two active sites per polypeptide instead of the one found in the pea enzyme, indicating a gene duplication occurred (Mitsuhashi and Miyachi, 1996; Mitsuhashi *et al.*, 2000). Interestingly, two β-CA cDNAs from the $C_4$ monocot *Zea mays* appear to encode unusually large monomers of 60 and 74 kDa (Burnell *et al.*, 1999). As in the red alga, each of these large polypeptides appears

to be a fusion of two dicot-type monomers since it contains two sets of active site residues. The quaternary structures of the maize CAs have not yet been resolved, but they are likely to be similar to that of the red alga.

The notion that the $CO_2$ residing in a hydrophobic pocket is required for activity is underscored by the recent comparison between the α-CA and β-CA crystal structures. The three dimensional structure of the active site of the β-CA is a mirror image of that of the active site of the α-CA (Kimber and Pai, 2000). β-CAs are generally less sensitive to inhibition by sulfonamide compounds than the α-CAs.

### C. γ-Carbonic Anhydrases

A γ-CA was first discovered in the archaebacterium *Methanosarcina thermophila* (Alber and Ferry, 1994). Since that time genes encoding putative γ-CA proteins have been found in eubacteria and plants (Newman *et al.*, 1994). The structure of the γ-CA is remarkably different from that of α-CA or β-CA. The γ-CA functions as a trimer of identical subunits. Each monomer is a left-handed β-helix (Kisker *et al.*, 1996). The trimer contains three zinc atoms, one at each subunit interface. As in α-CAs, three histidines and a water molecule coordinate the zinc atom but the histidines are provided by two adjacent subunits. In spite of the fact that each active site is at the interface between two subunits, architecturally the active site of γ-CA resembles that of β-CA (Kisker *et al.*, 1996). Under anaerobic conditions an enzyme containing iron rather than zinc at the active site can be isolated (Tripp *et al*., 2004). Recently, studies of the mitochondrial proteome demonstrated that the Arabidopsis γ-CA homologues are localized in mitochondria, and transport of one of them into isolated intact mitochondria has been demonstrated (Heazlewood *et al*., 2004; Parisi *et al*., 2004).

### D. δ-Carbonic Anhydrases

To date members of the δ-CA family have been found only in two species of the marine diatom *Thalassiosira* (Lane *et al*., 2005). The δ-CA gene from *T. weissflogii* appears to encode three highly similar repeats, while that from *T. pseudonana* encodes a single unit similar to each repeat. The δ-CA purified from *T. weissflogii* contains cadmium rather than zinc.

### E. ε-Carbonic Anhydrases

Recently, a member of a new CA family was shown to occur in *Halothiobacillus neapolitanus* (So *et al.*, 2004). This CA is the product of the *CsoS3* gene that lies in the carboxysome gene cluster and the protein is tightly associated with the carboxysome shell. The protein has no sequence homology with any member of the other CA families. The ε-CA appears to be narrowly distributed among some chemolithoautotrophic and marine bacteria that have carboxysomes similar to those of *H. neapolitanus*. The *H. neapolitanus* ε-CA is inhibited by ethyxoyzolamide similar to members of the other families, strongly suggesting that zinc is involved in catalysis. The zinc ligands of the ε-CA have not yet been identified.

CA genes and cDNAs have been isolated from virtually all photosynthetic organisms studied to date. However, function has been demonstrated for only a few of them. For example, the Arabidopsis genome contains seven α-CA, six β-CA, and three γ-CA genes, but the specific function(s) of the gene products remains obscure. Since CAs also are found in non-photosynthetic organisms, it cannot be assumed that all of those found in photosynthetic organisms function to accelerate $CO_2$ fixation by Rubisco. In the following sections, we will discuss only CAs for which there is evidence of participation in Ci accumulation.

## III. Cyanobacterial Model of $CO_2$ Concentrating Mechanisms

### A. Overall Organization of $CO_2$ Concentrating Mechanisms

While the focus of this article is on the role of plastids in CCMs, it is instructive to first look at the CCM in cyanobacteria. In the past few years a very detailed model of the cyanobacterial CCM has emerged (Maeda *et al.*, 2002; Price *et al.*, 2002). This model will likely be a roadmap for future research on the CCM in eukaryotic organisms, and specifically the contribution of the chloroplast to the accumulation of Ci (Fig. 1).

Cyanobacteria have evolved a remarkable environmental adaptation for survival at limiting $CO_2$ conditions by concentrating inorganic carbon internally using a CCM. Their ability to accumulate Ci allows cyanobacteria to have forms of Rubisco that have exceedingly low affinities for $CO_2$ compared with Rubisco from higher plants (Badger and Price, 2003). The

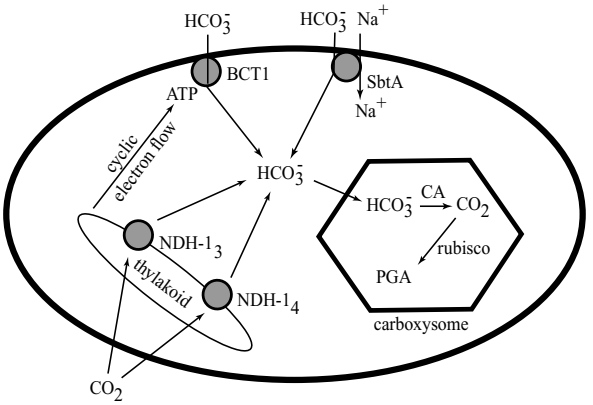


*Fig. 1.* A proposed model for CCM in cyanobacteria. BCT1, bicarbonate uniporter; NDH-$1_3$ and NDH-$1_4$, thylakoid-bound/NADPH dehydrogenase/$CO_2$ uptake systems; SbtA, sodium-dependent bicarbonate transporter. See text for details

$K_m(CO_2)$ of cyanobacterial Rubisco is about 150 μM (Jordan and Ogren, 1981; Badger *et al.*, 1998). This compares with a $K_m(CO_2)$ of 8 to 25 μM measured in Rubisco from higher plants or a dissolved $CO_2$ concentration of 11 μM for an aqueous solution in equilibrium with atmospheric $CO_2$. Without a functioning CCM, Rubisco in cyanobacteria would be operating at less than 10% of its maximum velocity.

A distinguishing feature of the cyanobacterial CCM is the existence of a constitutive form of CCM in cells grown even at hyper-normal levels of $CO_2$ (2 to 5% of $CO_2$). These high Ci cells have the ability to utilize both $CO_2$ and $HCO_3^-$ as substrates and to accumulate significant levels of Ci relative to the external environment. Cyanobacterial cells exposed to Ci limitation (typically 20 to 50 ppm $CO_2$ in solution and 350 ppm $CO_2$ in air) have the ability to express an enhanced level of CCM activity. This change is accompanied by an increase in Rubisco activity (Price *et al.*, 1992), about a two-fold increase in carboxysome content (McKay *et al.*, 1993; Turpin *et al.*, 1984) and an increase in the apparent affinities for $CO_2$ and $HCO_3^-$ (Badger and Price, 1992; Kaplan and Reinhold, 1999; Kaplan *et al.*, 1994; Price *et al.*, 1998). The apparent affinity of algal cells for $C_i$ is estimated by the amount of $C_i$ required for 50% of the maximal levels of photosynthesis and is expressed as $K_{0.5}(C_i)$. A high $K_{0.5}(C_i)$ value reflects cells with a poor ability to use external $CO_2$. A small $K_{0.5}(C_i)$ reflects cells that have the ability to fix $CO_2$ even at very low $C_i$ concentrations. Typically a 20-fold decrease in the $K_{0.5}$ (Ci) is seen when cyanobacteria are grown at 20 to 30 ppm $CO_2$ and more than half of this rise in affinity for $C_i$ is due to an increase in $HCO_3^-$ uptake (Sültemeyer *et al.*, 1995; Yu *et al.*, 1994).

Most of the studies of physiological and molecular aspects of the cyanobacterial CCM have been done on the freshwater cyanobacterium *Synechococcus* sp. PCC7942. This is largely because this strain is easily grown in the laboratory under the desirable defined conditions of rapid aeration and high light intensity and its excellent suitability for genetic modification using recombinant technologies. With the availability of a number of cyanobacterial genomes researchers have now investigated the CCM from a number of these organisms. While many of the details of the CCM will vary between species, there are a number of fundamental properties that cyanobacterial CCMs have in common.

The function of the CCM is to increase the concentration of $CO_2$ at the site of carbon fixation. Since $CO_2$ can cross biological membranes fairly readily, cyanobacteria package Rubisco internally in proteinaceous bodies called carboxysomes. All of a cell's Rubisco can be found in its carboxysome. In addition, the highest concentration of $CO_2$ found in the cell is thought to be in the carboxysome. All cyanobacteria have multiple transporters for inorganic carbon. These include $HCO_3^-$ transporters as well as mechanisms for $CO_2$ uptake. The form of inorganic carbon that is accumulated in the cytoplasm of the cyanobacterial cell appears to be $HCO_3^-$. This makes sense as $CO_2$ would be expected to readily leak out of the cell. Since $HCO_3^-$ is the accumulated Ci species, cyanobacteria must convert the accumulated $HCO_3^-$ to $CO_2$ before it can be fixed by Rubisco. Therefore cyanobacteria have a carbonic anhydrase that specifically coverts accumulated $HCO_3^-$ to $CO_2$. This β-CA is localized to the carboxysome and is thought to create a microenvironment high in $CO_2$ within the carboxysome (Fukuzawa *et al.*, 1992; Yu *et al.*, 1992). Finally, cyanobacteria have developed ways to recapture $CO_2$ if it leaks from the carboxysome. Some of the $CO_2$ uptake processes are thought to play important roles in the recapture of accumulated $CO_2$. The roles of the different transport proteins as well as the carboxysome are discussed in the next few sections.

Only a few α-CAs have been identified to date in cyanobacteria. One, encoded by the *ecaA* gene, has been identified in *Anabaena* and in *Synechococcus* (Soltes-Rak *et al.*, 1997). In each case, the CA is localized to the periplasmic space. Preliminary analysis of *Synechococcus* mutants in which *ecaA* has been inactivated by insertion of a drug-resistance cassette

suggests that extracellular carbonic anhydrase plays arole in inorganic-carbon accumulation by maintaining equilibrium levels of$CO_2$ and $HCO_3^-$ in the periplasm (Soltes-Rak *et al.*, 1997). In contrast, *Synechocystis* PCC6803 does not have an α-CA.

## B. Bicarbonate Transporters

Currently, evidence indicates that there are four modes of Ci transport in cyanobacteria, two for bicarbonate and two for carbon dioxide (Fig. 1). BCT1, a high affinity bicarbonate uniporter was the first to be identified and characterized. In *Synechococcus* PCC7942, BCT1 is encoded by the *cmpABCD* operon and is a member of the ABC transporter family (Omata *et al.*, 1999). CmpA is a lipoprotein proposed to be a bicarbonate scavenger outside the cytoplasmic membrane. CmpA is highly specific for bicarbonate binding and has a $K_d$ of about 5 μM. The CmpB polypeptides are thought to dimerize in the cytoplasmic membrane, while CmpC and CmpD, both of which have ATP binding sites, are exposed on the cytoplasmic side of the membrane. The stoichiometry of CmpA is high relative to the other BCT1 polypeptides, suggesting that CmpA can diffuse laterally through the cell membrane in order to capture bicarbonate, which it can then deliver to the CmpB subunits. The *cmpABCD* operon is expressed under severe Ci limitation. Database searches indicate that cmpABCD homologues are not found in marine cyanobacteria. However, since seawater is bicarbonate-rich relative to most fresh water, this operon would likely be non-functional in marine cyanobacteria even if homologues were present.

The second bicarbonate transport system is sodium-dependent, and may be a bicarbonate-sodium symporter (Shibata *et al.*, 2002). This transporter is encoded by the *sbtA* gene in *Synechocystis* PCC6803. Similar to the components of BCT1, expression of the SbtA protein is induced by low Ci; however, SbtA is a medium-affinity transporter. Homologues of SbtA can be found in the genomes of many, but not all, freshwater cyanobacteria. However, only weak SbtA homologues have been found in genomes of marine cyanobacteria.

A third bicarbonate transporter was discovered recently in marine cyanobacteria by Price and his colleagues (Price *et al.*, 2004). This transporter was originally thought to transport sulfate and was designated as a SulP-type transporter. This finding suggests that other transporters thought to carry anions may be bicarbonate carriers instead.

## C. $CO_2$ Uptake

Two $CO_2$ uptake systems, one constitutive and one inducible when cells are grown on low $CO_2$, have also been identified in some cyanobacteria (Maeda *et al.*, 2002; Shibata *et al.*, 2001). These uptake systems are based on specialized forms of thylakoid-based Type 1 NADPH dehydrogenase complexes (NDH-1). The constitutive, low affinity system, designated NDH-$1_4$, contains polypeptides encoded by the *ndhF4*, *ndhD4* and *chpX* (*cupA*) genes. The inducible, high affinity system, designated NDH-$1_3$, contains polypeptides encoded by the *ndhF3*, *ndhD3* and *chpY* (*cupB*) genes.

Both the NDH-$1_3$ and NDH-$1_4$ systems exhibit CA activity, and Price *et al.* (2002) speculate that the ChpX and ChpY proteins contain this activity. Although the ChpX and ChpY proteins contain no homology to any known CA family, sequence alignment of ChpX and ChpY homologs from several cyanobacteria reveals that each contains two highly conserved histidines and one highly conserved cysteine, which could coordinate zinc at the active site. Several amino acids that could form a proton wire are also conserved in this region of the protein (Price *et al.*, 2002).

## D. Carboxysomes

Carboxysomes are proteinaceous, polyhedral inclusion bodies found in all cyanobacteria and most other autotrophic bacteria that play an important role in the CCM of cyanobacteria. Immunogold labeling shows Rubisco is localized to the carboxysome (McKay *et al.*, 1993). Ci is delivered into the cell in the form of $HCO_3^-$ and remains largely as $HCO_3^-$ except in the carboxysome where a β-CA converts the $HCO_3^-$ to $CO_2$ (Badger and Price, 1992). This increases the concentration of $CO_2$ at the site of Rubisco, ensuring efficient $CO_2$ fixation.

Recent analysis of seven cyanobacterial genomes has led to the identification of two types of carboxysomes in cyanobacteria (Price *et al.*, 1993; Badger, 2003). One type has the typical cyanobacterial carboxysomes and the other type has carboxysomes more like autotrophic β-proteobacteria such as *Thiobacillus* species. In most cyanobacteria, the *ccmKLMN* genes are clustered. The peptides encoded by the *ccmK* and *ccmO* genes in cyanobacteria are homologous to one another and to the CsoS1 peptides which are Thiobacillus carboxysomal shell proteins (Cannon *et al.*, 2002). The *ccmL* gene product may function in assembly or structure of the carboxysome. The *ccmM* gene encodes a fusion protein

described below, while the function of the *ccmN* gene product is not known.

Mutations in some genes outside the *ccmKLMN* cluster also result in high $CO_2$-requiring (HCR) phenotypes. For example, mutation of the *ccmA* gene results in a failure to assemble carboxysomes. Cells without carboxysomes exhibit an HCR phenotype even though they are capable of Ci transport (Badger and Price, 1994; Ronen-Tarazi *et al.*, 1995). $CO_2$ leakage out of the carboxysome has been hypothesized to be prevented by the spatial arrangement of CA and Rubisco in the carboxysomes (Reinhold *et al.*, 1991), which ensures that the $CO_2$ produced is fixed before it can diffuse to the cytoplasm. $CO_2$ leakage is further minimized by the carboxysomal proteinaceous shell, which is selectively permeable to $HCO_3^-$ and not to $CO_2$.

One of the carboxysomal shell proteins, CcmM, has been found to have a strong N-terminal sequence homology with the CA active site from γ-CAs. The C-terminal region of CcmM has three to four 87 amino acid repeats that are very similar to the cyanobacterial Rubisco small subunit. The function of CcmM is not clear. Cells deleted in *CcmM* have an HCR phenotype and lack carboxysomes (Ludwig *et al.*, 2000). From these results it is clear that CcmM is required for correct carboxysome assembly and for optimal growth on low levels of $CO_2$. It is not clear if CcmM has CA activity or its enzymatic activity is needed for correct assembly of carboxysomes.

Cyanobacterial Rubisco is involved in the functional organization of the carboxysome. A number of alterations in the structure, localization and conformation of Rubisco result in an HCR phenotype (Badger *et al.*, 1998). Replacement of *Synechocystis* PCC6803 Rubisco with that of *Rhodospirillum rubrum* resulted in an HCR phenotype and absence of carboxysomes, although cells could accumulate Ci (Pierce *et al.*, 1989). *Rhodospirillum* Rubisco lacks small subunits and has low specificity for $CO_2$ compared to $O_2$($S_{c/o}$). A *Synechococcus* HCR mutant, EK6, contains a Rubisco small subunit with a thirty amino acid extension and lacks mature carboxysomes. *In vitro* analysis showed that EK6 Rubisco has the same $K_m$ ($CO_2$) as the wild type enzyme (Schwarz *et al.*, 1995; Orús *et al.*, 1995). Another HCR mutant, Mu28, lacks carboxysomes and immunolabelling showed that the Rubisco is distributed throughout the mutant cell (Friedberg *et al.*, 1993). Taken together, these results indicate that the structure of Rubisco dictates the organization of the carboxysome.

### *E. Carboxysomal Carbonic Anhydrase*

Characterization of a *Synechococcus* PCC7942 mutant containing a disruption in ORF 272 led to the simultaneous isolation of the first carboxysomal β-CA gene *icfA* and its product (Fukuzawa *et al.*, 1992; Yu *et al.*, 1992). Later a homologue was described from another cyanobacterium, *Synechocystis* 6803 (So and Espie, 1998).

The carboxysomal CA has a 60 to 70 amino acid extension at the carboxyl end of the protein (Badger and Price, 1994) when compared with other β-CAs, giving a monomer size of 31 kDa, compared to 24 kDa for the chloroplast enzyme of higher plants. Mutagenesis experiments have shown that the carboxyl terminal extension may be required for the oligomerization of the CA and this oligomerization is essential for catalysis (So *et al.*, 2002).

Mutants having defective or missing carboxysomal CA can no longer grow on limiting $CO_2$ and show an HCR phenotype. These cells can still accumulate Ci to high levels but cannot rapidly convert the $HCO_3^-$ to $CO_2$ for fixation. These observations support the notion that the carboxysomal CA converts accumulated $HCO_3^-$ to $CO_2$ for Rubisco. The location of the carboxysomal CA is considered essential to its function. To prevent the dissipation of the $HCO_3^-$ pool, CA activity should be absent in the cytosol of cyanobacteria. This is supported by the fact that the expression of the human CA in the cytoplasm of *Synechococcus* PCC7942 cells results in a massive leakage of $CO_2$ from the cells expressing an HCR phenotype and implicates the carboxysomes as the site of $CO_2$ elevation (Price and Badger, 1989). The *Synechocystis* 6803 genome encodes no other β-CA, indicating that the carboxysomal β-CA is the only β-CA in this organism. Other cyanobacterial genomes also appear to lack genes encoding cytoplasmic CAs (Price and Badger, 1989).

Carboxysomes of some marine cyanobacteria, such as *Prochlorococcus marinus* MED4 and MIT9313, were thought to lack CA since their genomes encoded no homologues of the α-, β-, or γ-CA families (Badger *et al.*, 2002). The β-CA of the marine cyanobacterium *Synechococcus* WH8102 lacks the carboxyl terminal extension found in other carboxysomal CAs (Badger *et al.*, 2002) and so it also was thought to lack a carboxysomal CA. However, genomes of all of these organisms encode homologues of the recently discovered ε-CA from *H. neapolitanus* (So *et al.*, 2004). Thus it would appear that CA is a component of all cyanobacterial carboxysomes.

## IV. $CO_2$ Uptake in Eukaryotic Algal Cells

### *A. Overall Organization of the CCM of Unicellular Green Algae*

Unicellular green algae also have the ability to accumulate Ci and enhance $CO_2$ fixation by Rubisco. CCMs have been demonstrated in a number of green algae including species of *Chlamydomonas*, *Chlorella*, *Dunaliella* and *Scenedesmus* (Tsuzuki and Miyachi, 1989, 1991). The CCM is clearly not limited to green algae as it is thought to occur is a very wide range of aquatic organisms (Colman *et al.*, 2002; Raven *et al.*, 2002). However most of the research on eukaryotic CCMs has been done using green algae and the working models are based on this work.

Many of the fundamental properties of the CCM of cyanobacteria are also present in eukaryotic algae. As in cyanobacteria, Rubisco is compartmentalized in eukaryotic algae. In the case of eukaryotic algae, Rubisco is located in the chloroplast pyrenoid. Isolated chloroplasts are capable of accumulating Ci implying there are Ci transporters on either the chloroplast envelope or the thylakoid membrane or both.

A general model of the *C. reinhardtii* CCM is shown in Fig. 2. In the model Ci must cross the plasma membrane and the chloroplast envelope to get to the site of carbon fixation. Ci transporters have been postulated to be localized to both the plasma membrane and the chloroplast envelope although these transporters remain to be identified. What has been found is that there are CAs for many compartments of the cell. CAs have been localized in the mitochondria, the periplasmic space, the chloroplast stroma and the chloroplast thylakoid lumen (Fujiwara *et al.*, 1990; Eriksson *et al.*, 1996; Karlsson *et al.*, 1998; Mitra *et al.*, 2004).

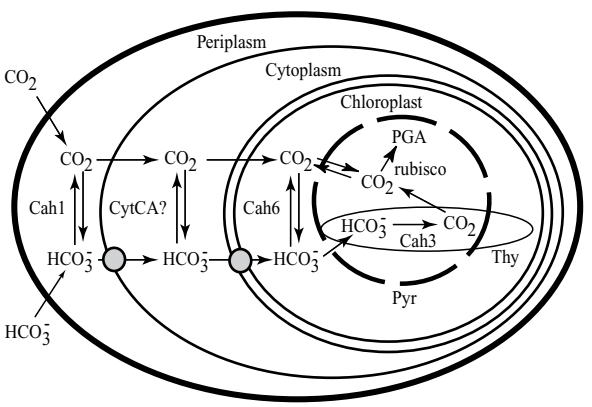


*Fig. 2.* A model showing the potential roles of carbonic anhydrases in the operation of CCM in *C. reinhardtii.* Cah1, periplasmic carbonic anhydrase; Cah3, thylakoid carbonic anhydrase; Cah6, chloroplastic carbonic anhydrase; CytCA, cytoplasmic carbonic anhydrase; PGA, phosphoglyceric acid; Pry, pyrenoid; Thy, thylakoid. Putative $HCO_3^-$ transporters are denoted by small grey circles. See text for details

### *B. Ci uptake Across the Plasma Membrane*

In green algae, carbonic anhydrases are thought to play a number of roles in the delivery of $CO_2$ to Rubisco. One function of CA is to facilitate the diffusion of $CO_2$ across the plasma membrane. *C. reinhardtii* has two α-CAs, Cah1 and Cah2, located in the periplasm. The genes encoding these CAs were the first α-CA genes cloned from a photosynthetic organism (Fukuzawa *et al.*, 1990; Fujiwara *et al.*, 1990). These two genes encode very similar proteins although they are differentially regulated. *Cah1* is expressed under low $CO_2$ conditions but not under high $CO_2$ conditions. In contrast, *Cah2* is poorly expressed under low $CO_2$ and slightly upregulated under high $CO_2$ conditions. In addition, the expression of *Cah2* under high $CO_2$ appears low compared to the expression of *Cah1* under low $CO_2$ (Fujiwara *et al.*, 1990; Rawat and Moroney, 1991). Possibly, *Cah2* resulted from a gene duplication event and has a poorly functioning promoter.

Cah1 facilitates the diffusion of both $CO_2$ and $HCO_3^-$ to the cell surface by promoting rapid equilibrium of these inorganic carbon species. $CO_2$ is then available for both passive diffusion and active uptake across the cell membrane and $HCO_3^-$ is available for $HCO_3^-$ uptake systems. It is thought that Cah1 contributes to the delivery of $CO_2$ to the cell when the external pH is high or when there is a large unstirred boundary layer around the cell (Moroney *et al.*, 1985). Cah2 is not expressed under low $CO_2$ conditions and is never expressed at high levels. The function of Cah2 is not clear. An HCR mutant of *C. reinhardtii*, *cia5*, fails to produce the low $CO_2$-induced periplasmic CA and any of the proteins known to be associated with the CCM. These results seem to support the hypothesis that Cah1 is required for the functional operation of CCM in *C. reinhardtii* (Moroney *et al.*, 1985).

The halophilic green alga *Dunaliella salina* also has two CAs that are located in the periplasm (Fisher *et al.*, 1996; Yang *et al.*, 1999). One of the CAs, p60, appears to have two active sites, possibly the result of gene duplication and fusion events (Fisher *et al.*, 1996). Increasing salinity, an alkaline shift, or removal of bicarbonate induces the expression of p60. This enzyme may enhance $CO_2$ uptake by cells growing in hypersaline media.

Some eukaryotic algae can take up $HCO_3^-$ from the environment. Like cyanobacteria, *Scenedesmus* can take up Ci from the medium even when the external pH is greater than 9 (Thielmann *et al.*, 1989). There is also evidence that *C. reinhardtii* can take up $HCO_3^-$ as well as $CO_2$, but there are no reports of $HCO_3^-$ transporters in the plasma membrane from any alga. Identifying these transporters remains an important unanswered question in this field.

### C. Ci Transporters of Algal Plastids

Intact chloroplasts from green algae can accumulate Ci (Goyal and Tolbert, 1989; Moroney and Mason, 1991) and like cyanobacteria this accumulated Ci is thought to be largely in the form of $HCO_3^-$ as the stromal pH is typically close to pH 8 in the light. A strong indication that $HCO_3^-$ is accumulated is the observation that *C. reinhardtii* mutants deficient in the chloroplast carbonic anhydrase Cah3 still accumulate inorganic carbon but can no longer grow on low $CO_2$ (Karlsson *et al.*, 1998). These observations suggest that plastids have functioning Ci transporters. These transporters could be located on the chloroplast envelope or the thylakoid membrane

At this time no plastid Ci transporter has been definitively identified, but LIP-36 is a candidate. LIP-36 is localized to the chloroplast envelope and is very strongly up regulated when *C. reinhardtii* cells are grown on low $CO_2$ (Ramazanov *et al.*, 1993). LIP-36 is encoded by two very closely related genes known as *Ccp1* and *Ccp2* for Chloroplast Carrier Protein (Chen *et al.*, 1997). LIP-36 is clearly a transport protein belonging to the mitochondrial carrier protein family. Proteins in this class include the brown fat uncoupler protein as well as antiporters. The presence of LIP-36 correlates very well with the presence of a CCM in *C. reinhardtii*.

Recently an RNAi approach was used to determine whether LIP-36 participates in Ci uptake. Cells transformed with the RNAi construct did have greatly reduced levels of *Ccp1* and *Ccp2* transcripts and had reduced levels of LIP-36 (Pollock *et al*., 2004). These cells also had reduced growth on low $CO_2$ implying that some aspect of the CCM or photorespiratory pathway had been adversely affected by the lack of LIP-36. However, these cells had an apparently normal affinity for Ci and could accumulate Ci as well as wild-type cells (Pollock *et al*., 2004). The observation that the affinity of the cells lacking LIP-36 was unchanged argues against the hypothesis that LIP-36 is a Ci transporter. However, it should be noted that Ci transport in cyanobacteria is highly redundant and no growth defects were observed until more than one Ci uptake mechanism was lost (Maeda *et al.*, 2002; Price *et al.*, 2002). It may be possible that LIP-36 is one of a number of Ci transporters and more than one must be decreased before changes in Ci affinity and uptake are affected. The fact that the strains carrying the *Ccp1/Ccp2* RNAi construct showed reduced growth on low $CO_2$ does support the idea that LIP-36 is required for optimal growth on low $CO_2$.

Besides LIP-36, another candidate for a plastid Ci transporter has emerged. In *C. reinhardtii*, there is an ABC-type transporter that is induced when cells are grown on low $CO_2$ (Im *et al.*, 2003). This protein also has the potential to be involved in Ci uptake although the location of the transporter and its substrate affinity remain to be determined. No clear homologues to ChpX or ChpY have been noted in eukaryotic algae and there is no evidence for $Na^+$-$HCO_3^-$ cotransport as is observed in cyanobacteria. The identification of Ci transporters in plastids remains an important goal in the understanding of CCMs in eukaryotes.

### D. Pyrenoids

The pyrenoid is an electron-dense, proteinaceous structure found in chloroplasts of most types of algae and several species of lichens and hornworts (Morita *et al.*, 1998; Morita *et al.*, 1999; Smith and Griffiths, 1996a,b, 2000). The pyrenoid is surrounded by a sheath of carbohydrates such as starch, amylose or paramylon (Gibbs, 1962; Okada, 1992) and is the structural equivalent of the carboxysome. Pyrenoids purified from both *Eremosphera* (Okada, 1992) and *C. reinhardtii* (Kuchitsu *et al.*, 1991) consist primarily of Rubisco. *C. reinhardtii* cells with a mutation in the rbcL gene (rubisco large subunit) that leads to a truncated large subunit have no pyrenoids (Rawat *et al.*, 1996). In general, there is a correlation between the operation of a CCM and the presence of a pyrenoid (Morita *et al.*, 1997; Smith and Griffiths, 1996a,b)

Although it is accepted that Rubisco is the major constituent of the pyrenoid, there are conflicting findings regarding what proportion of Rubisco is in the pyrenoid. Cryofixation and immunogold studies using *C. reinhardtii* indicated that the pyrenoids in low $CO_2$ grown cells contain almost 90% of the Rubisco (McKay and Gibbs, 1991; McKay *et al.*, 1993; Morita *et al.*, 1997; Borkhsenious *et al.*, 1998). *In vitro* measurements of Rubisco activity imply that the enzyme in the pyrenoid must be active to account for $CO_2$ fixation rates observed in *C. reinhardtii*. Under high $CO_2$ conditions, only 50% of the Rubisco is immunolocalized

in the pyrenoid (Borkhsenious *et al.*, 1998). A specific location of Rubisco is also compatible with the view that organisms that have CCMs specifically package Rubisco.

Some arctic species of the unicellular volvocalean green alga *Chloromonas*, some bryophytes, and some lichen photobionts such as *Coccomyxa* lack both pyrenoids and the CCM (Palmqvist *et al.*, 1994; Morita *et al.*, 1997; Smith and Griffiths, 1996a,b). The CCM might not be necessary for survival of the slow growing arctic algae (Honegger, 1991). On the other hand, biochemical analysis indicates that *Coccomyxa* Rubisco is more efficient than Rubisco of *C. reinhardtii* (Palmqvist *et al.*, 1995), so perhaps an efficient Rubisco compensates for an inefficient CCM.

The correlation between presence of a pyrenoid and a CCM is not absolute. Two strains of *Chloromonas*, *C. rosae* UTEX 1337 and *C. serbinowii* (Starr and Zeikus, 1993), lack pyrenoids but have a CCM (Morita *et al.*, 1998). Low $CO_2$ acclimated cells of these two strains have the high photosynthetic affinity for $CO_2$ and high CA activity that are features of the CCM (Badger and Price, 1994; Tzusuki and Miyachi, 1989, 1991), although their intracellular inorganic carbon pools are rather small compared with those of the typical pyrenoid-containing *Chlamydomonas reinhardtii* (Morita *et al.*, 1998). These results suggest that such a small Ci pool is related to absence of Rubisco compartmentalization in pyrenoids.

The question remains whether a pyrenoid is required for an operational CCM in those species that have both. Given the number of algal species, there likely is diversity in the mechanisms used to generate $CO_2$ and concentrate it in the chloroplast. (Raven, 1997; Badger *et al.*, 1998). The possibility that $CO_2$ may be elevated within the entire chloroplast rather than just the pyrenoid has been suggested (Badger, 2003). Algae that lack a pyrenoid might have a less efficient CCM but compensate with a Rubisco with a high $CO_2/O_2$ selectivity. On the other hand, strains with a more active CCM might have a Rubisco with a lower selectivity but might need to put fewer resources into synthesis of Rubisco.

## *E. Carbonic Anhydrases of the Algal Plastid*

In *C. reinhardtii* there are two chloroplast CAs, one in the thylakoid lumen (Karlsson *et al.*, 1995, 1998) and one in the stroma (Mitra *et al.*, 2004). The thylakoid α-CA, Cah3, contains an amino terminal extension characteristic of a bipartite leader sequence. Cah3 is constitutively expressed and is slightly upregulated under low $CO_2$ conditions (Karlsson *et al.*, 1998). Cah3 has been overexpressed in *E. coli* (Karlsson *et al.*, 1998), and recently significant CA activity was detected in the purified recombinant protein (Mitra *et al*., 2005).

It was initially suggested that Cah3 accelerates the formation of $CO_2$ from $HCO_3^-$ and is needed for the delivery of $CO_2$ to Rubisco (Raven, 1997; Karlsson *et al.*, 1998; Moroney and Somanchi, 1999). The hypothesis was that $HCO_3^-$ is transported into the thylakoid lumen where it is converted to $CO_2$ by Cah3. The $CO_2$ produced in the thylakoid lumen could then diffuse across the thylakoid membrane and be fixed by Rubisco in the pyrenoid. In fact, the thylakoid membranes of many algae invaginate into the pyrenoid (Raven, 1997).

Physiological studies using *C. reinhardtii* mutants with defects in the *Cah3* gene provide evidence in support of this hypothesis. The *C. reinhardtii* mutant *cia3* has two point mutations in the region coding for the transit peptide of the Cah3 protein. This strain grows poorly under low $CO_2$ but grows well under high $CO_2$. Another mutant of *Cah3*, *ca-1*, shows the same phenotype. The sequence of the *ca-1* gene revealed a point mutation in the 5′ end of the gene that created a stop codon. Both the *cia3* and *ca-1* mutants can grow well on low $CO_2$ when complemented by the wild type *Cah3* gene (Karlsson *et al.*, 1998; Funke *et al.*, 1997). Clearly Cah3 is required for the growth of *C. reinhardtii* in air levels of $CO_2$ (Funke *et al.*, 1997).

A recent study of wild-type and mutants lacking Cah3 by Hanson *et al.* (2003) strongly supports the idea that Cah3 is required to deliver $CO_2$ to Rubisco. When Cah3 is missing, cells can still accumulate Ci but can no longer efficiently fix $CO_2$. A thylakoid-associated α-CA has been found also in two other green microalgae, *Tetrahedron minimum* and *Chlamydomonas noctigama* (van Hunnik *et al.*, 2001). To date, there is no report of a thylakoid-located CA in higher plants and sequence analysis has not identified a CA with a bipartite targeting sequence.

Recently a new β-CA, Cah6, was identified in *C. reinhardtii* (Mitra *et al.*, 2004). Cah6 is located in the chloroplast stroma. Cah6 is expressed both under high and low $CO_2$ conditions. Immunolocalization reveals that Cah6 is four-fold more abundant in the area around the pyrenoid, particularly in the starch sheath surrounding the pyrenoid, compared to other areas in the chloroplast stroma. Cah6 might play an indirect role in the CCM by trapping unfixed $CO_2$ that diffuses from the pyrenoid and converting it to $HCO_3^-$. This conversion of $CO_2$ to $HCO_3^-$ would increase the $HCO_3^-$ pool in the stroma, which is essential for the operation of CCM. Generation of mutants will clarify the role of Cah6 in the chloroplast of *C. reinhardtii*.

### *F. Is the Thylakoid Membrane Involved With $CO_2$ Uptake?*

The discovery that Cah3 is localized to the thylakoid lumen might explain why it has been impossible to demonstrate Ci uptake in the dark in algae. Spalding and Ogren (1982) first demonstrated that light is required for Ci uptake in *C. reinhardtii* and that electron transport inhibitors block this uptake. The recent discovery of a second chloroplast carbonic anhydrase (Mitra *et al.*, 2004) might help explain the tight linkage between light energy and Ci accumulation.

In the model shown in Fig. 3, an increase in the chloroplast $CO_2$ concentration is a direct result of the pH differential generated by light-driven electron flow in the thylakoid membrane. Cah3 is in the lumen of the thylakoid membrane (Karlsson *et al.*, 1998; M.M. Mitra and J.V. Moroney, unpublished data), and Cah6 is in the chloroplast stroma (Mitra *et al.*, 2004). $CO_2$ diffusing across the chloroplast envelope or escaping from the pyrenoid would be rapidly converted to $HCO_3^-$ by Cah6. Thus if the pH of the stoma is 8 and the initial level of $CO_2$ is close to ambient levels (10 μM), the initial stromal $HCO_3^-$ concentration would be close to 300 μM if the $CO_2$ and $HCO_3^-$ in the stroma were in equilibrium. If the $HCO_3^-$ accumulated in the chloroplast stroma is transported across the thylakoid membrane in the light, the presence of Cah3 in the thylakoid lumen would rapidly convert that $HCO_3^-$ to $CO_2$ in the acidic thylakoid lumen. If the thylakoid lumen had a pH of 5.3 or less, a localized $CO_2$ concentration of greater than 1 mM could be generated (Fig. 3). This $CO_2$ could readily diffuse from the lumen to the pyrenoid, raising the $CO_2$ concentration in the compartment where Rubisco is localized. Since the pH gradient across the thylakoid membrane is set up by light-driven electron transport, it becomes clear why the presence of light appears to be so important to the functioning of the CCM.

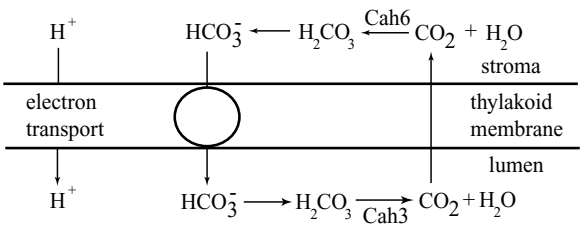


*Fig. 3.* Possible role of the thylakoid membrane in the CCM of green algae. Cah3, thylakoid carbonic anhydrase; Cah6, chloroplastic carbonic anhydrase. See text for details.

## V. $CO_2$ Uptake in Higher Plants

$CO_2$ enters a leaf through stomates, and then diffuses through the air spaces surrounding mesophyll cells. Since $CO_2$ diffuses about 1000 times faster in air than in water, having large air spaces in the leaf likely facilitates Ci uptake. Since the apoplastic space is acidic (pH ≈ 5.5 to 6.0), $CO_2$is the predominant Ci in the cell wall water, although some $HCO_3^-$ also is present.

While cyanobacteria have been shown to contain two bicarbonate transporters and two $CO_2$ transporters, there is no evidence for active transport of either into cells of higher plants. In fact, no homologues of any of the cyanobacterial transporters can be identified in searches of various databases. Since the algal and cyanobacterial CCMs evolved at about the same time as land plants did, it may well be that each group of organisms evolved different strategies for acquisition of inorganic carbon.

A homologue of animal bicarbonate transporters has been identified as a boron transporter in Arabidopsis (Takano *et al.*, 2002). While this transporter exhibits a strong preference for boron, the Arabidopsis genome contains at least 6 homologs of the boron transporter, one of which could encode a bicarbonate transporter. If that is not the case, then $CO_2$ must diffuse through the plasma membrane. Alternatively, it may be carried into the cell by aquaporins (Tyerman *et al.*, 2002). Once in the cytoplasm, the fate of $CO_2$ depends on whether the plant carries out the $C_3$, $C_4$, or CAM pathway.

### *A. $CO_2$ Uptake in $C_3$ Plants*

Once in the cytoplasm, with a pH ≈7.5, the $CO_2/HCO_3^-$ equilibrium could rapidly be attained by the activity of cytoplasmic carbonic anhydrases (Fett and Coleman, 1994). $CO_2$ could then diffuse across organelle membranes. In chloroplasts, stromal carbonic anhydrases (Burnell *et al.*, 1990; Fawcett *et al.*, 1990) would rapidly convert the $CO_2$ not used by Rubisco into $HCO_3^-$, the predominant form of Ci in the stroma (pH≈8.0), thus minimizing diffusion of $CO_2$ back into the cytoplasm.

In this scenario of $CO_2$ transport by diffusion, the large central vacuole would play a critical role as a reservoir of Ci. $CO_2$ could diffuse across the vacuolar membrane, or be transported by aquaporins. The pH of the vacuole is similar to that of the apoplast, and so the thin layer of cytoplasm in a typical higher plant cell is bounded by compartments where the $CO_2/HCO_3^-$ equilibrium would favor $CO_2$. On the other hand, the

alkaline pH of the cytoplasm and chloroplasts would favor $HCO_3^-$ as reservoir of Ci.

Transgenic tobacco plants expressing a chloroplast CA in the antisense orientation have been generated (Price *et al.*, 1994; Majeau *et al.*, 1994). CA activity in some of these plants was reduced by 98 to 99% compared with wild type plants, but net $CO_2$ fixation as measured by gas exchange was unaffected. However, differences in carbon isotope discrimination indicated that antisense plants had a 15-μbar reduction in the partial pressure of $CO_2$ at Rubisco active sites. This difference could lead to a modest 4.4% reduction in the rate of $CO_2$ assimilation (Price *et al.*, 1994). However, since we now know that Arabidopsis contains 16 genes encoding CAs, it may be impossible to obtain an altered phenotype without ablation of a number of these genes.

### *B. The $C_4$ CCM*

$C_4$ plants achieve higher photosynthetic capacity and more efficient water use than $C_3$ plants by spatially separating $CO_2$ assimilation and $CO_2$ fixation. In a typical $C_4$ plant with Kranz anatomy this is accomplished by expression of enzymes involved in $CO_2$ assimilation and regeneration of the acceptor phospho*enol*pyruvate (PEP) in thin-walled mesophyll cells and expression of Rubisco and decarboxylating enzymes in thick-walled bundle sheath cells. $CO_2$ diffuses through the stomates and into the cytoplasm of mesophyll cells. The alkaline pH of the cytoplasm would favor $HCO_3^-$, and a cytoplasmic CA facilitates the reaction (Ku *et al.*, 1996) PEP carboxylase uses $HCO_3^-$ rather than $CO_2$ as a substrate and has no affinity for $O_2$, so it is well suited for the initial step in Ci assimilation in the mesophyll. The $HCO_3^-$ is used by PEP carboxylase in the conversion of PEP to the $C_4$ acid oxaloacetate. The activity of PEP carboxylase is regulated by reversible phosphorylation, with the phosphorylated form being less sensitive to inhibition by malate and more sensitive to stimulation by glucose-6-phosphate (Vidal and Chollet, 1997). In $C_4$ plants, the enzyme is phosphorylated in the light and dephosphorylated in the dark. The oxaloacetate formed by carboxylation of PEP can be reduced to malate or transaminated to aspartate, either of which is then exported to bundle sheath cells, depending on the type of $C_4$ metabolism exhibited (Day and Siedow, 2000). In $NADP^+$-malic enzyme plants, malate is decarboxylated to pyruvate in bundle sheath chloroplasts. In $NAD^+$-malic enzyme plants, aspartate enters the mitochondrion where it is converted in two steps to malate which is decarboxylated to pyruvate which is then converted to alanine. In PEP carboxykinase plants, aspartate in the cytoplasm is converted to oxaloacetate which is then decarboxylated to PEP. Alternatively, plants in this group can transport malate which, using mitochondrial $NAD^+$-malic enzyme, is decarboxylated to pyruvate. Regardless of the mode of decarboxylation, the end result is that $CO_2$ is concentrated in the bundle sheath cells where it is used as a substrate by Rubisco. Thus, the high rates of photorespiration typical of $C_3$ plants are avoided.

While CA clearly plays a role in facilitating PEP carboxylase activity in the cytoplasm of mesophyll cells, Burnell and Hatch (1988) proposed that bundle sheath cells must lack CA activity since its presence would result in excess leakiness of $CO_2$. Indeed, transgenic *Flaveria bidentis* expressing a tobacco CA had three to five times more CA activity in bundle sheath cells and had increased C isotope discrimination, indicating that $CO_2$ did leak from the bundle sheath cells (Ludwig *et al.*, 1998). The transgenic plants had approximately 15% lower rate of $CO_2$ assimilation than control plants.

Results of recent studies indicate that some photosynthetic organisms lacking Kranz anatomy carry out $C_4$ photosynthesis. For example, the diatom *Thalassiosira weissflogii* (Reinfelder *et al.*, 2001) and the siphonaceous green alga *Udotea flabellum* (Reiskind and Bowes, 1991; Bowes *et al.*, 2002) have $C_4$-like photosynthesis. The freshwater monocot *Hydrilla verticillata* carries out $C_4$ photosynthesis in submersed leaves (lacking Kranz anatomy) and $C_3$ photosynthesis in aerial leaves (Bowes *et al.*, 2002). Finally, the chenopod *Bienertia cycloptera* carries out $C_4$ photosynthesis in the absence of Kranz anatomy (Voznesenskaya *et al.*, 2002). Although this latter plant clearly lacks Kranz anatomy, enzymes involved in Ci assimilation and fixation are compartmentalized at the cellular level. Chlorenchyma cells contain a large central cytoplasmic compartment containing granal chloroplasts and mitochondria. The enzymes Rubisco, $NAD^+$-malic enzyme, and glycine decarboxylase are in this central compartment. The central cytoplasmic compartment is surrounded by the vacuole and connected to the peripheral cytoplasmic compartment by channels. The peripheral compartment contains pyruvate Pi dikinase and most of the PEP carboxylase. Thus, the enzymes involved in the initial steps of Ci assimilation are at the cell periphery and the Calvin-Benson cycle operates at the cell interior.

### *C. The Crassulacean Acid Metabolism CCM*

Plants that have Crassulacean acid metabolism (CAM) temporally separate $CO_2$ assimilation and fixation.

Stomata are open at night, and $CO_2$ is converted to $HCO_3^-$ by a cytosolic CA. A cytosolic PEP carboxylase converts $HCO_3^-$ and PEP to oxaloacetate, and a cytosolic malate dehydrogenase converts oxaloacetate to malate, which is transported into the vacuole (Dodd *et al.*, 2002). As is the case for $C_4$ plants, PEP carboxylase activity is regulated by reversible phosphorylation by a specific kinase. However, in contrast to $C_4$ plants, the kinase is more active at night (Chollet *et al.*, 1996).

During the day, stomates are closed, and malate exits the vacuole and is decarboxylated to pyruvate by a cytosolic NADP-linked malic enzyme. The pyruvate ultimately is used to make starch and Calvin-Benson cycle intermediates, and the $CO_2$ is used as a substrate by Rubisco. Since all of these reactions occur when stomata are closed, photorespiration is reduced and water loss is minimized. Indeed, CAM plants can have water use efficiencies 5 to 10 times greater than $C_3$ plants (Cushman and Bohnert, 1997); however, their photosynthetic capacity is very low (Black, 1973). In some species, CAM is obligate, while in others it can be induced by environmental or developmental triggers. Water deficit, high salinity, and photoperiod are among environmental conditions known to trigger a shift from $C_3$ to CAM metabolism (Cushman and Bohnert, 1997).

## VI. The Significance of the $CO_2$ Concentrating Mechanism and Future Research Directions

### *A. Potential Consequences of the CCM for Plant Productivity*

Since $C_4$ plants have higher photosynthetic capacity, as well as more efficient use of water and nitrogen, than $C_3$ plants, several groups of researchers have attempted to make $C_3$ plants more $C_4$-like by transfer of $C_4$ genes into $C_3$ plants. These approaches have been recently reviewed (Leegood, 2002; Miyao, 2003; Rademacher, 2002).

Typical $C_4$ plants contain a number of morphological adaptations in addition to Kranz anatomy that facilitate the CCM. For example, bundle sheath cells in $C_4$ plants typically have thick, suberized cell walls, while $C_3$ bundle sheath cells lack suberization; $C_4$ mesophyll cells have more plasmodesmata than $C_3$ mesophyll cells; and the distance between veins is less in $C_4$ leaves than in $C_3$ leaves. However, the genetic bases for these morphological differences are not understood, so efforts have focused on reducing photorespiration in $C_3$ plants by expression of enzymes that function in Ci accumulation or regeneration in $C_4$ plants.

Remarkably, all of the enzymes for the $C_4$ pathway are found in $C_3$ plants (Hausler *et al.*, 2002). However, none of these enzymes functions in photosynthesis. They are generally expressed at much lower levels, in different patterns, and with different modes of regulation than their counterparts in $C_4$ plants.

A number of transgenic $C_3$ plants overexpressing PEP carboxylase have been generated and results have been recently reviewed (Hausler *et al.*, 2002; Leegood, 2002). The transgenic plants accumulated more malate or oxaloacetate than control plants. However, maize PEP carboxylase in rice was more active at night than during the day, indicating that the maize enzyme was phosphorylated in the same pattern as the endogenous enzyme. Thus, other efforts at overexpressing PEP carboxylase have used bacterial (non-phosphorylated) or mutated $C_4$ enzymes in order to obtain an enzyme that is active during the day. Regardless, no increased growth has been observed in the transgenic plants. In addition, perturbation of stomatal movement and decreased flavenoid synthesis also have been observed in some transgenic plants (summarized in Hausler *et al.*, 2002 and Miyao, 2003). Transgenic $C_3$ plants overexpressing PEP carboxykinase, and NADP-linked malic enzyme, either alone or in combination with one another or with PEP carboxylase, also have been generated. However, to date these plants have either shown no improvement in photosynthesis or have displayed phenotypes indicative of perturbations in metabolism (Hausler *et al.*, 2002).

Leegood (2002) speculated that efforts to engineer $C_4$ photosynthesis in $C_3$ plants would be unsuccessful without introduction of a compartment in which $CO_2$ can be concentrated. However, our knowledge of the genetic bases of $C_4$ characteristics such as number of plasmodesmata, pattern of venation, or suberization of the bundle sheath cell wall precludes broad scale transfer of $C_4$ genes encoding these characteristics into $C_3$ plants. Leegood proposed several creative alternatives including introduction of genes encoding pyrenoid or carboxysome components into $C_3$ plants and transfer of $C_3$ photorespiratory glycine metabolism to bundle sheath cells.

Efforts have been made to express a Rubisco with a higher $S_{c/o}$ in $C_3$ plants. Whitney *et al.* (2001) attempted this approach using the rbcLS genes from the diatom *Phaeodactylum tricornutum* and the rhodophyte *Galdieria sulphuraria*. In each case the genes were introduced into the chloroplast genome and were shown to be transcribed abundantly. However, all

of the large subunits and most of the small subunits were insoluble. It is not clear why chloroplast chaperones failed to fold these algal Rubisco subunits.

Recently, Lieman-Hurwitz *et al.* (2003) generated transgenic Arabidopsis and tobacco plants expressing the *Synechococcus* PCC 7942 *ictB* gene product fused to a Rubisco small subunit transit sequence. The *ictB* gene was identified in a screen for high $CO_2$-requiring mutants, but its exact function has not been clearly defined (Bonfil *et al.*, 1998). However it has no homology with any subunits of the know cyanobacterial Ci transporters.

Transgenic plants expressing the *ictB* fusion gene had slightly lower $CO_2$ compensation points than control plants. The transgenic Arabidopsis plants also had slightly higher relative growth rates, and slightly more dry weight than control plants when grown under low (30%) relative humidity (Bonfil *et al.*, 1998). However, the location and amount of IctB protein produced in the transgenic plants could not be verified for lack of specific antibodies. Clearly an understanding of the function of the IctB protein would facilitate interpretation of these results.

In summary, efforts to genetically engineer $C_3$ plants that are more $C_4$-like and have significantly greater rates of photosynthesis and/or growth have not yet been successful. Perhaps the major lesson we have learned to date is how little we still know about even the most basic of plant metabolic pathways.

## B. The CCM and the Global Carbon Cycle

The discovery that almost 50% of the world's photosynthesis occurs in organisms that employ a CCM (Falkowski *et al.*, 1998; Field *et al.*, 1998) raises two questions regarding the global carbon cycle. The first question is how big an impact does the CCM make on $CO_2$ uptake from the atmosphere? The second question is how will plants and algae that have a CCM be affected by rising $CO_2$ levels? Since marine aquatic photosynthetic organisms make up the bulk of the organisms that have a CCM, this section will concentrate on the effect of the CCM on marine algae.

The annual oscillations in atmospheric $CO_2$ levels clearly show that photosynthesis plays a key role in the global carbon cycle. There is a decrease in atmospheric $CO_2$ as the plants and algae in the Northern hemisphere grow each summer and an increase as these photosynthetic organisms die back during the winter. Would global photosynthetic uptake of $CO_2$ be different if algae did not have functioning CCMs? The answer to this question must be yes because without a CCM the growth of algae in the North Atlantic and North Pacific each summer would not occur since loss of the CCM due to mutation inevitably leads to algal strains that do not grow on air levels of $CO_2$ (Price *et al.*, 1998; Moroney and Somanchi, 1999). It is clear that a functioning CCM is required for efficient photosynthesis in an aquatic environment. In the absence of algal blooms the annual decrease in atmospheric $CO_2$ would not be as significant and the increase in $CO_2$that occurs each year would likely be somewhat greater. Since marine organisms account for 50% of the world's photosynthesis, anything that decreases algal growth (such as the loss of the CCM) would also lead to a decrease in $CO_2$ uptake.

The second question is how will algae respond to increasing $CO_2$ levels? Will they have increased rates of photosynthesis, resulting in greater seasonal declines in atmospheric $CO_2$? Will algal blooms be more widespread? While it is impossible to answer these questions with certainty, it appears unlikely that the present increase in $CO_2$ will have a major impact on algal growth since there is evidence that growth of marine algae is usually limited by nutrients other than carbon. The nutrients Fe, N and P are most often cited as limiting growth of marine algae (Behrenfeld *et al.*, 1996; Falkowski *et al.*, 1998). So even as the atmospheric $CO_2$ concentration increases, without increases in the other nutrients it seems unlikely that there will be a major change in the growth of these algae.

One interesting idea that has been proposed is to encourage algal blooms by seeding the ocean with Fe or other limiting nutrient (Boyd *et al.*, 2000). The idea is that the phytoplankton will bloom and algae such as coccoliths will deposit a significant amount of carbon in calcium carbonate shells. As the bloom subsides and the algae die the shells will sink to the bottom of the ocean effectively pulling that amount of carbon out of the global carbon cycle (at least for the short-term). Seeding experiments indicate that this idea might be feasible (Boyd *et al.*, 2000) although the long-term ecological effects of this approach are uncertain.

## C. Future Research Directions

Perhaps the greatest challenge for researchers in the CCM area is to establish which photosynthetic organisms have CCMs and how these organisms will respond to rising levels of atmospheric $CO_2$. At the present time it is assumed that most algae have a CCM of some sort, but this assumption should be tested by analysis of Ci uptake in a large number of algal species.

A second challenge is to identify membrane proteins that may be involved in Ci uptake, particularly in eukaryotes. For example, no Ci transporter localized to the plasma membrane or the chloroplast envelope has been identified in eukaryotic algae or higher plants. While some candidate transporters have been proposed for the chloroplast envelope, none of these proteins have been shown to transport $HCO_3^-$. In addition, do plant aquaporins play a role in $CO_2$ transport? This question remains to be resolved.

A third challenge is to determine roles of the various carbonic anhydrases in the CCM. Examination of genomes of higher plants and algae reveal that the number of carbonic anhydrase in these organisms has been greatly underestimated. There are at least 16 CA or CA-like genes in Arabidopsis for example. What are the spatial and temporal patterns of expression of these CAs? What are their subcellular locations? Do any or all of them participate in delivery of $CO_2$ to Rubisco? There are still numerous unresolved questions regarding the number and distribution of CA genes and gene families, structure, localization and function. The challenge for future researchers will be to determine the expression patterns, localization and physiological roles for each of these CA isoforms.

The availability of Arabidopsis, rice and *Chlamydomonas* genome sequences and EST databases can be used to find out the exact number of expressed CA isoforms in these organisms. These genomes can be exploited to identify candidates for bicarbonate transporters. Additional genome sequences will become available in the near future. It will be especially interesting to compare the genomes of diatoms and other eukaryotic algae with those of higher plants. This explosion of information should keep CCM researchers busy for the foreseeable future.

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# Chapter 14

# Synthesis, Export and Partitioning of the End Products of Photosynthesis

Andreas P.M. Weber*
*Department of Plant Biology, Michigan State University, East Lansing, MI 48824, U.S.A.*

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*Author for correspondence, email: aweber@msu.edu

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## Summary

Chloroplasts are the metabolic factories of plant cells. They are the site of various biosynthetic pathways such as carbon, nitrogen and sulfur assimilation, fatty acid biosynthesis, amino acid biosynthesis, isoprenoid biosynthesis and secondary metabolite biosynthesis, to mention just a few. Many of these metabolic pathways require the rapid and controlled exchange of precursors, intermediates and end products between the chloroplast stroma and the surrounding cytosol. However, two lipid bilayer membranes, the inner and outer chloroplast envelope membranes, form a permeation barrier between plastid stroma and the cytosol. Transporters in the inner plastid envelope membrane catalyze the efficient and specific exchange of metabolites between plastid stroma and other cellular compartments, thereby integrating plastidal metabolism into the metabolic networks in plant cells. Metabolite transporters not only catalyze the flux of metabolites between compartments, they also represent information pathways that communicate the metabolic status between compartments. A classic example is the coordination of sucrose biosynthesis in the cytosol with starch biosynthesis in the stroma by the triosephosphate/phosphate translocator (Flügge, 1995; Flügge, 1999).

The compartmentation of metabolic pathways augments options for regulation, permits the simultaneous operation of pathways that compete for the same substrates and helps avoiding futile cycles. Recent advances in the molecular identification of plastid envelope transporters and forward and reverse genetic approaches have increased our understanding of the impact that transport processes have on the synthesis of end products of photosynthesis.

In this chapter, I will review the synthesis of end products of photosynthesis, with particular emphasis on metabolite transporters in the chloroplast envelope membrane that interconnect the metabolic pathways in the plastid stroma with those in the cytosol.

*Abbreviations:* AGPase – ADP-glucose pyrophosphorylase; C2 Cycle – C2 carbon oxidation cycle (photorespiratory carbon cycle); CGT1 – cytosolic 4-$\alpha$-glucanotransferase 1; DAP – dihydroxyacetone 3-phosphate; D-enzyme – disproportionating enzyme; DiT1 – 2-oxoglutarate/malate translocator; DiT2 – glutamate/malate translocator; DOXP – 2-dexoyxylulose-5-phosphate pathway; Ery 4P – erythrose 4-phosphate; FBPase – fructose 1,6-*bis*phosphatase; Fru – fructose; Fru 1,6*b*P – fructose 1,6-*bis*phosphate; Fru 6P – fructose 6-P; GAP – glyceraldehyde-3-phosphate; GAPDH – NADPH-dependent glyceraldehyde phosphate dehydrogenase; Glc – glucose; Glu 1P – glucose 1-phosphate; Glu 6P – glucose 6-phosphate; GOGAT– glutamate synthase; GPT – glucose 6-phosphate/phosphate translocator; GS – glutamine synthetase; IPP – isopentenyldiphosphate; Mal – maltose; MalP – maltodextrin phosphorylase; MalQ – 4-$\alpha$-glucanotransferase (amylomaltase); MEP – 2-methylerythritol-4-phosphate; PCO Cycle – photosynthetic carbon oxidation cycle; PEP –phospho*enol*pyruvate; 2-PG – 2-phosphoglycolate; PGI – phosphoglucoisomerase; PGM – phosphoglucomutase; Pi – inorganic phosphate; PPDK – pyruvate:phosphate dikinase; PPi –pyrophosphate; PPT – phospho*enol*pyruvate/phosphate translocator; 3-PGA – 3-phosphoglyceric acid; RPPP – reductive pentose phosphate pathway (Calvin-Benson cycle); Ru-1,5-*b*P – ribulose 1,5-*bis*phosphate; Rubisco – ribulose 1,5-*bis*phosphate carboxylase/oxygenase; SPP – sucrose-phosphate phosphatase; SPS – sucrose-phosphate synthase; Suc – sucrose; TIM – triosephosphate isomerase; TK – transketolase; TP – triose phosphate; TPT – triose phosphate/phosphate translocator; UGPase – UDPG-pyrophosphorylase; UDPG – UDP-glucose; YFP – yellow fluorescent protein; Xul – xylulose; Xul 5-P – xylulose 5-phosphate.

## I. Introduction

Chloroplasts are the sites of photosynthetic carbon assimilation in plant cells. The net product of carbon assimilation by the reductive pentose phosphate pathway, triose phosphate, serves as principle precursor for all biosynthetic reactions in plants. Recently assimilated carbon is shuttled into starch and sucrose biosynthesis, nitrogen and sulfur metabolism, fatty acid biosynthesis, cell wall biosynthesis, secondary metabolism and a puzzling variety of other metabolic routes. A key question in plant physiology is how the allocation of carbon to the individual pathways is regulated. Levels of control include, but are not limited to, transcriptional, translational, posttranslational and allosteric regulation of enzyme activity, sub-cellular compartmentation of metabolic pathways, and the distribution of specific pathways between different plant tissues. Environmental factors such as temperature, light intensity and water supply need to be integrated with developmental programs, nutrient status, effects of biotic and abiotic stresses, and source-sink interactions.

The initial step in the allocation of recently assimilated carbon to different metabolic routes is the partitioning of triose phosphates between the plastid stroma and the plant cell cytosol. In addition, reduced carbon

can be withdrawn from the regenerative phase of the Calvin-Benson cycle, e.g., to fuel the plastid-localized Shikimic acid pathway.

## II. Biosynthesis of Sucrose and Transitory Starch

Carbohydrates that are synthesized in chloroplasts by the reductive pentose phosphate pathway during the day are mainly exported to the cytosol as triose phosphate by the triose phosphate/phosphate translocator (TPT; Fliege *et al.*, 1978; Flügge *et al.*, 1989; Flügge, 1999). The predominant fate of triose phosphate in the cytosol is the conversion to sucrose, which serves as the transport form of photoassimilates that are allocated to sink tissues. Under conditions when the rate of photosynthesis exceeds the rate of sucrose biosynthesis, a significant portion of recently assimilated carbon dioxide (up to 50%) is stored within the chloroplast as transitory starch. During the following dark period, this transitory starch is remobilized to support a continuous supply of carbon to developing sink organs and energy metabolism in leaves.

In the following sections, the biosynthesis of sucrose and starch will be briefly summarized to provide the background for the integration of these pathways by transporters in the chloroplast envelope membrane. For a more detailed treatise, the reader is referred to several recent reviews (Huber and Huber, 1996; Smith, 1999; Kossmann and Lloyd, 2000; Zeeman *et al.*, 2002; Lunn and MacRae, 2003; Koch, 2004).

### *A. Synthesis and Export of Triosephosphates*

#### *1. Reduction of 3-PGA to Triosephosphates*

Carbon dioxide is assimilated into organic C-compounds by the stroma-localized reductive pentose phosphate pathway (RPPP; Calvin-Benson cycle). The carboxylation of the carbon dioxide acceptor ribulose 1,5-*bis*phosphate (Ru-1,5-*b*P) is catalyzed by the enzyme ribulose 1,5-*bis*phosphate carboxylase/oxygenase (Rubisco), yielding the unstable C6 intermediate 2-carboxy-3-ketoarabinitol-1,5-*bis*phosphate that hydrolyses non-enzymatically into two molecules of 3-phosphoglyceric acid (3-PGA). 3-PGA represents the first stable intermediate of carbon fixation. After completion of the carboxylation phase of the RPPP, 3-PGA is reduced to glyceraldehyde-3-phosphate (GAP) in the reducing phase of the RPPP. In an ATP-consuming reaction that is catalyzed by phosphoglycerate kinase, 3-PGA is activated to 1,3-*bis*phosphoglycerate and then reduced to GAP by NADPH-dependent glyceraldehyde phosphate dehydrogenase (GAPDH). Triosephosphate isomerase (TIM) catalyzes the reversible inter-conversion between GAP and dihydroxyacetone 3-phosphate (DAP). GAP and DAP represent the end products of the reducing phase of the RPPP. Only one out of six synthesized triose phosphates can be withdrawn from the Calvin-Benson cycle. Five out of six TPs are recycled to Ru-1,5-*b*P in the regenerative phase of the RPPP.

#### *2. Export of Triosephosphates to the Cytosol*

Triose phosphates not required for the regeneration of the $CO_2$ acceptor Ru-1,5-*b*P represent the first important branch point in the allocation of recently assimilated carbon dioxide to different metabolic routes. They can either remain inside the chloroplast to enter starch biosynthesis or other plastid-localized pathways, or they can be exported to the cytosol (Fig. 1A). Chloroplasts are bound by a double membrane, the chloroplast envelope. Hence, solutes cannot freely permeate between the chloroplast stroma and the surrounding cytosol. Plastid metabolism is interfaced with cytosolic metabolism by solute transporters that reside in the inner chloroplast envelope membrane. These transport proteins ensure the specific exchange of solutes between plastid and cytosol. The export of the triose phosphates GAP and DAP to the cytosol is mediated by the triose phosphate/phosphate translocator. This transporter catalyzes the strict counter-exchange of phosphorylated C3 compounds such as triose phosphates and 3-PGA with inorganic phosphate (Pi; Fig. 1B). The strict stoichiometry of the counter-exchange is important for the maintenance of phosphate homeostasis in the stroma because inorganic phosphate is required for the biosynthesis of ATP from ADP and Pi in the light reaction of photosynthesis. Depletion of the plastidal phosphate pool by unbalanced export of phosphate in the form of triose phosphates would lead to inhibition of photosynthetic electron transport (Sharkey, 1985) and ultimately to damage of the photosynthetic machinery.

#### *3. Possible Role of the 3-PGA/Triosephosphate Shuttle*

TPT not only catalyzes the strict counter-exchange of TPs with Pi but also the counter-exchange of 3-PGA with TPs. TP exported to the cytosol can be oxidized

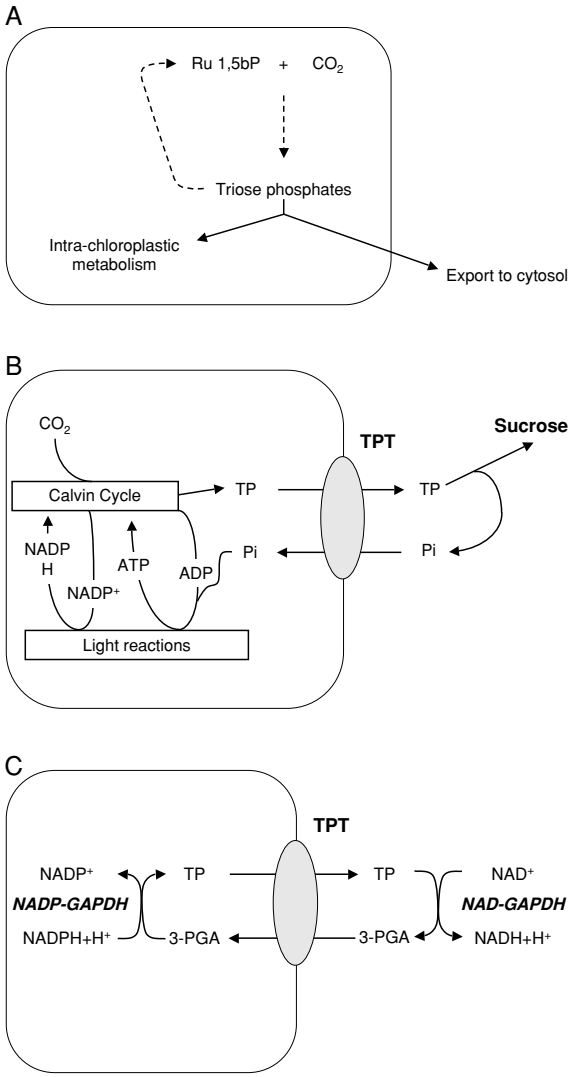


*Fig. 1.* The central role of triose phosphates in plant metabolism. (A) Triose phosphates not required for regeneration of the carbon dioxide acceptor molecule ribulose 1,5-bisphosphate can be either exported to the cytosol to drive, e.g., sucrose biosynthesis or they can remain inside the plastid where they can enter metabolic pathways such as starch and terpenoid biosynthesis. Triose phosphates thus represent the first important branch point in the allocation of recently allocated carbon dioxide to different metabolic routes. (B) Coupling of cytosolic sucrose biosynthesis with light and dark reactions by the triose phosphate/phosphate translocator (TPT). Export of organically bound phosphate from plastids in the form of triose phosphates (TP) must be coupled to import of inorganic phosphate (Pi) to prevent depletion of the plastidal phosphate pool. Pi is required inside the stroma as substrate for photophosphorylation. (C) Export of redox equivalents from plastids by the 3-phosphoglycerate (3-PGA)/TP shuttle. Redox equivalents can be indirectly exported from the plastid stroma in form of reduced TPs that can be oxidized to 3-PGA in the cytosol by NAD-dependent glyceraldehyde-phosphate dehydrogenase (NAD-GAPDH), yielding one NADH per TP. 3-PGA is shuttled back to the stroma by TPT to be reduced to TP in the Calvin-Benson cycle.

to 3-PGA by the cytosolic NAD-GADPH, yielding NADH and 3-PGA. 3-PGA is transported back into the stroma by TPT and reduced to TP by NADP-GAPDH. Thus, redox power can be transferred from the stroma to the cytosol by the 3-PGA/TP shuttle (Fig. 1C).

## *B. Sucrose Biosynthesis*

### *1. Overview of Sucrose Biosynthesis During the Day*

During photosynthesis, sucrose (Suc) is synthesized in the cytosol from the triose phosphate that is exported from the plastid. Two molecules of TP are combined to form one molecule of fructose 1,6-*bis*phosphate (Fru 1,6*b*P) in a reversible reaction catalyzed by aldolase. Fru 1,6*b*P is dephosphorylated to fructose 6-P (Fru 6P) by fructose 1,6-*bis*phosphatase (FBPase). Thereby Fru 6P enters the cytosolic hexose phosphate pool. Phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM) catalyze the conversion of Fru 6P to glucose 6-phosphate (Glu 6P) and Glu 1P, respectively. Glc 1P is activated to UDP-glucose (UDPG) by UDPG-pyrophosphorylase (UGPase). The first committed step of Suc biosynthesis is catalyzed by sucrose phosphate synthase (SPS) that converts UDPG and Fru 6P to sucrose-phosphate and pyrophosphate (PPi). Sucrose-phosphate is subsequently dephosphorylated to Suc by sucrose-phosphate phosphatase (SPP). During Suc biosynthesis, all the organically bound phosphate is converted to Pi that is available as counter-exchange substrate for the export of additional TPs from the stroma. Hence, the export of TPs from the stroma is coupled to the rate of Suc biosynthesis by the availability of Pi in the cytosol (Fig. 1B).

### *2. Critical Role of Cytosolic Fructose 1,6-Bisphosphatase*

The conversion of TP to Fru 6P is controlled by the regulatory metabolite fructose 2,6-*bis*phosphate (Fru 2,6*b*P), which is a strong inhibitor of FBPase (Stitt *et al.*, 1987; Stitt, 1990). The concentration of Fru 2,6*b*P increases markedly upon transition from light to dark, essentially switching off FBPase activity (Servaites *et al.*, 1989b). Hence, TP cannot be converted to Suc in the dark.

## *C. Transitory Starch Biosynthesis*

Up to one-half of the carbon dioxide that is assimilated in the chloroplasts by photosynthesis during the day is not exported to the cytosol in the form of triose

phosphate but is maintained in the chloroplast as transitory starch. During the following dark period, transitory starch is mobilized to sustain a continuous supply of carbon, mainly in the form of sucrose, for export to sink organs as well as for energy metabolism in leaves. Transitory starch shows a relatively high turnover. Depending on environmental conditions and plant species, all transitory starch that has accumulated during the day can be remobilized during the dark period.

Starch synthesis and degradation are important for plant growth and development and for the adaptation of plants to the environment. Mutants deficient in starch synthesis (Caspar *et al.*, 1985; Hanson and McHale, 1988; Lin *et al.*, 1988a, b; Huber and Hanson, 1992; Kofler *et al.*, 2000) or starch degradation (Caspar *et al.*, 1991; Zeeman *et al.*, 1998a; Zeeman *et al.*, 1998b; Critchley *et al.*, 2001) show reduced growth and delayed flowering under conditions in which photosynthesis is restricted. The effects of deficiencies in starch biosynthesis and degradation have mainly been studied in model plant systems (i.e. *Arabidopsis*) under controlled environmental conditions – even more dramatic effects on plant performance are to be expected under natural growth conditions. The phenotype of these mutants (reduced growth and delayed flowering) becomes more severe with decreasing day length but is alleviated in continuous light, emphasizing the importance of the transitory starch pool for carbon supply in the dark. Suc levels remain relatively constant throughout the diurnal cycle in wild type plants whereas they drop dramatically during the dark period in mutants with impaired turnover of transitory starch (Zeeman and ApRees, 1999) and in starch-free mutants (A.P.M. Weber, unpublished).

## III. Breakdown of Transitory Starch and Export of Breakdown Products

Until very recently, surprisingly little was known about the degradation of transitory starch in leaves. In contrast, the biochemical and molecular processes involved in the degradation of cereal endosperm starch are understood in great detail. The cellular structure and the amyloplasts in cereal endosperm disintegrate during desiccation of the endosperm tissue. Hence, the starch-storing cereal endosperm represents an acellular tissue that is directly accessible to $\alpha$-amylases that are secreted by the aleurone layer of the seed coat during germination, and the resulting glucose can be taken up by the growing embryo via the scutellum. However, transitory starch in leaves and storage starch found in potato and many other tubers is stored within intact plastids and the integrity of plastids is maintained during starch degradation. Consequently, starch degradation in cereal endosperm cannot serve as a model for the degradation of transitory and storage starch in other tissues. In the following sections, the recent advances in our understanding of the breakdown of transitory starch will be reviewed.

### *A. Nocturnal Breakdown of Transitory Starch*

#### *1. Breakdown of Transitory Starch Allows Sucrose Biosynthesis at Night*

During the day, recently fixed carbon is exported from chloroplasts in the form of TP, which is converted in the cytosol to Suc. This Suc serves as the predominant photoassimilate that is allocated to sink tissues and, as such, is the major osmolyte driving phloem translocation. During the dark period, TP production by photosynthesis ceases and the supply of carbon for Suc production comes from the breakdown of transitory starch. Hence, in addition to providing reduced carbon for sink tissues, the continued production of sucrose in source tissues is also required to maintain phloem transport during the dark period.

#### *2. The Breakdown of Transitory Starch Occurs Mainly by the Hydrolytic Pathway*

The actual pathway for the breakdown of transitory starch was mostly unknown until recently. It was frequently stated that phosphorolysis of starch represents the major pathway for starch degradation in the dark and that triose phosphates are the major export products from chloroplasts at night. However, during the past two decades, compelling evidence for a predominant role of the hydrolytic pathway has accumulated (see Smith *et al.*, 2003 and Sharkey *et al.*, 2004 for recent reviews).

Phosphorolytic starch breakdown would lead to the export of TP from plastids and the conversion of TP to sucrose would require cytosolic FBPase. However, as outlined above, cytosolic FBPase is switched off in the dark by the regulatory metabolite Fru 2,6*b*P, effectively blocking the conversion of TP to Fru 1,6*b*P (Servaites *et al.*, 1989a; Servaites *et al.*, 1989b; Stitt, 1990). In addition, it was demonstrated that TP and Fru 1,6*b*P levels decrease to almost zero in the dark (Gerhard *et al.*, 1987), essentially ruling out a major flux from TP to Suc in the dark. The critical role of cytosolic FBPase in the conversion of TP to Suc was further emphasized by *Flaveria linearis* plants deficient in this enzyme

(Sharkey *et al.*, 1992; Micallef *et al.*, 1996; Micallef and Sharkey, 1996) and by transgenic potato plants in which the expression of FBPase was repressed by an antisense construct (Zrenner *et al.*, 1996). These mutant and transgenic plants do not export sucrose from source tissues during the day because the conversion of TP to Suc is blocked. In stead, assimilated carbon is stored as transitory starch and starch is mobilized during the dark period by a pathway that obviously does not involve cytosolic FBPase.

An FBPase-independent pathway of Suc biosynthesis during the dark period was also demonstrated in transgenic potato plants showing antisense repression of TPT (Riesmeier *et al.*, 1993; Heineke *et al.*, 1994). Similar to the FBPase mutants described above, these transgenic potato plants synthesize less sucrose during the day and allocate a higher portion of recently assimilated $CO_2$ into transitory starch. This is compensated by a higher rate of starch turnover at night that is accompanied by increased sucrose export from source tissues. Interestingly, tobacco plants showing antisense repression of TPT (Häusler *et al.*, 1998; Häusler *et al.*, 2000a,b) and an Arabidopsis mutant carrying a T-DNA insertion in the TPT-gene (Schneider *et al.*, 2002) did not show massively reduced Suc biosynthesis in the light although TPT activity was reduced by 70% in tobacco or absent in the Arabidopsis mutant, respectively. Obviously sucrose biosynthesis occurs by a pathway that does not involve TPT in these plants (see below). Suc metabolism at night was unaltered, indicating that TPT does not play a role during starch breakdown.

Schleucher *et al.* (1998) found that the labeling pattern of sucrose observed after deuterium feeding of bean leaves in the dark can only be explained by a hydrolytic breakdown of starch, demonstrating that starch conversion to Suc does not involve TP. However, the labeling pattern did not rule out the possibility of hexoseP export from chloroplasts. This seems unlikely because the same study demonstrated sub-cellular concentrations of Pi and Glc 6P that are not suggestive of Glc 6P export from chloroplasts. Furthermore, the activity of a glucose 6-phosphate/phosphate translocator (GPT) was only detectable in non-green plant tissues and its expression was restricted to heterotrophic tissues (Kammerer *et al.*, 1998). Further support for the absence of significant Glc 6P transport from mesophyll chloroplasts comes from the observation that a mutant in the plastidal isozyme of phosphoglucoisomerase (conversion of Fru 6P to Glc 6P) contains substantial amounts of starch in non-green tissues whereas photosynthetically active tissues are essentially starch free (T. Yu *et al.*, 2000). In addition, at least in Arabidopsis, no pathway exists for Glc 1P transport into plastids because a mutation in the plastidal isozyme of phosphoglucomutase (conversion of Glc 6P to Glc 1P) leads to a starch free phenotype (Caspar *et al.*, 1985; Kofler *et al.*, 2000). These findings make it unlikely that Glc 6P or Glc 1P can be exported to the cytosol as a product of starch breakdown (see also reviews by Fischer and Weber, 2002 and Weber, 2004).

Striking evidence against a significant role of the phosphorolytic pathway in nocturnal starch turnover comes from the observation that an Arabidopsis T-DNA insertion knockout mutant deficient in plastidal starch phosphorylase is not affected in sugar or starch metabolism (Smith *et al.*, 2003). In contrast, an Arabidopsis mutant deficient in plastidal endoamylase (*sex4*; Zeeman *et al.*, 1998a; Zeeman and ApRees, 1999) displays a starch excess phenotype that is caused by reduced turnover of transitory starch during the night. This demonstrates that endoamylase ($\beta$-amylase) is required for efficient breakdown of transitory starch. Moreover, antisense repression of exoamylase ($\alpha$-amylase) in transgenic potato plants also led to a starch excess phenotype in leaves (Scheidig *et al.*, 2002), demonstrating that $\alpha$-amylase is essential for nocturnal starch degradation. The plastidal disproportionating enzyme (D-enzyme) was also recently shown to play an important role in starch degradation (Critchley *et al.*, 2001). An Arabidopsis T-DNA insertion mutant deficient in D-enzyme had reduced rates of starch degradation. In addition, large amounts of maltotriose accumulated after onset of the dark period. Obviously, D-enzyme is required for effective starch degradation. Its role is most likely the to metabolize glucans that are too short to be attacked by $\alpha$-amylase or phosphorylase (Critchley *et al.*, 2001).

The cumulative evidence outlined above strongly indicates that transitory starch is broken down by the concerted action of endo- and exoamylases, and the D-enzyme.

### *3. The Critical Role of Starch Phosphorylation for Transitory Starch Degradation*

The presence of monoesterified phosphate groups in starch was known for long time. However, the functional relevance of and the mechanism for starch phosphorylation were unknown (see Blennow *et al.*, 2002 for review). Lorberth *et al.* (1998) demonstrated that antisense-repression of a gene encoding a starch-granule bound protein from potato tuber starch of

previously unknown function (R1) led to a starch-excess phenotype in leaves and to decreased starch degradation in cold-stored tubers. Surprisingly, the reduced degradation of starch was correlated with a strong reduction in starch phosphate content, indicating that (i) R1 is involved in determining the degree of starch phosphorylation, and that (ii) the degree of starch phosphorylation is important for degradation of starch. Later, it was shown that R1 reversibly binds to starch during starch degradation in the dark and dissociates from starch granules in the light and therefore might be involved in the regulation of starch turnover (Ritte *et al.*, 2000). The Arabidopsis starch excess mutant *sex1* (Caspar *et al.*, 1991) was found to be deficient in R1 (T. Yu *et al.*, 2001). *Sex1* accumulates up to five-times higher starch levels than the wild type and shows no appreciable turnover of starch (Trethewey and ap Rees, 1994). Starch isolated from *sex1* has drastically reduced phosphate content and the extent of starch accumulation was inversely correlated with the degree of starch phosphorylation (T. Yu *et al.*, 2001). A careful comparison of R1 to proteins of known function led to the proposal that R1 might catalyze the phosphorylation of starch in a dikinase-type reaction using ATP as phosphoryl donor (T. Yu *et al.*, 2001). Subsequently, Ritte *et al.* (2002) demonstrated that recombinant R1 has the activity of a starch-water dikinase.

Although the precise role of starch phosphorylation by R1 is still unknown, the phenotype of R1 antisense and knockout plants clearly underlines the importance of phosphorylation for transitory starch degradation.

### *4. Export of Starch Breakdown Products from Chloroplasts*

The end products of hydrolytic starch breakdown are glucose and maltose and these end products need to be exported from the chloroplast to the cytosol. Schäfer *et al.* (1977) found the activity of a glucose transporter in the chloroplast envelope membrane, and Herold *et al.* (1981) and Beck (1985) reported that isolated chloroplasts have the activity of a maltose transporter. The maltose transporter was not inhibited by glucose and the glucose transporter was not inhibited by maltose or maltooligosaccharides, indicating that chloroplasts posses two distinct transport systems for Glc and Mal (Rost *et al.*, 1996). Moreover, isolated chloroplasts were found to be permeable to Glc and Mal, but not to larger maltodextrins (Rost *et al.*, 1996). Several recent studies showed that starch-laden chloroplasts export Glc and Mal in the dark (Servaites and Geiger, 2002; Ritte and Raschke, 2003; Weise *et al.*, 2004) and it was concluded that Mal is the major form of carbon export from chloroplasts at night (Weise *et al.*, 2004).

A putative chloroplast envelope glucose transporter (pGlcT) was identified using a differential labeling procedure and the corresponding gene was cloned from a number of plant species (Weber *et al.*, 2000). pGlcT is distantly related to monosaccharide/proton co-transporters of the plant plasma membrane but similar to facilitative glucose transporters of mammalian red blood cells and liver cells (Weber *et al.*, 2000). A recent study showed that pGlcT is not only expressed in starch-containing tissues, but also in starch-free tissues such as ripening olive fruits (Butowt *et al.*, 2003). This finding indicates that pGlcT may have additional functions other than glucose export from plastids. We have recently identified a T-DNA insertion mutant for pGlcT in Arabidopsis. So far, these mutants have not revealed the in planta role of pGlcT because the mutant exhibits an embryo lethal phenotype and it was therefore not possible to isolate homozygous knockout mutants (J. Truchina and A.P.M. Weber, unpublished). Additional studies and the isolation of additional mutant alleles will be required to unravel a potential role of pGlcT in starch metabolism. In addition to pGlcT (At1g16150), the Arabidopsis genome encodes two other putative plastidal monosaccharide transporters (J. Truchina and A.P.M. Weber, unpublished). Peptide-tags for pGlcT and for one of the other putative plastidal monosaccharide transporters (At5g59250) have been reported from isolated Arabidopsis chloroplast envelope membranes (Ferro *et al.*, 2003), confirming the presence of at least two distinct putative monosaccharide transporters in the chloroplast envelope membrane.

The plastidal maltose transporter was recently identified and the importance of maltose export from chloroplasts at night was demonstrated by the severe phenotype of the corresponding knockout mutant (Niittylä *et al.*, 2004). The transporter was identified by isolating two allelic mutants (*mex1*-1, *mex1*-2) exhibiting high starch levels and very high maltose levels. The gene affected in these mutants (*MEX1*, At5g17520) was identified by positional cloning and was shown to encode a novel type of maltose transporter (MEX1) that is unrelated to other sugar transporters. MEX1 is a hydrophobic membrane protein with nine $\alpha$-helical transmembrane domains and it carries a chloroplast transit peptide. The targeting of MEX1 to the chloroplast was demonstrated by a MEX1-YFP fusion (Niittylä *et al.*, 2004) and a peptide tag derived from MEX1 independently confirmed the plastidal localization of MEX1 (Ferro *et al.*, 2003). MEX1 was previously known as RCP1 (Tsugeki and Fedoroff, 1999)

and shown to be expressed in leaves and roots of Arabidopsis plants; however, its function was unknown. *Mex1* plants were smaller in comparison to the wild type and their chlorophyll content was 60% lower than in the wild type while maltose levels were 40-fold higher. A double mutant generated by a genetic cross of *mex1* with *dpe1* (knockout in the plastidal D-enzyme) shows an even more dramatic phenotype, characterized by extremely slow growth and very low chlorophyll content (Niittylä *et al.*, 2004). In *mex1*, some carbohydrate resulting from starch breakdown can still be exported in the form of Glc. Glc is formed in the stroma during starch degradation by the action of the D-enzyme (Critchley *et al.*, 2001). In the *mex1/dpe1* double mutant, however, both the export of maltose from chloroplasts and the metabolism of maltotriose to glucose are blocked and little or no carbohydrate from starch can be exported.

The severe growth retardation and the pale green phenotypes of *mex1* plants and of *mex1*/*dpe1* double mutants emphasizes the importance of maltose export from chloroplasts at night and provides compelling evidence for the predominant role of the hydrolytic pathway of starch breakdown.

## 5. Fate of Starch Breakdown Products in the Cytosol

Hydrolytic starch breakdown yields glucose and maltose as end products and these metabolites are exported to the cytosol by specific, distinct glucose and maltose transporters in the chloroplast envelope. In the cytosol, Glc is phosphorylated to Glc 6P by hexokinase and thereby enters the cytosolic hexoseP pool. A hexokinase activity on the cytosolic side of the chloroplast outer envelope membrane was shown previously (Stitt and ap Rees, 1979) and it was demonstrated that spinach hexokinase 1 is anchored in the chloroplast outer envelope membrane (Wiese *et al.*, 1999). It was proposed that the chloroplast-associated hexokinase is involved in the energization of Glc export from chloroplasts, thereby efficiently coupling Glc export with downstream Glc metabolism (Wiese *et al.*, 1999; Weber *et al.*, 2000). Evidence for an important role for hexokinase in nocturnal starch metabolism comes from the observation that antisense repression of hexokinase in potato plants leads to a starch excess phenotype in leaves (Veramendi *et al.*, 1999).

In contrast to cytosolic Glc metabolism, not much is known about the fate of maltose in the cytosol. Maltase and maltose-phosphorylase activities have been reported from leaves of several plant species. However, a search of the Arabidopsis genome did not reveal candidate genes encoding the corresponding proteins. In *E. coli*, Mal is metabolized to Glc and Glc 1P by the concerted action of two enzymes: amylomaltase (MalQ) and maltodextrin phosphorylase (MalP; see Boos and Shuman, 1998 for review). MalQ transfers maltosyl and longer dextrinyl residues onto Glc, Mal, or longer maltodextrins; the smallest substrate is maltotriose. It releases Glc from the reducing end, and transfers a maltosyl residue on the reducing end of the acceptor (Glc, Mal, or larger maltodextrin). MalP forms Glc 1P by phosphorylysis of the reducing end Glc-residue from larger maltodextrins. The concerted action of MalQ and MalP converts Mal to one molecule of Glc and one molecule of Glc 1P (Boos and Shuman, 1998). The reaction mechanism of MalQ is similar to that of the plastidal D-enzyme. Lu and Sharkey (2004) have searched the Arabidopsis genome for genes encoding proteins with similarity to MalQ from *E. coli* and found two proteins in the Arabidopsis genome are related to MalQ from *E. coli*: (i) the plastidal disproportionating enzyme that was previously shown to have a critical role in starch breakdown in Arabidopsis (At5g64860; Critchley *et al.*, 2001) and (ii) a second gene of unknown function (At2g40840). Both proteins can be clearly recognized as type II 4-$\alpha$-glucanotransferases. As expected for the plastidal D-enzyme, TargetP (Emanuelsson *et al.*, 2000) predicts plastidal localization of At5g64680. However, according to TargetP, At2g40840 is most likely a cytosolic enzyme. Hence, At2g40840 was called cytosolic 4-$\alpha$-glucanotransferase 1 (CGT1; Lu and Sharkey, 2004). Based on its similarity to *E. coli* MalQ, Lu and Sharkey (2004) hypothesized that CGT1 might be involved in cytosolic maltose metabolism in plants. The hypothesis was tested by isolation of two independent knockout alleles for *CGT1* and analysis of starch and carbohydrate metabolism in these mutants. *Cgt1* mutants exhibit a dwarf phenotype, are pale green, and accumulate large amounts of maltose. This phenotype resembles that of the maltose transporter mutant *mex1* and indicates that CGT1 is in fact involved in maltose metabolism. A massive accumulation of maltose was also observed in *E. coli* mutants lacking the activity of MalQ (Szmelcman *et al.*, 1976), further supporting the hypothesis that CGT1 is required for maltose metabolism in plants. Additional mutant alleles of *CGT1* were recently reported and the localization of CGT1 in the cytosol was demonstrated by immunological methods (Chia *et al.*, 2004).

However, as outlined above, the conversion of maltose to Glc and Glc 1P in *E. coli* requires two proteins, the 4-$\alpha$-glucanotransferase MalQ (amylomaltase) and the maltodextrin phosphorylase MalP. Lu and Sharkey (2004) proposed that the cytosolic isozyme of starch phophorylase in plants has the function similar to maltodextrin phosphorylase in *E. coli*. In fact, a search of the Arabidopsis genome with MalP from *E. coli* revealed two proteins with high similarity to MalP: (i) At3g29320 and (ii) At3g46970. At3g29320 represents the plastid-targeted starch phosphorylase isozyme B whereas At3g46970 represents the cytosolic starch phosphorylase isozyme H. Cytosolic isoforms of starch phosphorylase have been reported many times, however, their functions in plant metabolism remained unclear. The proposal that starch phosphorylase H has maltodextrin phosphorylase activity and is involved in maltose metabolism in the cytosol is attractive and reasonable. However, antisense repression of phosphorylase H in potato did not reveal any significant influence on starch or carbohydrate metabolism (Duwenig *et al.*, 1997), thus weakening the above-mentioned hypothesis. However, the lack of an appreciable effect on starch and sugar metabolism in the transgenic potato plants may be due to residual activity of starch phosphorylase H. Interestingly, antisense repression of D-enzyme in potato to almost undetectable levels did not lead to measurable effects on starch metabolism (Takaha *et al.*, 1998) but a complete knockout of D-enzyme in Arabidopsis led to a massive accumulation of maltotriose and reduced rates of nocturnal starch degradation (Critchley *et al.*, 2001). Hence, knockout plants in Arabidopsis will have to be analyzed for further clarification of the putative role of starch phosphorylase H in plants.

The proposed pathway for maltose metabolism in the cytosol by CGT1 and phosphorylase H would also elegantly explain the presence of a cytosolic polysaccharide fraction that binds tightly to the cytosolic phosphorylase (Yang and Steup, 1990). In analogy to the MalQ/MalP system in *E. coli*, the amylomaltase MalQ would produce a maltodextrin by subsequently adding Glc residues from Mal onto the reducing end of the maltodextrin. This maltodextrin is proposed to be identical to the cytosolic polysaccharide fraction described by Yang and Steup (Yang and Steup, 1990). The plant ortholog of MalP, starch phosphorylase H, would subsequently release Glc 1P from the maltodextrin as soon as the chain length had reached the critical length of five Glc residues (Lu and Sharkey, 2004; see also Weber, 2004 for a recent review).

### *6. The Severe Phenotype of Mutants Defective in Maltose Metabolism is Surprising*

It is surprising that mutants defective in plastidal and cytosolic maltose metabolism display a much more severe phenotype than starch-free mutants. As outlined above, mutants deficient in starch biosynthesis are characterized by slow growth and delayed flowering and the phenotype can be alleviated by decreasing the length of the dark period. The same holds true for mutants that are severely impaired in starch degradation and do not accumulate maltose such as *sex1*. However, the maltose-accumulating mutants *mex1* and *cgt1* grow even slower than starch-free mutants and are pale green, although they show considerable turnover of transitory starch. Most likely, the severe phenotypes of *mex1* and *cgt1* can be attributed to the large accumulation of maltose. This may be due to osmotic effects or to a potential triggering of sugar-responsive signaling pathways by maltose that leads to reduced expression of photosynthetic genes (Niittylä *et al.*, 2004; Sharkey *et al.*, 2004).

## *B. Breakdown of Transitory Starch in the Light—The Starch Pool is Accessible During the Day*

It is generally accepted that transitory starch is synthesized during the day and broken down at night. The transitory starch pool does not seem to be accessible during active photosynthesis and it was recently demonstrated by pulse-chase labeling experiments that Arabidopsis plants do not turn over transitory starch in the light (Zeeman *et al.*, 2002). However, results from transgenic plants indicate that transitory starch can be broken down in the light and that the starch pool can act as a dynamic carbon buffer during active photosynthesis under certain conditions.

As outlined above, the triosephosphate/phosphate translocator TPT represents the day path for carbon export and does not play a significant role in the export of starch breakdown products at night.

However, when the regular day path for carbon export from chloroplasts is blocked by antisense repression of TPT expression or knockout of the TPT-gene, an alternative pathway for carbon export to the cytosol involving the transitory starch pool comes into play.

Antisense repression of TPT in transgenic tobacco plants reduced its activity by more than 70% in comparison to the wild type (Häusler *et al.*, 1998). It was demonstrated by pulse-chase studies and analysis of

starch and soluble sugars throughout the photoperiod that tobacco plants with reduced TPT activity allocate relatively more recently fixed carbon dioxide into transitory starch pool. This is accompanied by increased starch breakdown in the light, increased activity of starch degrading enzymes, an increase in glucose export capacity from chloroplasts, and by increased hexokinase activity (Häusler *et al.*, 1998). Thus, recently assimilated carbon, instead of being exported to the cytosol in form of triose phosphates, is converted into glucose and maltose in the stroma by shuttling through the transitory starch pool. Subsequently, the neutral sugars are exported to the cytosol by specific transporters, thereby bypassing the bottleneck generated by the reduction in TPT activity. In a follow-up study, it was demonstrated that TPT activity does not limit photosynthetic carbon dioxide assimilation under standard growth conditions (Häusler *et al.*, 2000a; Häusler *et al.*, 2000b). However, maximal rates of carbon dioxide assimilation were severely reduced in antisense-TPT tobacco plants at saturating $CO_2$ and light conditions. Overexpression of TPT increased photosynthetic capacity under saturating conditions, indicating that wild type levels of TPT activity can limit $CO_2$ assimilation under saturating conditions (Häusler *et al.*, 2000a; Häusler *et al.*, 2000b).

The results obtained with transgenic tobacco plants were recently complemented by Arabidopsis T-DNA insertion mutants in the TPT gene (*tpt-1*; (Schneider *et al.*, 2002). Similar to the transgenic tobacco plants, *tpt-1* did not display an apparent phenotype under standard growth conditions. The loss of TPT activity in *tpt-1* was bypassed by increased allocation of recently assimilated carbon into the starch pool in the light. However, a simultaneous knockout of starch biosynthesis by crossing *tpt-1* to a mutant lacking AGPase-activity led to a dramatic dwarfish phenotype, clearly demonstrating that starch biosynthesis is the major salvage pathway for the compensation of a TPT knockout. Astoundingly, a genetic cross of *tpt-1* to the starch excess mutant *sex1* caused only minor growth retardation and a moderate phenotype. As outlined above, *sex1* is deficient in a starch, water dikinase activity that is required for the turnover of transitory starch in Arabidopsis and transitory and storage starch in potato (Lorberth *et al.*, 1998; T. Yu *et al.*, 2001). Interestingly, the abundance of a (most likely plastidal) water-soluble polydextran was increased in the *tpt-1/sex1* double mutant. This indicates the presence of a highly mobile maltodextrin pool that may serve as a temporary carbon store in the absence of starch turnover (Schneider *et al.*, 2002).

These findings clearly demonstrate that transitory starch can be broken down in the light when triose-phosphate export from chloroplasts becomes limiting or is abolished. However, this effect seems to be species-dependent. Transgenic potato plants showing antisense repression of TPT use a different strategy. These plants hyper-accumulate starch during the day and show increased carbon export from plastids (and consequently sucrose export from source leaves) at night (Heineke *et al.*, 1994). Moreover, a naturally occurring *Flaveria linearis* mutant deficient in cytosolic FBPase does not synthesize sucrose during the day. Instead, assimilated carbon is allocated into transitory starch and converted to sucrose via hydrolytic starch breakdown at night (Sharkey *et al.*, 1992). These species-dependent (and probably also growth-condition dependent) strategies for bypassing deficiencies in triose-phosphate export open up interesting possibilities for studying the regulation of transitory starch turnover.

## IV. Photosynthetic Carbon Oxidation Cycle (PCO)

Although the photosynthetic carbon oxidation (PCO) cycle does not lead to net assimilation of carbon, it constitutes a major carbon flux in photosynthetic cells and has considerable impact on gross end product synthesis. Therefore the carbon oxidation cycle will be reviewed with particular emphasis on the transport steps involved in this highly compartmentalized metabolic pathway.

### *A. Oxidation of Ribulose 1,5-Bisphosphate*

#### *1. Enzymes and Products*

Rubisco is a bifunctional enzyme. It not only catalyzes the productive carboxylation of Ru*b*P but also its non-productive oxygenation (Bowes *et al.*, 1971; Ogren and Bowes, 1971; Bowes and Ogren, 1972). Oxygenation of Ru*b*P leads to the production of one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). Two molecules of 2-PG are recycled to one molecule of 3-PGA by the photosynthetic carbon oxidation cycle. Because this pathway leads to consumption of oxygen (oxygenation of Ru*b*P) and production of carbon dioxide (during the recycling of 2-PG) in the light, it is also called photorespiration.

The pathway and the use of mutants in the study of photorespiration have been reviewed previously

(Ogren, 1984; Leegood *et al.*, 1995; Douce and Neuburger, 1999; Wingler *et al.*, 2000; Somerville, 2001); hence I will provide only a brief outline as background for the following paragraphs. The photorespiratory pathway involves three cellular compartments, namely chloroplasts, peroxisomes and mitochondria. 2-PG produced by Rubisco is dephosphorylated by a specific phosphatase in the chloroplast. The resulting glycolate is exported to the cytosol and imported into peroxisomes where it is oxidized to glyoxylate, which is then transaminated to glycine by either glutamate or serine. Glycine is transported to the mitochondria where two molecules of glycine are converted to one molecule each of serine, ammonia and carbon dioxide by the joint actions of glycine decarboxylase and serine hydroxymethyltransferase (see recent reviews by Douce *et al.*, 2001; Bauwe and Kolukisaoglu, 2003). Serine is transported back to the peroxisomes, converted to glycerate by the joint action of serine glyoxylate aminotransferase and hydroxypyruvate reductase and glycerate is shuttled to the chloroplasts where it is phosphorylated to 3-PGA by glycerate kinase. In summary, the reaction sequence converts two molecules of 2-PG (four carbon atoms) into one molecule of each 3-PGA (three carbon atoms) and carbon dioxide and thus leads to the net loss of one carbon.

### *2. Energy Requirements and Flux Considerations*

The specificity factor of the bifunctional enzyme Rubisco for the carboxylation reaction versus the oxygenation reaction is in the range of 80–100 for most land plants (Spreitzer and Salvucci, 2002). Under current atmospheric conditions this leads to a carboxylation-to-oxygenation ratio of approximately 3 to 1. Hence, the rate of photorespiration would be 25% of the rate of gross $CO_2$ assimilation. These rates have been independently confirmed by short-term labeling studies of the intermediates glycolate, glycine and serine with $^{18}O_2$ (de Veau and Burris, 1988), by using $^{13}CO_2$ and mass spectrometry to determine $CO_2$ fluxes under conditions of steady state photosynthesis (Haupt-Herting *et al.*, 2001), and from the post-illumination photorespiratory $CO_2$ burst (Laisk and Sumberg, 1994).

Photorespiration is an energetically wasteful process. The assimilation of twelve molecules of $CO_2$ (as required for the synthesis of one molecule of sucrose) by the Calvin-Benson cycle requires 36 ATP and 24 NADPH. One NADPH is energetically equivalent to 2.5 ATP, hence an amount of energy equivalent to 96 ATPs is required for the assimilation of twelve molecules of $CO_2$ in the absence of photorespiration. Operating the C2 cycle costs approximately 8.5 ATP per fixed $O_2$: 2 ATP for the glycerate kinase and glutamine synthetase reactions, plus two reduced ferredoxins for the GOGAT reaction, which is energetically equivalent to 2.5 ATP. Another four ATP are required for re-assimilating the $CO_2$ lost by the glycine decarboxylase reaction. Given a ratio of 3:1 for carboxylation to oxygenation reaction, 130 ATP would be required to fix twelve molecules of $CO_2$ in presence of 25% photorespiration. This is 35% more than what is required in the absence of photorespiration. Hence, photorespiration represents a major energy drain.

It also represents a major carbon flux. For each molecule of $CO_2$ evolved by photorespiration, four carbon atoms are moved through the C2 cycle. Thus, a conservative estimate for the carbon flux through the C2 cycle would be 60 to 70% of the photosynthetic $CO_2$ uptake (Siedow and Day, 2000).

## *B. Multiple Plastidal Transport Steps in the PCO Cycle*

As outlined above, the photorespiratory C2 cycle generates a massive flux of carbon in photosynthetic plant cells. The C2 cycle involves chloroplasts, peroxisomes and mitochondria. Pathway intermediates hence need to be transported across several biological membranes (Fig. 2). However, very little is known about the proteins involved in these transport steps.

### *1. Overview of Subcellular Transport Steps*

Glycolate is exported from chloroplasts, taken up by peroxisomes and converted to glycine. Glycine is exported to the cytosol and taken up by mitochondria where two molecules of glycine are converted to serine. Serine is exported to the cytosol and imported into peroxisomes where it is converted to glycerate. Glycerate is released from the peroxisomes and imported into chloroplasts to finally close the photorespiratory carbon cycle. The conversion of serine to glycerate via hydroxypyruvate in peroxisomes requires one NADH that is indirectly imported from the cytosol via an oxaloacetate/malate shuttle. The oxidative decarboxylation of glycine in the mitochondria generates NADH that can be exported to the cytosol by an oxaloacetate/malate shuttle in the mitochondrial membrane. Furthermore, the transamination of glyoxylate to glycine in peroxisomes requires the transfer of glutamate from chloroplasts to peroxisomes and

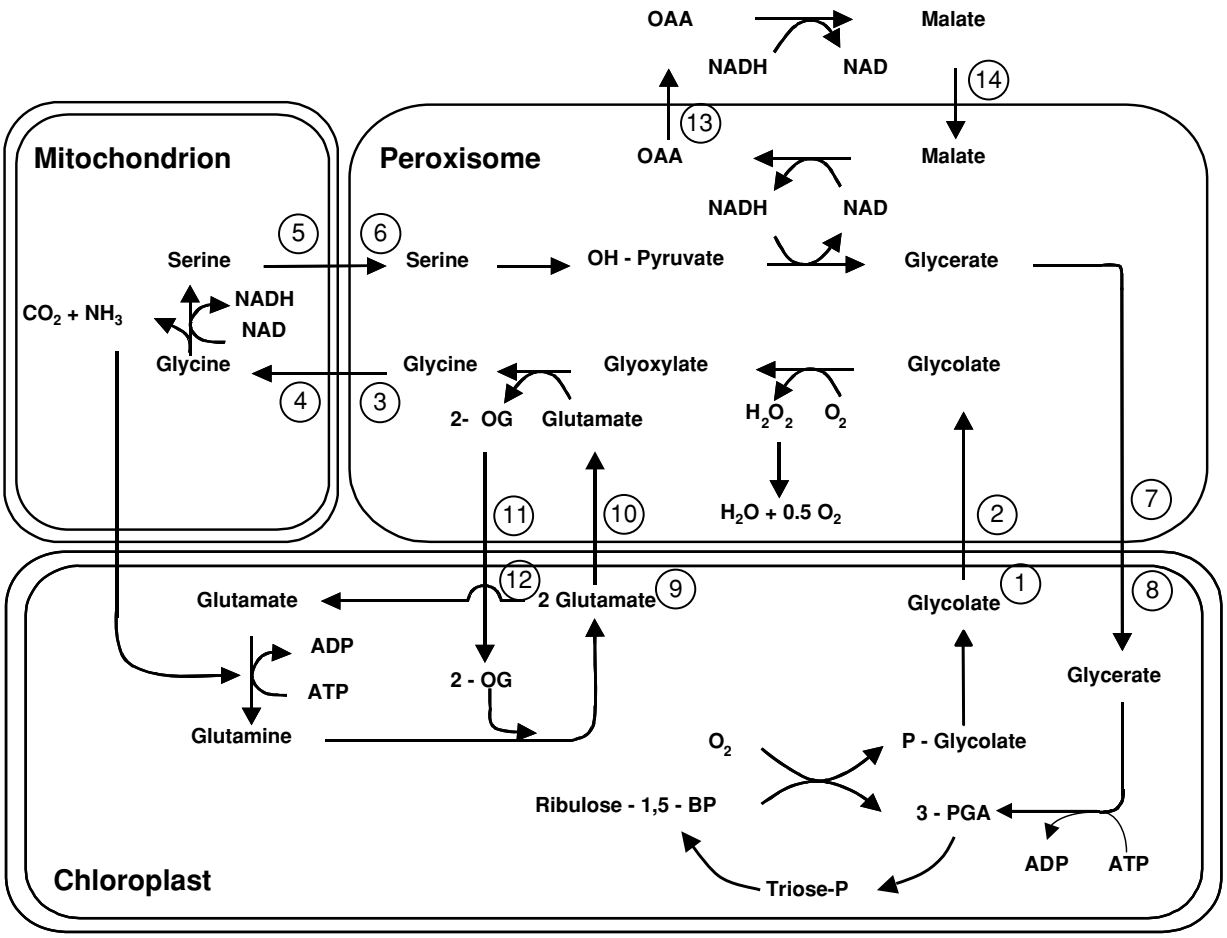


*Fig. 2.* Transport steps in the photorespiratory pathway. The photorespiratory pathway causes a massive flux of metabolites between chloroplasts, peroxisomes, and mitochondria. Not considering ammonia transport, fourteen additional membrane transport steps are involved: (1) export of glycolate from plastids and (2) its import into peroxisomes; (3) export of glycine from peroxisomes and (4) its import into mitochondria; (5) export of serine from mitochondria and (6) its import into peroxisomes; (7) export of glycerate from peroxisomes and (8) its import into chloroplasts. Additional transport steps are required for shuttling glutamate and 2-oxoglutarate between chloroplasts and peroxisomes (9–12) and for the oxaloacetate/malate shuttle between peroxisomes and the cytosol (13–14). With the exception of the plastidal glutamate/malate (9; Renné *et al.*, 2003) and 2-oxoglutarate/malate (12; Weber *et al.*, 1995) translocators, none of the other transporters has been identified at the molecular level to date.

the back-transport of the resulting 2-oxoglutarate to chloroplasts (Fig. 2).

## *2. Transport of 2-Oxoglutarate and Glutamate*

Of the multiple transport steps involved in the photorespiratory carbon cycle, only the plastidal transporters for glutamate and 2-oxoglutarate are known at the molecular level. The uptake of 2-oxoglutarate into plastids and the export of glutamate from plastids are catalyzed by a malate-coupled two-translocator system (Woo *et al.*, 1987; Flügge *et al.*, 1988; J. Yu and Woo, 1992). 2-Oxoglutarate is imported into the chloroplast stroma in counter-exchange with malate by the 2-oxoglutarate/malate translocator (DiT1) and 2-oxoglutarate is converted into glutamate by the concerted actions of glutamine synthetase (GS) and glutamate synthase (GOGAT). The resulting glutamate is exported to the cytosol by the glutamate/malate translocator (DiT2), also in counter-exchange with malate (see Weber and Flügge, 2002 for a recent review). Consequently, 2-oxoglutarate is exchanged for glutamate without net malate transport. DiT1 has been purified from spinach chloroplast envelope membranes, the corresponding cDNA was cloned

and the substrate specificity of yeast-expressed recombinant DiT1 was analyzed in reconstituted liposomes (Weber *et al.*, 1995). The cDNA encodes a protein that accepts dicarboxylic acids such as malate, fumarate and 2-oxoglutarate but not the amino acids glutamate and aspartate (Weber *et al.*, 1995). Recently, it was demonstrated that DiT1 is able to catalyze the counter-exchange of oxaloacetate with malate *in vitro* (Taniguchi *et al.*, 2002; Renné *et al.*, 2003). However, whether DiT1 functions as an oxaloacetate/malate exchanger *in vivo* remains to be demonstrated. The glutamate/malate translocator DiT2 was recently identified from Arabidopsis (Taniguchi *et al.*, 2002), maize (Taniguchi *et al.*, 2004), spinach, sorghum and *Flaveria* (Weber and Flügge, 2002; Renné *et al.*, 2003). DiT2 is a broad-specificity dicarboxylate translocator that accepts the dicarboxylic acids malate, 2-oxoglutarate, fumarate and succinate as well as the amino acids glutamate and aspartate as substrates (Taniguchi *et al.*, 2002; Renné *et al.*, 2003). However, DiT2 has a relatively high $K_m$ for 2-oxoglutarate that lies 10-fold above the actual concentration of 2-oxoglutarate in the cytosol. In addition, transport of 2-oxoglutarate by DiT2 is strongly inhibited by malate and glutamate (Renné *et al.*, 2003). Hence, under physiological conditions, DiT2 is likely not able to exchange 2-oxoglutarate with glutamate. DiT1, however, is a high-affinity 2-oxoglutarate transporter that is not inhibited by malate or glutamate (Renné *et al.*, 2003). Thus, the complex malate-coupled, two-translocator system is required because of the kinetic constants and substrate specificities of DiT1 and DiT2.

The plastidal dicarboxylate transport system is essential for survival in ambient air as was demonstrated by the isolation of the photorespiratory Arabidopsis mutant *dct* that is deficient in plastidal dicarboxylate transport (Somerville and Ogren, 1983). The mutant lacks a chloroplast envelope membrane protein with an apparent molecular mass of 45 kDa (Somerville and Somerville, 1985). Based on the physiological and biochemical characterization of *dct*, both DiT1 and DiT2 are candidates for the defective gene in *dct*. It was recently demonstrated that *dct* is deficient in the plastidal glutamate/malate translocator DiT2 (Renné *et al.*, 2003). Hence, DiT2 is essential for a functional photorespiratory pathway.

Whether or not DiT1 is required for photorespiration is not clear. Taniguchi's lab has reported two knockout alleles of DiT1 that do not display a photorespiratory phenotype (Taniguchi *et al.*, 2002). However, an additional knockout allele isolated in the author's lab displays a photorespiratory phenotype, although it is less pronounced than in *dct*. Moreover, antisense repression of DiT1 in transgenic tobacco plants leads to a clear photorespiratory phenotype that is reversible by high $CO_2$ (J. Schneidereit and A.P.M. Weber, unpublished). Further analysis is required to unequivocally define the role of DiT1 in the photorespiratory pathway.

### 3. Transport of Glycolate and Glycerate

The second plastidal transport system involved in photorespiration is the glycolate/glycerate transporter. Although this transporter likely catalyzes a carbon flux approximating that of triose phosphates (see above), the corresponding protein is not known at the molecular level. The kinetic constants and the substrate specificity of the transporter from pea chloroplast envelope membranes have been determined (Howitz and McCarty, 1985a, b) and the spinach chloroplast envelope membrane transporter was analyzed in reconstituted liposomes (Howitz and McCarty, 1991). Transport of glycerate was inhibited by glycolate and vice versa. Both transport activities had similar sedimentation coefficients in glycerol gradients. These findings indicate that the same protein transports glycolate and glycerate.

None of the previously characterized photorespiratory mutants was found to be defective in the glycolate/glycerate transporter. The recently finished Arabidopsis and rice genomes did not reveal obvious candidate genes encoding this transporter. The most promising approach towards identifying this protein currently is the systematic knockout analysis of putative plastidal envelope membrane transporters that can be predicted by bioinformatic approaches from the genome sequences (Schwacke *et al.*, 2003) and analysis of these knockouts for a photorespiratory phenotype.

### 4. Other Transport Systems Involved in Photorespiration

Photorespiration requires transporters for glycolate, glycerate, glycine, serine, oxaloacetate, glutamate, 2-oxoglutarate and malate in the peroxisomal membrane and transporters for glycine and serine in the mitochondrial inner membrane. None of these transporters is known at the molecular level. Biochemical evidence indicates that a specific porin is involved in the peroxisomal malate shuttle (Reumann *et al.*, 1996), and that porins might be involved in the transport of 2-oxoglutarate (Reumann *et al.*, 1998; Reumann, 2000).

## V. Keeping the Balance—Partitioning of Recently Assimilated Carbon into Multiple Pathways

Intermediates of the reductive pentose phosphate pathway are not only withdrawn to fuel starch and sucrose biosynthesis but also used as precursors for plastid-localized metabolic pathways such as the Shikimic acid pathway (Schmid and Amrhein, 1995) and the DOXP pathway (Schwender *et al.*, 1996; Lichtenthaler *et al.*, 1997; Schwender *et al.*, 1997).

### *A. Shikimic Acid Pathway*

The plastid-localized Shikimic acid pathway produces the aromatic amino acids tryptophan, phenyalanine and tyrosine. Moreover, numerous secondary compounds such as anthocyanins, flavonoids, alkaloids, lignin, phytoalexins and the plant hormone auxin are derived from the Shikimic acid pathway. Depending on environmental conditions, up to 50% of recently assimilated carbon can be directed into lignin biosynthesis. Thus, the Shikimic acid pathway can represent a major carbon sink in plants. Two precursors are required for the Shikimic acid pathway, erythrose 4-phosphate (Ery 4P) and phospho*enol*pyruvate (PEP).

#### *1. The Critical Role of Transketolase*

The Shikimic Acid pathway precursor Ery 4P can be withdrawn from the pentose phosphate cycle. Together with xylulose 5-P (Xul 5P) it is produced from Fru 6P and GAP in a reversible reaction catalyzed by transketolase (TK). TK thereby represents an important branch point in metabolism: the substrate Fru 6P is the precursor for starch biosynthesis, GAP can be exported to the cytosol by TPT, Ery 4P can serve as precursor for the Shikimic acid pathway but also for the production of Xul 5P. Hence, TK is also critical for the regeneration of the $CO_2$ acceptor Ru*b*P and for thus for the continued operation of the Calvin-Benson cycle.

The role of TK as an important determinant of photosynthetic and phenylpropanoid metabolism was recently demonstrated by antisense repression of TK in transgenic tobacco plants (Henkes *et al.*, 2001). A relatively small decrease of TK activity (20 to 40%) inhibited the regeneration of RuBP and consequently photosynthesis. Soluble sugars decreased whereas starch remained high. This effect can be attributed to an accumulation of Fru 6P and a decrease in Ery 4P in the antisense plants. Ery 4P competitively inhibits the first step in starch biosynthesis, the conversion of Fru 6P to Glc 6P by phosphoglucose isomerase (PGI; Kelly and Latzko, 1980). A decrease in Ery 4P as caused by reduced TK activity will thus relieve the competitive inhibition of PGI and increase the carbon flux into starch biosynthesis. In addition, TK catalyzes the first enzymatic step downstream of Fru 6P in the Calvin-Benson cycle and thereby cause accumulation of Fru 6P. The combined effects of a decreased inhibition of PGI by Ery 4P and the massive accumulation of Fru 6P lead to increased carbon flux into starch biosynthesis and a decreased carbon supply to sucrose biosynthesis. Decreased TK activity hence alters photosynthate allocation in favor of starch biosynthesis.

Reduced TK activity also led to pronounced decreases in aromatic amino acids levels, and of intermediates and end products of phenylpropanoid metabolism (Henkes *et al.*, 2001). Levels of Tyr, Phe, Trp, caffeic acid, tocopherol and hydroxycinnamic acids were inversely correlated with TK activity. A massive decrease in lignin content was observed if TK activity was reduced by 40% or more.

These results demonstrate that TK is an important determinant of photosynthetic and phenylpropanoid metabolism. TK activity clearly limits the supply of precursors to the Shikimic acid pathway and thereby colimits the flux into phenylpropanoid metabolism.

#### *2. The Critical Role of Phospho*enol*pyruvate and the Phosphoenolpyruvate Translocator*

Most plastid types, apart from plastids in lipid storing tissues, are unable to convert triose phosphates to PEP via the glycolytic pathway. For example, chloroplasts from pea, spinach, and Arabidopsis (Stitt and ap Rees, 1979; Schulze-Siebert *et al.*, 1984; Bagge and Larsson, 1986; Van der Straeten *et al.*, 1991) and non-green plastids from pea roots and cauliflower buds (Journet and Douce, 1985; Borchert *et al.*, 1993) lack sufficient activity of phosphoglyceromutase and/or enolase to catalyze a significant flux from TP to PEP. Hence, PEP needs to be imported from the cytosol.

A phospho*enol*pyruvate/phosphate translocator (PPT) was first identified from cauliflower buds (Fischer *et al.*, 1997) and related sequences have been described from a large variety of plant species (Knappe *et al.*, 2003a). *In vitro*, recombinant, reconstituted PPT catalyzes the strict counter-exchange of PEP with Pi or 2-PGA whereas it operates as a PEP/Pi-antiporter under physiological conditions (Fischer *et al.*, 1997). In cauliflower and maize, PPT transcripts were detected in green and non-green tissue, with steady-state transcript levels being highest in non-green tissues

(Fischer *et al.*, 1997). The Arabidopsis genome encodes two PPT-proteins, AtPPT1 and AtPPT2 (Knappe *et al.*, 2003a; Knappe *et al.*, 2003b). The corresponding genes are differentially expressed: *AtPPT1* expression is confined to the leaf vasculature and to roots (with the highest expression in xylem parenchyma cells) whereas *AtPPT2* is expressed ubiquitously in leaves, but not in roots (Knappe *et al.*, 2003b).

A knockout mutant in *PPT1* (*cue1*) was recently isolated. It displays a reticulate phenotype that is characterized by pale leaf interveinal regions and green periveinal regions. Mesophyll cells in *cue1* are aberrantly shaped and contain abnormal chloroplasts, whereas bundle sheath cells and chloroplasts appear like the wild type (Streatfield *et al.*, 1999). It was hypothesized that the phenotype is caused by reduced import of PEP into plastids, thereby reducing the supply of precursors to the Shikimic acid pathway and possibly also to other metabolic pathways such as the isopentenyl biosynthesis. This hypothesis was supported by the observation that the contents of and the flux into metabolites derived from the Shikimic acid pathway, such as phenylpropanoids, were severely reduced in *cue1* (Streatfield *et al.*, 1999; Voll *et al.*, 2003). The bottleneck in PEP import into plastids in *cue1* could be bypassed by constitutive overexpression of pyruvate:phosphate dikinase (PPDK) in the stroma of *cue1* (Voll *et al.*, 2003). PPDK generates PEP from pyruvate that obviously can be taken up from the cytosol, thereby detouring the defective *PPT1*. These findings also provide strong evidence for a substantial pyruvate transport capacity across the chloroplast envelope membrane. Importantly, PPDK overexpression did not alleviate all aspects of the *cue1* phenotype. This indicates that the *cue1* phenotype cannot be exclusively attributed to a reduction in PEP transport capacity (Voll *et al.*, 2003) but may also involve metabolic signals that regulate mesophyll cell development (Knappe *et al.*, 2003b).

The import of PEP from the cytosol increases the potential for coordinated regulation of carbon flux into the different sinks. In contrast to Ery 4P, which can be directly withdrawn from the plastidal pentose phosphate cycle, PEP synthesis occurs in the cytosol and is thus dependent on the Pi-controlled export of TP from the plastid or flux through the glycolytic pathway (and on PEP usage by e.g. PEP carboxylase). Together with Ery 4P availability, PEP supply co-limits flux into the Shikimic acid pathway because PEP is required as precursor at two steps in the pre-chorismate pathway.

### *B. Synthesis of Isoprenoids by the DOXP Pathway*

Isoprenoids such as carotenoids and the phytol side chain of chlorophyll are synthesized in the plastid stroma from the C5-precursor isopentenyldiphosphate (IPP). As we now know, most or probably all plastid-synthesized isoprenoids are derived from the recently discovered 2-dexoyxylulose-5-phosphate pathway (DOXP pathway) that is also known as the 2-methylerythritol-4-phosphate (MEP) pathway (Schwender *et al.*, 1996; Lichtenthaler *et al.*, 1997). Allocation of carbon to isoprenoid biosynthesis can represent a significant carbon sink and therefore needs to be considered in the context of photosynthetic end product synthesis. For example, during ripening of tomato or pepper fruits, large amounts of carotenoids are synthesized within a relatively short time. However, carbon allocation to carotenoid biosynthesis can be dwarfed by flux into isoprene biosynthesis (Sharkey and Yeh, 2001). For example, aspen and oak typically emit isoprene at 2% of the actual rate of photosynthesis. Under non-physiological conditions, however, the rate of isoprene emission can exceed the rate of photosynthesis, leading to a negative carbon balance (Sharkey and Yeh, 2001). Plant-derived isoprene emission represents a major carbon flux from the biosphere to the atmosphere. Approximately 500 Tg C per year are emitted in the form of isoprene by plants, a flux that equals biogenic methane emissions (Sharkey and Yeh, 2001).

Isoprenoid biosynthesis by the plastid localized DOXP-pathway starts with the precursors pyruvate and GAP (Lichtenthaler *et al.*, 1997). GAP can be withdrawn from the reductive pentose phosphate pathway whereas pyruvate has to be imported from the cytosol because photosynthetic plastids lack the glycolytic sequence from GAP to PEP. Pyruvate transporters have been characterized in a number of C4 (Huber and Edwards, 1977; Aoki *et al.*, 1992) and C3 (Proudlove and Thurman, 1981) plants. Additional evidence for efficient uptake of pyruvate into chloroplasts from C3 plants comes from the observation that isolated spinach chloroplast are able to synthesize pyruvate-derived amino acids upon external supply of pyruvate (Schulze-Siebert *et al.*, 1984). Moreover, the PPT-deficient mutant *cue1* could be complemented by overexpression of plastidal PPDK, demonstrating that pyruvate uptake into chloroplasts also occurs in vivo (Voll *et al.*, 2003). Another route for pyruvate import into chloroplast is uptake of PEP and conversion of PEP to pyruvate by pyruvate kinase.

Both the Shikimic acid and the DOXP pathways withdraw intermediates from the Calvin-Benson cycle. To avoid depletion of the acceptor for carbon dioxide assimilation, Ru*b*P, anaplerotic reactions are required to replenish the reductive pentose phosphate pathway.

## VI. Conclusions and Future Directions

Recently assimilated carbon dioxide can be allocated to a multitude of biochemical pathways in plant cells. Over the last few decades, much knowledge about the partitioning of recent photosynthate between sucrose and starch biosynthesis has accumulated. However, the regulation of carbon partitioning between primary and secondary metabolism is much less well understood. Some of the major routes for carbon export from and import into plastids have been recently elucidated at the molecular level. The molecular basis of many other transport processes, in particular those involved in the photorespiratory carbon cycle, remains to be unraveled.

The advent of complete genome sequences from Arabidopsis and rice, together with the comprehensive collections of knockout mutants, enables systematic reverse genetics approaches to identify novel transporters and potential regulatory elements (Flügge *et al.*, 2003; Weber *et al.*, 2004). For example, the power of reverse genetic approaches was demonstrated by the elucidation of cytosolic maltose metabolism (Lu and Sharkey, 2004) and the role of the plastidal disproportionating enzyme in starch turnover (Critchley *et al.*, 2001). In addition, forward genetic screens remain powerful tools to study photosynthetic carbon metabolism. The recent identification of the plastidal maltose transporter MEX1 (Niittylä *et al.*, 2004) and the glutamate/malate translocator DiT2 (Renné *et al.*, 2003) underscores the usefulness of this approach.

The combination of classical physiological and biochemical techniques with novel genomics approaches will certainly lead to a deeper understanding of synthesis, transport and partitioning of the end products of photosynthesis.

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# Section IV

## Non-Photosynthetic Metabolism in Plastids

# Chapter 15

# Chlorophyll Synthesis

Robert D. Willows*
*Department of Chemistry and Biomolecular Sciences, Division of Environmental and Life Sciences, Macquarie University, NSW 2109, Australia*

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## Summary

Chlorophyll is the dominant pigment in a mature plant cell, whether in the leaf of a plant or in the abundant algal species. Chlorophyll is synthesized within the chloroplast from a plentiful precursor, the amino acid glutamate. From glutamate to the tetrapyrrole protoporphyrin IX, at which the pathway branches between chlorophyll and heme, the reactions occur in the plastid stroma and are catalyzed by soluble enzymes. The latter steps to chlorophyll, the first being the insertion of the central magnesium atom, occur with enzyme complexes that are at least partially if not entirely localized on membranes. Magnesium chelatase, the key enzyme in this pathway, is a complex of proteins that includes soluble and membrane-bound subunits. Subsequent reactions occur primarily on membranes and involve modification of structural groups on the periphery of the molecule. The pentultimate precursor of chlorophyll, protochlorophyllide, is reduced by NADPH to chlorophyllide in the only reaction in the pathway that requires light. This reaction, which in angiosperms is catalyzed by light-dependent NADPH:protochlorophyllide oxidoreductase, dramatically changes the property of the molecule and allows the product chlorophyllide, and its esterified product, chlorophyll, to interact with proteins. These chlorophyll-protein complexes become the building blocks of the photosynthetic apparatus. The biosynthetic pathway is tightly regulated, particularly at the key reactions that generate 5-aminolevulinic acid, magnesium-protoporphyrin IX and chlorophyllide. Expression of genes encoding critical enzymes is usually regulated markedly by light, and the activities of the enzymes are also regulated by end-products in typical feedback inhibition.

*Author for correspondence, email: Robert.Willows@mq.edu.au

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## I. Introduction: Overview of Chlorophyll Biosynthesis

This chapter emphasizes the reactions in chlorophyll synthesis in the latter part of the pathway. These latter intermediates become important in processes related to chloroplast development, thylakoid biogenesis and regulatory mechanisms of these processes. For a description of the earlier reactions in the pathway, see the forthcoming volume "Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics and Biological Function" (Editors: Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger and Hugo Scheer) in this series.

Although the plastid has a number of essential functions, photosynthesis occurs only in the chloroplast version of the organelle and requires chlorophyll. The importance of these molecules has attracted a great amount of effort to understand their characteristics and means of synthesis. A robust process must be in place to achieve the synthesis of chlorophyll—one of the most dominant substances in the plant cell—which occurs over the developmental stage of hours to a few days as a leaf matures. Chlorophyll is synthesized from the plentiful amino acid, glutamate. For biosynthetic reactions, including incorporation of glutamate into proteins, glutamate is converted to glutamyl-transfer RNA (tRNA) in a two-step reaction that requires ATP and is catalyzed by glutamyl-tRNA synthetase. The "activated" $\alpha$-carboxyl group, linked to the tRNA via a reactive ester bond, is then reduced by NADPH to another reactive product, glutamate 1-semialdehyde (GSA), in the reaction catalyzed by glutamyl-tRNA reductase (GTR). GSA is rapidly converted to 5-aminolevulinic acid (ALA), in which the carbon-1 of glutamate becomes carbon-5 in ALA (Fig. 1). This reaction is an example of a rare intramolecular transaminase, in which the amino group, formerly on carbon-2 of glutamate and now carbon-4 of GSA, is transferred to carbon-5 in a reaction that requires the cofactor pyridoxamine-phosphate. GSA, and its more stable isomer, ALA, are the committed precursors for chlorophyll synthesis. Synthesis of ALA, and specifically of GSA, is the rate-controlling step in the pathway, and thus the activities of the enzymes that catalyze these steps are tightly controlled (Vavilin and Vermaas, 2002; Eckhardt *et al.*, 2004). The primary means of feedback control on the activity of glutamyl-tRNA reductase is thought to be mediated by heme. The crystal structures of the key enzymes in ALA synthesis were resolved, glutamyl-tRNA reductase by Schubert *et al.* (2002) and GSA transaminase by Hennig *et al.* (1994, 1997).

Two molecules of ALA are condensed to porphobilinogen by the enzyme porphobilinogen synthase (also called ALA dehydratase). Four molecules of porphobilinogen then are linked to achieve the tetrapyrrole structure. Condensation of the pyrrole rings is catalyzed by porphobilinogen deaminase (also called hydroxymethylbilane synthase). This enzyme has an unusual feature of containing a protein-bound dipyrromethane cofactor. Four porphobilinogen molecules are added sequentially to the cofactor to generate a chain of six pyrrole rings. The outer four are then cyclized, with reversal of the orientation of the last unit added, by uroporphyrinogen III synthase (Jordan, 1994; Beale, 1999). Uroporphyrinogen III is converted in several steps, by trimming of the propionyl side chains and oxidation, to yield protoporphyrin IX.

The early steps in the biosynthetic pathway are catalyzed by soluble proteins in the stroma of the plastid. The latter reactions, which involve increasingly more hydrophobic products, are localized on membranes. Increasing evidence supports localization of the reactions between protoporphyrin IX to protochlorophyllide on the inner membrane of the chloroplast envelope (Joyard *et al.*, 1998; Beale, 1999; Eckhardt *et al.*, 2004). Chlorophyll biosynthesis from glutamyl-tRNA to chlorophyll *b* requires 15 enzymes and 27 genes. With the identification of the gene for 8-vinyl reductase, the enzyme that catalyzes conversion of divinyl-protochlorophyllide or divinyl-chlorophyllide to the monovinyl forms, all the enzymes and genes involved in the biosynthetic pathway in *Arabidopsis thaliana* are known (Nagata *et al.*, 2005).

## II. Protoporphyrin IX to Chlorophyll

The steps from protoporphyrin IX onwards are unique to the chlorophyll biosynthetic pathway. Figure 2 shows an overview of the enzymatic steps in the synthesis of chlorophyll from protoporphyrin IX onwards. The insertion of magnesium commits protoporphyrin IX to

*Abbreviations:* ALA – 5-aminolevulinic acid; CAO – chlorophyll *a* oxygenase; DPOR – dark NADPH:protochlorophyllide oxidoreductase; GSA – glutamate 1-semialdehyde aminotransferase; GTR – glutamyl-tRNA reductase; LHC – light-harvesting complex; Lhcb – apoprotein of light-harvesting complex; PLB – prolamellar body; POR – light-dependent NADPH:protochlorophyllide oxidoreductase; PORA, PORB, PORC – three forms of light-dependent, NADPH:protochlorophyllide oxidoreductase; tRNA – transfer RNA.

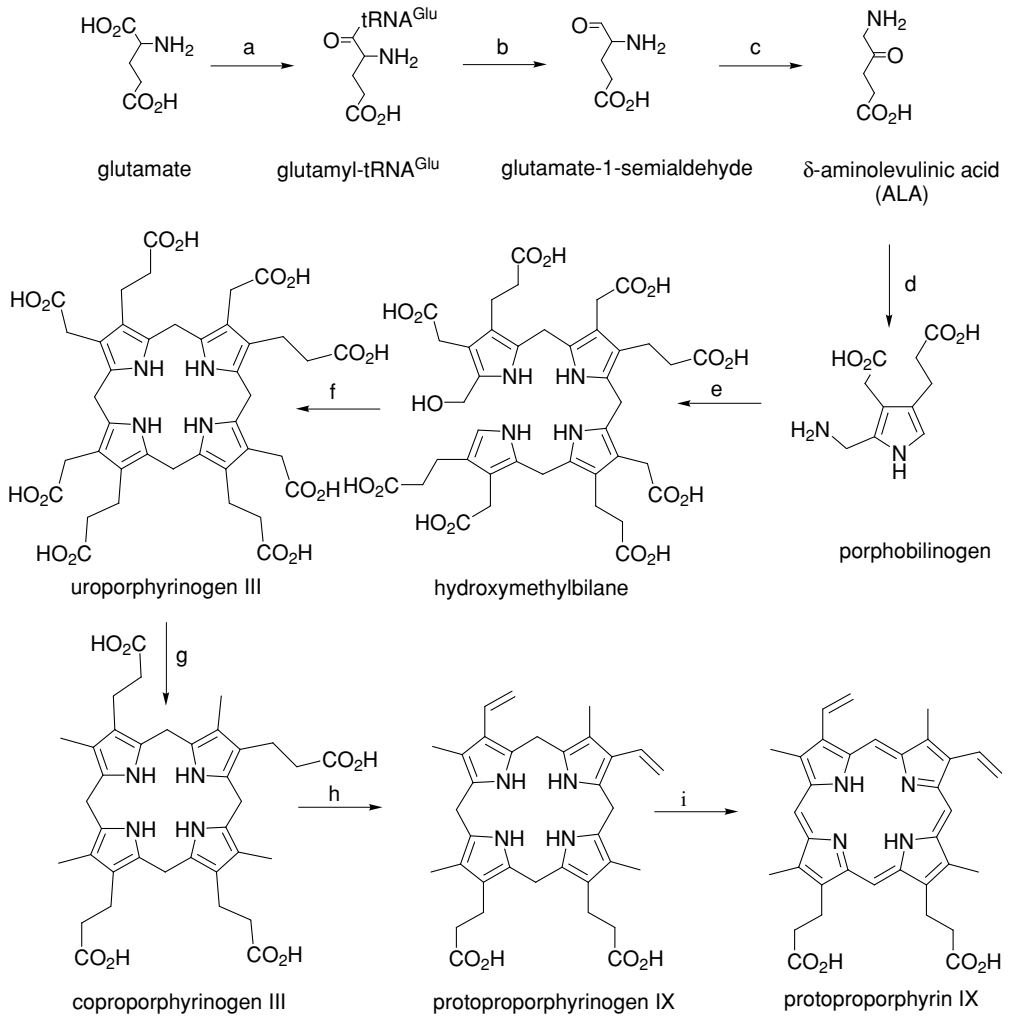


*Fig. 1.* Scheme showing the pathway for the synthesis of protoporphyrin IX from glutamate. (a) glutamyl tRNA synthetase; (b) glutamyl tRNA reductase (GTR); (c) glutamyl tRNA aminotransferase (GSAT); (d) ALA dehyratase (ALAD) or porphobilinogen synthase (PBGS); (e) porphobilinogen deaminase (PBGD) or hydroxymethylbilane synthase; (f) uroporphyrinogen IIII synthase; (g) uroporphyrinogen III decarboxylase (UROD); (h) coproporphyrinogen oxidase (COPOX); (i) protoporphyrinogen oxidase (PROTOX).

chlorophyll synthesis rather than heme synthesis. Thus there is a need for regulating the flux of intermediates between these two pathways. This chapter will deal with the overall regulatory mechanisms of chlorophyll biosynthesis in section III. This section will be confined to a discussion to the enzymes and the transcriptional regulation of the genes encoding these enzymes.

## *A. Magnesium Chelatase*

Magnesium chelatase is a complex enzyme consisting of three distinct types of subunits. It is the first committed step in chlorophyll biosynthesis, as the preceding steps in the pathway are shared by the heme biosynthetic pathway (Willows, 2003; Willows and Hansson, 2003). The porphyrin substrate for both magnesium chelatase and ferrochelatase is protoporphyrin IX and, as both enzymes are found in the chloroplast, there is a requirement for regulation of these activities so that the demand for the end products of each pathway is satisfied. These two enzymes are quite different in structure, cofactor requirement and mechanism of metal ion insertion, partly because it is more difficult to insert $Mg^{2+}$ than $Fe^{2+}$ into the tetrapyrrole macrocycle. Magnesium chelatase requires $Mg^{2+}$ ions and the hydrolysis of ATP for metal ion insertion into protoporphyrin IX, as shown in Fig. 2. Compared to the complex mechanism for magnesium chelatase, ferrochelatase is

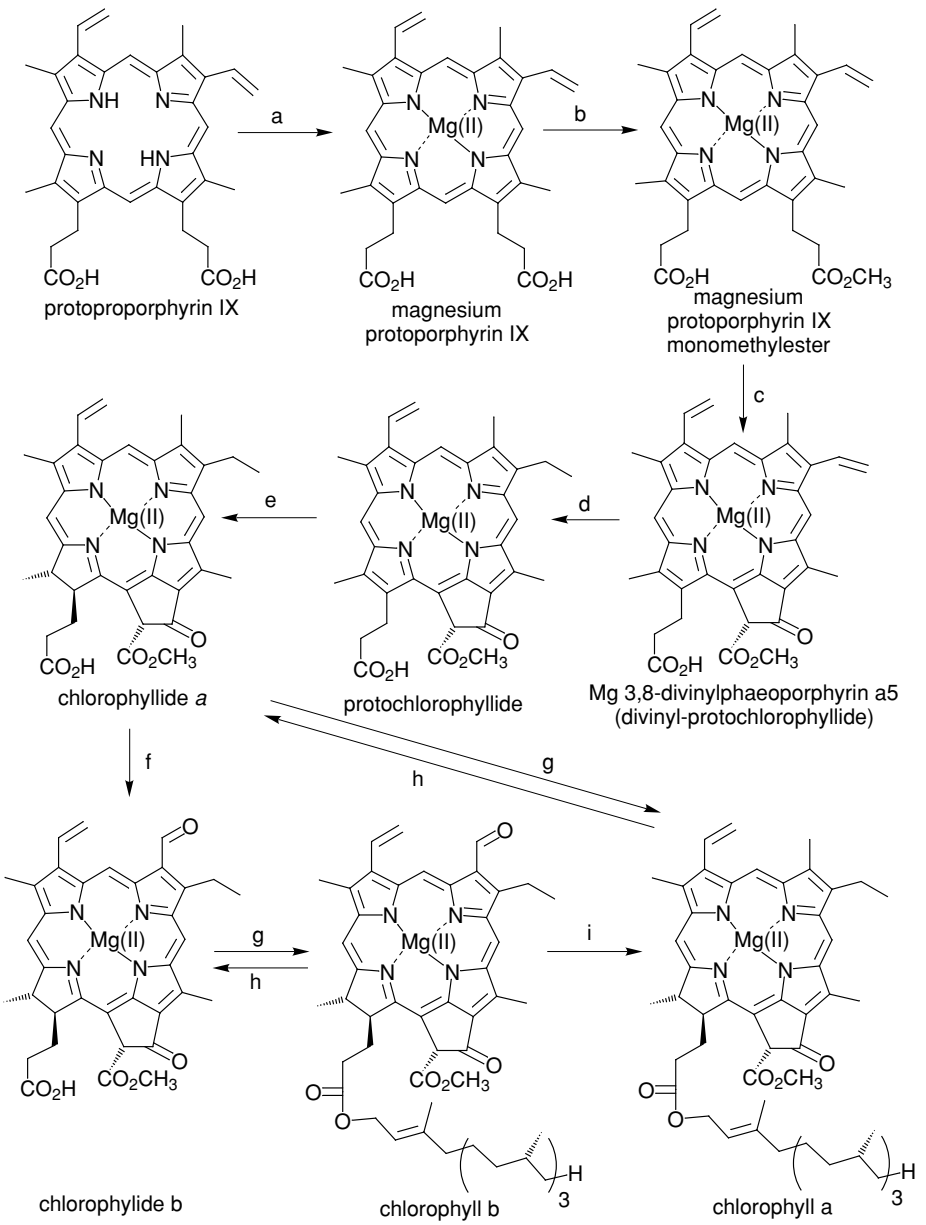


*Fig. 2.* Scheme showing the pathway for the synthesis of chlorophyll *a* and *b* from protoporphyrin IX. (a) magnesium chelatase; (b) S-adenosylmethionine:magnesium protoporphyrin IX O-methyltransferase; (c) magnesium protoporphyrin IX monomethylester oxidative cyclase; (d) 8-vinyl reductase; (e) protochlorophyllide oxidoreductase; (f) chlorophyll *a* oxidase; (g) chlorophyll synthase; (h) chlorophyllase; (i) chlorophyll *b* reductase.

a single subunit enzyme of about 40-kDa that catalyses $Fe^{2+}$ insertion into protoporphyrin IX without the involvement of any additional cofactors (Ferreira, 1999).

The magnesium chelatase subunits consist of a small ~40-kDa subunit, an intermediate sized subunit protein of ~70-kDa, and a large subunit protein of ~140-kDa. These subunits will be referred to as **I**, **D** and **H**, respectively, when referring to the proteins, and ***I***, ***D*** and ***H***, respectively, when referring to the genes encoding these proteins. The naming of the different subunits of the magnesium chelatase in the literature may be rather confusing for many people as various plant mutants have different names. The corresponding genes in these plants share the mutant name and the suffixes to the names are often

*Table 1.* Magnesium chelatase mutants

| Species | Subunit | Mutant, allele, and/or gene name/s | References |
|---|---|---|---|
| *Antirhinnum majus* | **H** | *olive-605* | Hudson *et al.*, 1993 |
| *A. thaliana* | **I** | *cs*, *ch-42* | Koncz *et al.*, 1990<br>Rissler *et al.*, 2002 |
| | **H** | *gun5*, *cch* | Larkin *et al.*, 2003<br>Mochizuki *et al.*, 2001<br>Strand *et al.*, 2003 |
| | **D** | *chlD* | Strand *et al.*, 2003 |
| | Unknown | *xan2*, *xan3* | Runge *et al.*, 1995 |
| *Chlamydomonas reinhardtii* | **H** | *chl1*, *brs-1* | Chekounova *et al.*, 2001 |
| | Unknown | *brs-2, brc-1, brc-2* | Wang *et al.*, 1974 |
| *Chlorella vulgaris* | Unknown | $W_5$-brown | Granick, 1948 |
| *Hordeum vulgare* | **I** | *xantha h* | Jensen *et al.*, 1996b |
| | **D** | *xantha g* | Petersen *et al.*, 1999 |
| | **H** | *xantha f* | Jensen *et al.*, 1996b |
| *Nicotiana tabacum* | **I** | *sulfur*, *chlI* | Nguyen, 1995<br>Papenbrock *et al.*, 2000b |
| | **H** | *chlH* | Papenbrock *et al.*, 2000a |
| *Rhodobacter* species | **H** | *bchH* | Bollivar *et al.*, 1994;<br>Coomber *et al.*, 1990<br>Gorchein *et al.*, 1993<br>Taylor *et al.*, 1983<br>Zsebo and Hearst, 1984 |
| | **D** | *bchD* | Bollivar *et al.*, 1994<br>Coomber *et al.*, 1990<br>Gorchein *et al.*, 1993<br>Taylor *et al.*, 1983<br>Zsebo and Hearst, 1984 |
| | **I** | *bchI* | Bollivar *et al.*, 1994<br>Coomber *et al.*, 1990<br>Gorchein *et al.*, 1993<br>Taylor *et al.*, 1983<br>Zsebo and Hearst, 1984 |
| *Triticum* species | Unknown | Driscoll's chlorina, *chlorina-1, CD3, chlorina-214* | Falbel and Staehelin, 1994<br>Freeman *et al.*, 1987<br>Pettigrew *et al.*, 1969<br>Sears and Sears, 1968 |
| *Zea mays* | Unknown | *l*-Blandy4, l*-Blandy3drk, l*-EMS1, oy-1039, oy-1040, l13-Neuffer2, l13-1050* | Mascia, 1978 |

contradictory, as detailed in Table 1. The molecular changes resulting from mutations in magnesium chelatase genes are known for mutants of barley, *Arabidopsis thaliana*, *Antirhinnum majus*, *Nicotiana tabacum*, *Chlamydomonas reinhardtii*, and the purple bacteria *Rhodobacter capsulatus*, and *Rhodobacter sphaeroides.* Putative magnesium chelatase mutants have also been identified in *Zea mays*, *Triticum* sp. and *Chlorella vulgaris*. A complete list of these magnesium chelatase mutants identifying the affected subunits was recently published (Willows and Hansson, 2003). All of the plant mutants have a chlorophyll-deficient, pale-green to yellow phenotype and accumulate protoporphyrin IX when fed ALA. Most of the mutants are recessive with the exception of three semi-dominant barley mutants that have single missense mutations in the **I** subunit (Hansson *et al.*, 1999). The algal mutants in contrast have a pale-brown phenotype due to the accumulation of protoporphyrin IX. This suggests that there is a difference in the regulation of chlorophyll biosynthesis in algae compared to plants, or at least angiosperms.

Mutants of the ***D*** and ***H*** genes of *A. thaliana* have a "genomes uncoupled" (*gun*) phenotype and

are defective in chloroplast to nucleus communication (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003; Strand *et al.*, 2003; also, see Chapter 9). The *gun* mutants were selected for their ability to express the chlorophyll *a*/*b* binding protein of photosystem II, Lhcb1, under conditions where it is normally not expressed. The *gun5* and *cch* alleles were found to have missense mutations resulting in an alanine to valine substitution in *gun5* and a proline to leucine substitution in *cch* (Mochizuki *et al.*, 2001). Subsequent work showed that magnesium protoporphyrin IX or its monomethyl ester is part of the chloroplast-nuclear signalling process and is possibly the primary signalling molecule (Strand *et al.*, 2003). A protein involved in the downstream signalling process called GUN4 was also shown to interact with the **H** subunit of magnesium chelatase (Larkin *et al.*, 2003). Thus the magnesium chelatase may have a dual role in chloroplast to nuclear signalling; it produces the nuclear signal *and* one of the subunits interacts with a downstream signalling component.

Transgenic tobacco plants that express the **I** and **H** gene in the antisense orientation were produced. These plants have a uniformly pale-green phenotype typical of some of the barley mutants in these two genes. Perhaps unsurprisingly, protoporphyrin IX did not accumulate to high levels in these mutants, which tends to support the theory that feedback inhibition of ALA biosynthesis by heme is a major controlling factor in the pathway. However, the transcript levels of the *Gtr* and *Alad* genes, encoding glutamyl-tRNA reductase and ALA dehydratase, respectively, were also reduced in these lines, suggesting that expression may be synchronised with magnesium chelatase transcripts in some way (Papenbrock *et al.*, 2000a,b). It is possible that this regulation of transcript levels is via magnesium protoporphyrin signalling.

In plants, all of the magnesium chelatase genes are nuclear encoded. In algae and cyanelles, with the exception of *C. reinhardtii*, the ***I*** gene of magnesium chelatase is located on the chloroplast genome while the ***H*** and ***D*** are located on the nuclear genome. The pattern of nuclear gene expression for magnesium chelatase genes is not consistent across plant and algal species. The only common features are that magnesium chelatase genes are regulated by a circadian clock when grown under normal day/night cycles and etiolated or dark adapted plants show light-induced expression of ***H*** and ***I*** (Koncz *et al.*, 1990; Jensen *et al.*, 1996b). The pattern of expression of ***H*** varies across species with *A. majus* having a maximum in the dark phase and a minimum in the light phase, with low to non-existent transcript levels at medium light intensity (Hudson *et al.*, 1993). The opposite pattern is observed for ***H*** of tobacco, soybean, barley and *A. thaliana,* with all species having maximum expression in the light phase (Gibson *et al.*, 1996; Jensen *et al.*, 1996b; Nakayama *et al.*, 1998; Papenbrock *et al.*, 1999). Expression of the ***I*** gene in tobacco, barley and *A. thaliana* follows a similar pattern to the ***H*** transcript (Gibson *et al.*, 1996; Jensen *et al.*, 1996b; Papenbrock *et al.*, 1999). The ***D*** and the ferrochelatase genes of tobacco have an inverse expression to ***H*** (Papenbrock *et al.*, 1999). In *C. reinhardtii* there are maxima in both the light and dark phases for all three genes (Lake and Willows, 2003). It was suggested that the differences in the pattern of expression of magnesium chelatase may be related to both light intensity and to the ability of plants or algae to adapt to various light conditions (Lake and Willows, 2003).

Most of the details of the mechanism for magnesium chelatase have been gleaned from studies of the *Rhodobacter* and *Synechocystis* enzymes. Although the enzymes from different sources have slightly different properties, the overall mechanism is likely to be similar and much of the information on the structure and catalytic mechanism discussed in this section is from studies with the cyanobacterial or purple bacterial enzymes. The magnesium chelatase reaction has been dissected into two phases. The first phase involves formation of an activation complex between subunits **I** and **D,** which is dependent on protein concentration and ATP (Walker and Weinstein, 1991; Jensen *et al.*, 1996a; Willows and Beale, 1998; Willows *et al.*, 1996; Guo *et al.*, 1998). This activation complex catalyses magnesium insertion into protoporphyrin only when combined with the **H** protein, Mg-ATP, protoporphyrin IX and $Mg^{2+}$. The **H** protein behaves as a substrate in the magnesium chelatase reaction and has a $K_m$ in the low micromolar range (Jensen *et al.*, 1998; Willows and Beale, 1998; Gibson *et al.*, 1999). The structure of the **I** protein from *R. capsulatus* was determined by X-ray crystallography (Fodje *et al.*, 2001). The **I** protein forms an ATP-dependent hexameric ring (Willows *et al.*, 2004), which is proposed to interact with a similar **D** hexameric ring to form a double-ring complex (Fodje *et al.*, 2001; Willows and Hansson, 2003). The **I** protein and a domain of the **D** protein belong to the extended class of triple-A proteins (AAA+) which are one of the largest and most diverse classes of proteins known and generally form ring-like structures (Confalonieri and Duguet, 1995; Vale, 2000). AAA+ proteins have also been called mechanoenzymes due to the mechanical nature of the large conformational changes that occur on ATP hydrolysis (Vale, 2000). The **I:D** double ring

structure presumably catalyses an ATP-dependent conformation change in **H** to effect magnesium insertion into a protoporphyrin IX bound to **H** (Hansson *et al.*, 2002; Willows and Hansson, 2003).

Studies that reported on compounds that specifically inhibit magnesium chelatase have been somewhat confusing, as inhibition of activity has been examined in a variety of ways ranging from *in vivo* studies, *in organello* studies, to true *in vitro* inhibition experiments. The *in vivo* and *in organello* studies suffer from problems of access of the inhibitor to the enzyme, which was highlighted in a recent review (Willows and Hansson, 2003). The inhibitors can be catalogued based on the mechanism of inhibition, being protein modifying agents, ATPase inhibitors, tetrapyrrole analogues and other inhibitors of undefined mechanism (Willows and Hansson, 2003). Light has also been shown to inhibit the magnesium chelatase of barley (Pöpperl *et al.*, 1997) and *Rhodobacter* (Willows and Beale, 1998), and this mode of inhibition probably occurs via photooxidative damage of the **H** subunit (Willows and Beale, 1998; Willows *et al.*, 2003). The inhibition by light of barley magnesium chelatase was also demonstrated with isolated chloroplasts. This contrasts with the situation *in planta* where isolated chloroplasts of barley from etiolated barley seedlings exposed to 4 h of light have considerably higher activity than chloroplasts from plants not exposed to light (Jensen *et al.*, 1996b). This increase in activity *in planta* can be attributed to the increased synthesis of the **I** and **H** subunits (Jensen *et al.*, 1996b) and is supported by data showing that magnesium protoporphyrin and magnesium protoporphyrin monomethyl ester levels increase dramatically in leaves from barley or tobacco when transferred from dark to light (Pöpperl *et al.*, 1997).

### *B. S-Adenosyl-L-Methionine:Magnesium Protoporphyrin IX-O-Methyltransferase*

S-Adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase catalyses the S-adenosylmethionine-dependent methylation of the carboxyl group of the 13-propionate on magnesium protoporphyrin IX. This enzyme is membrane associated and the activity has been characterised for a number of plant species (reviewed in Bollivar, 2003). The gene for S-adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase was cloned and sequenced from tobacco and *A. thaliana* (Block *et al.*, 2002), and antisense transgenic tobacco plants were produced and are the subject of a patent (Reindl *et al.*, 2001). Two barley mutants, *xantha-n* and *albina-e,* have no detectable S-adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase activity. These mutants also have defective membrane structure and the reduction in activity may be a pleiotrophic effect of the defective membrane structure (Moller *et al.*, 1997).

The enzymes from *Euglena gracilis* (Richards *et al.*, 1981; Hinchigeri and Richards, 1982), wheat (Hinchigeri *et al.*, 1981) and more recently *Synechocystis* (Shepherd *et al.*, 2003) have been kinetically characterised. The *Euglena* and *Synechocystis* enzymes operate via a random ternary mechanism where the porphyrin and substrate may bind in any order. However, the wheat enzyme appears to operate via a ping-pong mechanism with S-adenosylmethionine binding first and presumably methylating the enzyme. If this is confirmed, it would represent a novel mechanism for a methyltransferase.

### *C. Magnesium-Protoporphyrin IX Monomethylester Oxidative Cyclase*

An oxidative cyclization is required to create the fifth ring of chlorophyll, a reaction that is catalysed by magnesium protoporphyrin IX monomethyl ester oxidative cyclase. The origin of the oxygen atom in the fifth ring was studied by $^{18}O$ labelling using $^{18}O_2$ and/or $H_2^{18}O$. The oxo group in the fifth ring of chlorophyll is derived from molecular oxygen in cucumber (Walker *et al.*, 1989), while the oxo group in the fifth ring of bacteriochlorophyll of anaerobic photosynthetic bacteria is derived from water (Porra *et al.*, 1995, 1996; Porra and Scheer, 2001). This implies a completely different mechanism and subsequently a different type of enzyme that is required for formation of the fifth ring in these organisms.

There are only a limited number of reports demonstrating oxidative cyclase activity in oxygenic organisms. These reports include activity from chloroplasts of *C. reinhardtii* (Bollivar and Beale, 1996), developing chloroplasts from cucumber cotyledons (Vijayan *et al.*, 1992), lysed cucumber and *C. reinhardtii* chloroplasts (Walker *et al.*, 1991b; Whyte *et al.*, 1992; Whyte and Castelfranco, 1993; Bollivar and Beale, 1996) and cell-free extracts from cyanobacteria (Bollivar and Beale, 1996). The cucumber enzyme was resolved into membrane and soluble components, and inhibition studies suggested that the enzyme was probably not a member of the cytochrome P-450 family (Whyte and Castelfranco, 1993). In contrast to the cyclases from cucumber, *C. reinhardtii* cyclase activity did not require a soluble component and activity was found associated with

membranes. This enzyme is also not a member of the P-450 family based on inhibitor studies (Bollivar and Beale, 1995). The herbicide 2,2'-dipyridyl, which is a $Fe^{2+}$ chelator, inhibits most oxidative cyclases and reduced chlorophyll synthesis (Mostowska *et al.*, 1996). Other iron chelating inhibitors of this enzyme include 8-hydroxyquinoline, desferal mesylate (Walker *et al.*, 1991a) and $\beta$-thujaplicin (Oster *et al.*, 1996). Thus the one common feature of all known cyclases is that they are inhibited by chelators of $Fe^{2+}$, suggesting that non-heme iron or an iron sulphur cluster is involved in the reaction. As only hydrophobic $Fe^{2+}$ chelators appear to be effective inhibitors, it was suggested that the $Fe^{2+}$ requirement is associated with the cyclase membrane fraction (Bollivar and Beale, 1996).

Although no plant genes have been positively identified, hints at the identity of the plant oxidative cyclase genes come from *Chlamydomonas* mutants and the purple bacterium *Rubrivivax gelatinosus*. Unlike many purple bacteria, *Rx. gelatinosus* is able to synthesize bacteriochlorophyll *a* under both aerobic and anaerobic conditions. Disruption of the *AcsF* gene of *Rx. gelatinosus* prevents bacteriochlorophyll *a* synthesis and causes accumulation of magnesium protoporphyrin IX monomethyl ester under aerobic conditions but not under conditions of low aeration. The designation *acsF* stands for aerobic cyclization system Fe-containing subunit, as AcsF and its homologs have a conserved putative binuclear-iron-cluster motif (Pinta *et al.*, 2002). The AcsF protein is homologous to previously identified gene products in *C. reinhardtii* called Crd1 (Moseley *et al.*, 2000) and Cth1 (Moseley *et al.*, 2002) and homologs of AcsF were also identified in *A. thaliana* and *Synechocystis* (Pinta *et al.*, 2002).

*Crd1* and *Cth1* expression in *C. reinhardtii* is reciprocal and is regulated by copper and/or oxygenation conditions. *Crd1* is expressed under low aeration and/or low copper conditions and *Cth1* is expressed under oxygenated and copper sufficient conditions. Mutation of either of these genes and growth under conditions where the alternative protein is not expressed results in a chlorotic phenotype with reduced photosystem I and light-harvesting complex 1 accumulation (Moseley *et al.*, 2000, 2002). These results suggest that the Crd1 and Cth1 proteins probably encode two isoforms of the oxidative cyclase. Two mutant loci in barley called *xantha-l*$^{35}$ and *viridis-k*$^{23}$ also have defective cyclase activity (Walker *et al.*, 1997). Extracts of either *xantha-l* or *viridis-k* showed no activity in an *in vitro* assay nor did components in one extract complement the other when mixed. Fractionation studies showed that the *xantha-l* and *vividis-k* components are membrane-bound subunits and that cyclase activity also required a soluble, stromal component. The barley *Xantha-l* gene is homologous to the *Arabidopsis Crd1*, the ortholog of *AcsF* (Rzeznicka *et al.*, 2005). These putative cyclase encoding genes are nuclear encoded, but an ortholog of *AcsF* is found in the chloroplast genome of the red algae *Porphyra purpurea* (Reith and Munholland, 1995).

## D. 8-Vinyl Reduction

Virtually all photosynthetic organisms require reduction of the 8-vinyl group of chlorophyll to an ethyl group. 8-Ethyl and 8-vinyl derivatives of intermediates from protochlorophyllide to chlorophyllide *a* have been detected in a number of studies by low temperature fluorescence spectroscopy (Rebeiz *et al.*, 1994; JS Kim and Rebeiz, 1995; Parham and Rebeiz, 1995; JS Kim *et al.*, 1997). The relative amounts of 8-ethyl and 8-vinyl intermediates and the stage at which reduction occurs is complex and depends on numerous factors such as species, developmental stage, time in the dark or light, the age of the tissue, and light intensity (Rebeiz *et al.*, 1994). Separation of 8-vinyl-protochlorophyllide and 8-ethyl-protochlorophyllide using a solid phase polyethylene column was used to analyse the biosynthesis of these intermediates in wheat and cucumber cotyledons. The activity in wheat was higher than in cucumber and it was suggested that the reaction is reversible (Whyte and Griffiths, 1993). An 8-vinyl reductase activity was detected in plastid membranes from cucumber that converts 8-vinyl-chlorophyllide *a* to chlorophyllide *a* but was unable to convert 8-vinyl-protochlorophyllide to 8-ethyl-protochlorophyllide (Parham and Rebeiz, 1992, 1995). To explain the diversity of other 8-ethyl intermediates it was suggested that a soluble component may mediate the substrate specificity of the 8-vinyl reductase allowing other 8-vinyl intermediates to be converted to 8-ethyl forms (JS Kim *et al.*, 1997). A gene that encodes a 3,8-divinyl-protochlorophyllide *a* 8-vinyl reductase was recently identified and cloned. When expressed in *Escherichia coli*, the gene product reduced divinyl chlorophyllide *a* to monovinyl-chlorophyllide *a* (Nagata *et al.*, 2005).

## E. Protochlorophyllide Oxidoreductases

Two types of enzymes have been identified that reduce the D pyrrole ring of protochlorophyllide to form chlorophyllide. Of these two enzymes the light-requiring or light-dependent NADPH- protochlorophyllide oxidoreductase (EC 1.3.1.33 or EC 1.6.99.1,

abbreviated POR) has been the subject of a large number of reviews (Fujita, 1996; Reinbothe and Reinbothe, 1996; S Reinbothe *et al.*, 1996; Adamson *et al.*, 1997; Lebedev and Timko, 1998; Schoefs, 2001a,b; Rüdiger, 2003). POR is a single subunit enzyme that requires light as a substrate and it appears to be present in all organisms that synthesize chlorophyll. It has not been found in bacteriochlorophyll-synthesizing organisms. In the dark this enzyme forms a ternary complex with protochlorophyllide and NADPH and the bound protochlorophyllide is only reduced to chlorophyllide upon exposure to light.

The second type of enzyme, known as the light-independent protochlorophyllide oxidoreductase or DPOR, consists of three subunits (Armstrong, 1998; Fujita and Bauer, 2003). The multi-subunit DPOR has not been found in flowering plants (angiosperms) but appears to be present in most other chlorophyll and bacteriochlorophyll synthesizing organisms and allows these organisms to make chlorophyll in the dark. In contrast, flowering plants (angiosperms) are unable to synthesize chlorophyll in the dark, at least during the early stages of development, and thus do not appear to have a DPOR-type of enzyme. However, there are numerous reports that mature green leaves of some angiosperms can synthesize chlorophyll in the dark (reviewed in Adamson *et al.*, 1997). Thus DPOR may be present in mature leaves of some angiosperms or another as yet uncharacterised mechanism exists in these plants to allow chlorophyll synthesis in the dark.

### *1. Light-Dependent Protochlorophyllide Oxidoreductase (POR)*

The barley (*Hordeum vulgare*) *Por* gene encoding POR was the first to be sequenced (Schulz *et al.*, 1989). Since then many *Por* genes have been cloned and sequenced from a variety of sources and some plants have been found to contain multiple *Por* genes encoding different isoforms. *A. thaliana* has three *Por* genes encoding proteins termed PORA, PORB and PORC (Armstrong *et al.*, 1995; Oosawa *et al.*, 2000; Su *et al.*, 2001), while *Pinus taeda* (Skinner and Timko, 1998),*Pinus mugo* (Forreiter and Apel, 1993), barley (Holtorf *et al.*, 1995) and tobacco (Masuda *et al.*, 2002) have at least two genes encoding different POR isoforms. *Por* genes have also been identified in *Triticum aestivum* (Teakle and Griffiths, 1993), cucumber (Fusada *et al.*, 2000), *Pisum sativum* (Spano *et al.*, 1992), *C. reinhardtii* (Li and Timko, 1996) as well as from the cyanobacteria *Synechocystis* PCC6803 (Suzuki and Bauer, 1995). In barley and *A. thaliana* the isoforms are differentially expressed and the isoform called PORA is negatively regulated by light and appears to have a role only in the de-etiolation process (Armstrong *et al.*, 1995; Holtorf *et al.*, 1995; Holtorf and Apel, 1996). However, this type of differential regulation of isoforms does not appear to be universal as the two tobacco *Por* genes are regulated in a similar way and are not negatively regulated by light (Masuda *et al.*, 2002). Some plants such as cucumber have only a single *Por* gene indicating that multiple isoforms are not essential for plant growth and development (Fusada *et al.*, 2000).

POR is responsible for large crystalline-like membrane structures that form within chloroplasts known as prolamellar bodies (PLBs). These structures are visible by electron microscopy and are found in developing angiosperm chloroplasts that have not been exposed to light. These membrane-associated complexes consist of the protochlorophyllide:NADPH:POR ternary complex aggregated within a lipid matrix in the etioplast, which are poised, waiting for the final substrate, light, to allow photoconversion of the protochlorophyllide to chlorophyllide. The main spectral form of protochlorophyllide observed *in vivo* is due to these ternary complexes that make up the PLBs (Wiktorsson *et al.*, 1992, 1993, 1996b). Pigment binding to POR is reported to be essential for the formation of PLBs, as mutants that are unable to make protochlorophyllide do not make PLBs (Henningsen *et al.*, 1993) and PLB formation can also be inhibited by treatment of plants with gabaculine, which inhibits protochlorophyllide formation (Younis *et al.*, 1995). Import of PORA into chloroplasts has been reported to require the presence of protochlorophyllide within the chloroplast (S Reinbothe *et al.*, 1995a, 1995b, 2000). It has been suggested that this finding is an artifact (Aronsson *et al.*, 2000, 2003; Dahlin *et al.*, 2000). A recent paper reconciling these findings indicates protochlorophyllide is indeed required for the import of PORA but this only occurs in etioplasts within developing cotyledons (C Kim and Apel, 2004). A complicating factor in all of these analyses is that a light-induced protease that breaks down PORA is also present within developing chloroplasts (C Reinbothe *et al.*, 1995). Lipids are also required for the formation of PLBs (Klement *et al.*, 2000), and flavins (Belyaeva *et al.*, 2000), violaxanthin and zeaxanthin (Chahdi *et al.*, 1998) have been detected in PLBs and may be involved in their formation. PLBs have been detected in mutants of organisms that are normally able to synthesize chlorophyll in the dark such as the *yellow-in-the-dark* mutants of *C. reinhardtii*. This suggests that most PORs are capable of forming PLBs, and the demonstration that both PORA and PORB of

*A. thaliana* are able to form PLBs supports this suggestion (Sperling *et al.*, 1998; Franck *et al.*, 2000).

On exposure to light, protochlorophyllide bound to POR is converted to chlorophyllide and then rapidly to chlorophyll. The PLBs then disperse or disaggregate as the photosystems are assembled. Protein phosphorylation appears to be involved in both this disaggregation process and in the formation of the PLBs (Wiktorsson *et al.*, 1996a; Kovacheva *et al.*, 2000). Details of the fine structure of the PLBs and what occurs during the photoconversion process have been the source of much contention. It was proposed, based on *in vitro* experiments with zinc analogues of protochlorophyllide *a* and *b*, that in barley a ternary complex of NADPH:PORA:protochlorophyllide *b* and a ternary complex of NADPH:PORB:protochlorophyllide *a* form a 5:1 complex, respectively, within the PLBs. In this complex the NADPH:PORA:protochlorophyllide *b* acts as a light-harvesting complex transferring light to the NADPH:PORB:protochlorophyllide *a,* allowing photoconversion of protochlorophyllide *a* to chlorophyllide *a* (C Reinbothe *et al.*, 1999). Two of the problems with this model are that this was based on *in vitro* experiments with artificial substrates and that protochlorophyllide *b* had not been detected in the quantities required within developing chloroplasts of barley by other investigators (Willows, 1999; Armstrong *et al.*, 2000). Reinbothe *et al.* recently followed up these criticisms with two papers, one showing that protochlorophyllide *b* does in fact occur in barley and that it is rapidly converted to protochlorophyllide *a* by a reductase (S Reinbothe *et al.*, 2003) and the second showing that the *in vitro* produced 5:1 PORA:PORB complex can also be made using authentic protochlorophyllides *a* and *b* (C Reinbothe *et al.*, 2003). However, the controversy continues as Kolossov and Rebeiz (2003), using the methods of Reinbothe, found protochlorophyllide *b* only in mature green barley leaves and not in etiolated barley and thus proposed that the protochlorophyllide *b* complex does not occur *in vivo*.

### *2. Light-Independent (Dark) Protochlorophyllide Oxidoreductase (DPOR)*

Cyanobacteria, green algae and most non-flowering plants have both POR and DPOR. DPOR was reviewed by Armstrong (1998). Green algae and most nonflowering land plants are able to make chlorophyll in the dark with protein products of the chloroplast-encoded genes *ChlL*, *ChlN* and *ChlB.* Mutation or deletion of these chloroplast genes in the green algae *C. reinhardtii* prevented chlorophyll synthesis in the dark (Roitgrund and Mets, 1990; Suzuki and Bauer, 1992; Li *et al.*, 1993; Liu *et al.*, 1993). Seven *C. reinhardtii* nuclear mutants have a similar lack of chlorophyll in the dark and in all cases these mutations prevent the translation of mRNA from the chloroplast-encoded *ChlL* gene (Cahoon and Timko, 2000). The *ChlL* gene of *C. reinhardtii* hybridises to DNA from bacteria and nonflowering land plants, which can clearly synthesize chlorophyll in the dark, but no bands are evident when hybridised to DNA from the representative angiosperms, *Z. mays*, *A. thaliana*, *N. tabacum* and *Bougainvillea glabra* (Suzuki and Bauer, 1992). Unlike most other chlorophyll biosynthetic genes, when the *ChlL*, *ChlN* and *ChlB* genes are present, they are invariably found in the chloroplast genomes (Lidholm and Gustafsson, 1991; Burke *et al.*, 1993; Suzuki *et al.*, 1997; Armstrong, 1998).

### *F. Chlorophyll a Synthase*

Chlorophyll *a* synthase catalyses the final step in the synthesis of chlorophyll *a* with the esterification of a phytol group to the 17-propionate. Chlorophyll synthase genes, *ChlG*, have been cloned and the enzymes heterologously expressed in *Escherichia coli* from both oat (*Avena sativa*) and *A. thaliana.* The C*hlG* is nuclear encoded and encodes a chloroplast transit sequence for translocation of the enzyme into the chloroplast. Phytyl-pyrophosphate and geranylgeranyl-pyrophosphate are both substrates for chlorophyll synthases. *A. thaliana* chlorophyll synthase preferred geranylgeranyl-pyrophosphate as the substrate (Oster *et al.*, 1997; Oster and Rüdiger, 1997; Schmid *et al.*, 2001).

The *ChlP* gene product is required for the reduction of geranylgeraniol to phytol and it appears that reduction can occur either before or after esterification to chlorophyllide *a*. The *ChlP* genes in *N. tabacum* and *A. thaliana* are located in the nuclear genome and encode a putative 52-kDa precursor protein. Transgenic tobacco plants expressing antisense *ChlP* RNA have both reduced tocopherol and chlorophyll synthesis, indicating that this enzyme provides phytol and/or phytyl-pyrophosphate for both of these pathways (Tanaka *et al.*, 1999). Two types of reductase may be present in chloroplasts, as there is a reductase activity in the chloroplast envelope that converts geranylgeranyl-pyrophosphate to phytyl-pyrophosphate and a second in the thylakoids converts geranylgeraniol esterified to chlorophyllide *a* into chlorophyll *a* (Soll *et al.*, 1983).

### *G. Chlorophyll* a*-Chlorophyll* b *Cycle*

Chlorophyll *a* oxygenase (CAO) is the enzyme that catalyses the conversion of chlorophyll *a* to chlorophyll *b. CAO* genes have been identified in *C. reinhardtii*, *A. thaliana, Oryyza sativa, Marchantia polymorpha, Dunaliella salina, Prochlorothrix hollandica* and *Prochloron didemni* (Tanaka *et al.*, 1998; Espineda *et al.*, 1999; Tomitani *et al.*, 1999). The *A. thaliana* CAO was heterologously expressed in *E. coli* and required oxygen and reduced ferredoxin to convert chlorophyllide *a* to chlorophyllide *b*. Traces of a $7^1$-hydroxy intermediate were detected, and the enzyme could also use Zn-chlorophyllide *a* as a substrate but not pheophorbide or chlorophyll *a* (Oster *et al.*, 2000), indicating that the enzyme is a chlorophyllide *a* oxidase rather than a chlorophyll *a* oxidase.

Rüdiger (2002) reviewed the synthesis of chlorophyll *b* and suggested that the interconversion of chlorophyll *a* and *b* operates as a cycle. Chlorophyll *b* can be converted to chlorophyll *a* by a chloroplast localised reductase activity. The gene encoding this reductase has yet to be identified. This reductase activity is probably required for both alteration of the chlorophyll *a* to *b* ratio and in the degradation of chlorophyll. Both chlorophyll *a* and *b* can also be converted to their corresponding chlorophyllides by chlorophyllase. Thus the cycle exists from chlorophyllide *a* to *b,* followed by esterification to chlorophyll *b,* reduction to chlorophyll *a,* and deesterification back to chlorophyllide *a* (Rüdiger, 2002).

## III. Regulation of Chlorophyll Biosynthesis

The three main regulatory points in chlorophyll biosynthesis appear to be the steps involved in ALA biosynthesis, magnesium chelatase and protochlorophyllide reductase. Fig. 3 shows an overview of the regulatory mechanisms that affect these steps. The regulation of key steps in chlorophyll biosynthesis includes;

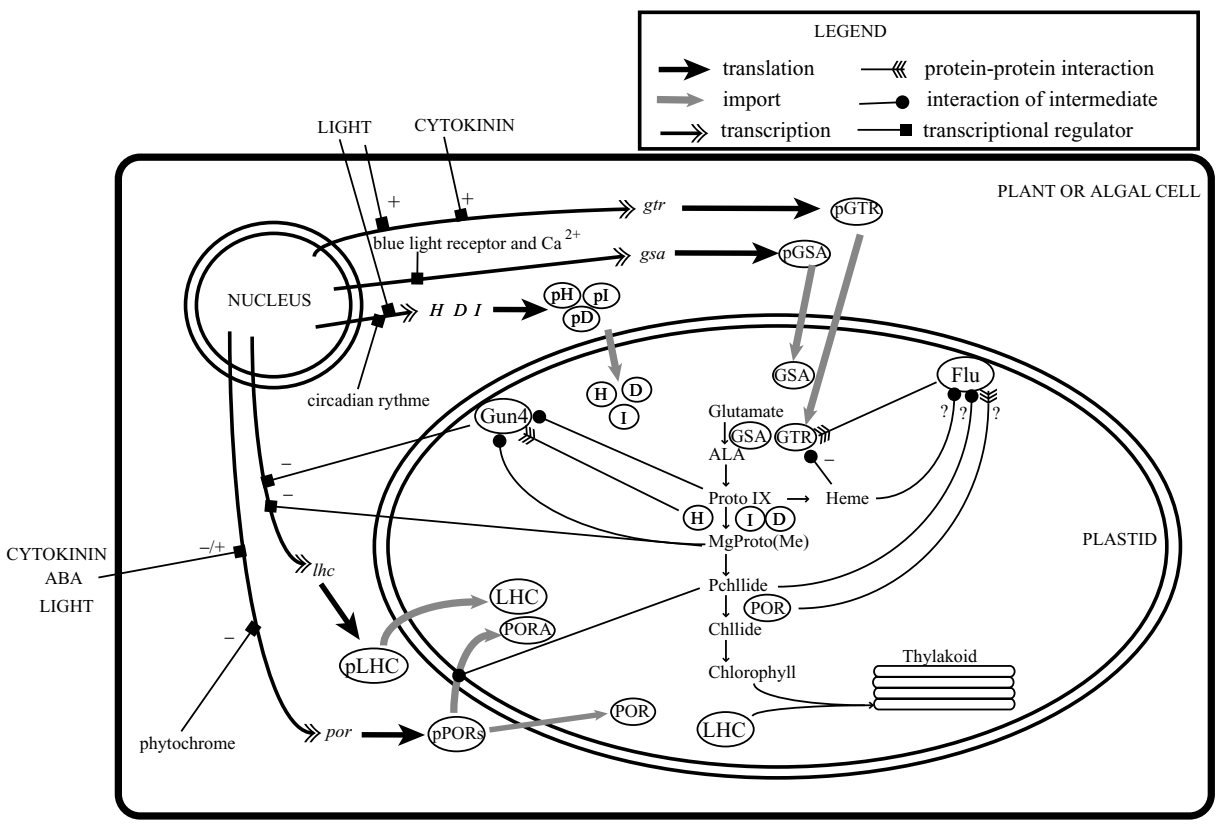


*Fig. 3.* Regulatory network within plant and algal cells involving the chlorophyll biosynthetic pathway enzymes and intermediates. (−) indicates inhibition, (+) indicates activation, and (?) indicate putative effects. Ellipses represent proteins or preproteins with small "p" prefix. H, D and I are magnesium chelatase subunits. For all other abbreviations see text.

(i) mechanisms to control quantities of individual enzymes using transcriptional or translational controls, and (ii) mechanisms to control activities of various enzymatic steps within the pathway using feedback inhibition or other modifiers of enzymatic activity. An additional feature that may or may not impact on regulation of the pathway but which is involved in plastid development is that both the product of the magnesium chelatase reaction and the magnesium chelatase itself are implicated in control of nuclear gene expression, specifically the control of LHC gene expression.

### A. Regulation of ALA Synthesis

The primary regulatory step in chlorophyll biosynthesis is at the level of ALA biosynthesis. This is clear from feeding studies with ALA, which causes the unregulated synthesis of chlorophyll in the light or of protochlorophyllide in the dark. In plants the enzyme glutamyl-tRNA reductase (GTR) is the rate determining step of the entire tetrapyrrole biosynthetic pathway (Grimm, 2003). Feedback inhibition and transcriptional regulation are both used to regulate the activity of this enzyme. Feedback regulation by heme is difficult to demonstrate conclusively because of the detergent like properties of heme and its low solubility. However, recombinant and natural barley GTR have been shown to be inhibited by heme and also appear to have a bound heme (Pontoppidan and Kannangara, 1994; Vothknecht *et al.*, 1998). The inhibition and heme binding were both abolished when the N-terminal end of the barley enzyme was truncated by 30 amino acids, which tends to confirm that the heme inhibition is not an artifact (Vothknecht *et al.*, 1998). Protochlorophyllide is known to limit its own synthesis in dark-grown plants and the feedback regulation occurs at the level of ALA synthesis. This inhibition is likely to be via the FLU protein, as mutations in the *Flu* gene result in deregulation of synthesis of protochlorophyllide, and FLU been shown to interact with GTR (Meskauskiene *et al.*, 2001; Meskauskiene and Apel, 2002).

All plants studied to date have multiple GTR genes, which are differentially expressed in various plant organs and under a variety of conditions, and both light and cytokinin have been shown to induce expression of one *Gtr* gene. Thus a *Gtr* of barley is induced by both light and cytokinin and is also circadian regulated (Bougri and Grimm, 1996). Light induced expression of both the *Gsa* and one of the *Gtr* genes of *A. thaliana* (Ilag *et al.*, 1994). Other regulators of *Gtr* transcription have included temperature, photooxidative stress, and sugar (Ujwal *et al.*, 2002; Grimm, 2003). These transcriptional studies are consistent with *in vivo* studies showing chlorophyll accumulation is enhanced by red-light and cytokinin treatment in developing cucumber cotyledons which involves $Ca^{2+}$ as a second messenger (Reiss and Beale, 1995).

The unicellular algae *C. reinhardtii* seems to regulate its ALA synthesis by controlling levels of the enzyme glutamate semi-aldehyde aminotransferase, as the transcript levels for this enzyme vary 26-fold in response to blue-light, ammonia and/or acetate (Matters and Beale, 1994, 1995; Im *et al.*, 1996). However, details of the regulation of *C. reinhardtii Gtr* have yet to be reported and it is conceivable that it may be similarly regulated. The change in *C. reinhardtii Gsa* transcript levels are mediated via inositol triphosphate-induced $Ca^{2+}$ release which activates calmodulin and a $Ca^{2+}$/calmodulin dependent protein kinase (Im and Beale, 2000). The similarity between aspects of this transcriptional regulation and that of some plant *Gtr* and *Gsa* genes indicate that this signalling pathway may be conserved between plants and algae although the sensor may have diverged.

### B. Magnesium Chelatase

Regulation of magnesium chelatase gene expression has been examined in a number of species. In etiolated barley, expression of ***I*** and ***H*** genes is induced by light, while in green barley seedlings grown in normal day light cycles the ***H*** transcript levels follows a circadian rhythm with maxima in the light phase (Jensen *et al.*, 1996b). The tobacco ***H*** and ***I*** transcripts follow a similar circadian pattern but the ***D*** transcript has an inverse expression pattern with maximal mRNA levels in the dark phase. In *A. thaliana* (Gibson *et al.*, 1996) and *A. majus* (Hudson *et al.*, 1993) the ***H*** transcript is at its maximal level in the dark and is down-regulated in the light. The ***I*** gene in barley and *A. thaliana* is constitutively expressed except during the initial phases of greening. In *C. reinhardtii* all three ***H***, ***I*** and ***D*** genes appear to be regulated the same way. The transcript levels of these genes follow a diurnal regulation with maxima in both the light and dark phases with rapid fall in transcripts at the start of the light phase followed by a rise to a maximum at about 4 hours light and fall to a minimum near the end of the light and an increase to a maxima near the end of the dark phase (Lake and Willows, 2003). Another factor regulating magnesium chelatase activity is the recent finding that an active **I** protein is required to stabilise the **D** protein *in vivo* (Lake *et al.*, 2004). Thus reduction in the amount of **I**

will cause a corresponding loss of the **D** subunit and a reduction of magnesium chelatase activity.

Magnesium chelatase proteins and magnesium protoporphyrin IX have been implicated in chloroplast-to-nuclear signalling. As mentioned previously, the *A. thaliana* genomes-uncoupled mutant, *gun-5*, is a result of a point mutation in the ***H*** gene; a mutation in the ***D*** gene gives a similar phenotype (Mochizuki *et al.*, 2001; Strand *et al.*, 2003). The recently identified GUN-4 protein binds protoporphyrin IX and also interacts with the porphyrin-binding **H** protein. This implicates GUN-4 as one of the downstream signalling components in chloroplast nuclear signalling (Larkin *et al.*, 2003).

### C. Protochlorophyllide Oxidoreductase (POR)

Phytochrome, circadian clocks, cytokinin, abscisic acid and leaf age have been implicated in control of *Por* gene expression. The amounts of POR protein and mRNA decrease rapidly in many species when etiolated plants are exposed to light (Forreiter *et al.*, 1990), suggesting phytochrome involvement in this process. Experiments using *A. thaliana* with red and far-red light treatments have confirmed phytochrome A regulates *PorA* mRNA levels (Barnes *et al.*, 1996; Sperling *et al.*, 1997, 1998). Phytochrome has also been shown to regulate the expression of the *Por* gene from a lower plant (*Marchantia paleacea*) (Suzuki *et al.*, 2001). In barley the phytochrome- and/or light-dependent regulation of *PorA* mRNA levels is dependent on a 3′-untranslated region in the mRNA (Holtorf and Apel, 1996). In addition to the reduction in message, a light-dependent degradation of the PORA bound to chlorophyllide, but not protochlorophyllide, occurs and a light-induced protease has been shown to be responsible (C Reinbothe *et al.*, 1995). In contrast, cucumber, which only has a single *Por* gene, shows an increase in *Por* message levels during the de-etiolation process (Kuroda *et al.*, 1995). Moreover, in fully green leaves of cucumber, this gene is expressed at very low levels in the dark and the amount of *Por* message increases dramatically when plants are transferred from dark to light (Kuroda *et al.*, 2000).

The effect of leaf age on *Por* gene expression has been studied in pea (He *et al.*, 1994), barley (Holtorf *et al.*, 1995; Schunmann and Ougham, 1996), wheat (Marrison *et al.*, 1996), and *A thaliana* (Armstrong *et al.*, 1995). In barley and *A. thaliana*, *PorA* mRNA is only expressed in young etiolated tissue while the *PORB* mRNA is expressed throughout development. In light-grown seedlings of pea and wheat the youngest leaves contained the highest POR message levels.

The plant hormones cytokinin and abscisic acid appear to have a role in regulation of *Por* gene expression. The involvement of cytokinin was inferred from the finding that cytokinins overcame the inhibition of greening caused by treatment with cadmium and mercury (Thomas and Singh, 1995, 1996), although cadmium and mercury also have a direct effect on POR enzyme activity (Boddi *et al.*, 1995; Lenti *et al.*, 2002). It was subsequently found that cytokinins directly activated *Por* gene expression in cucumber (Kuroda *et al.*, 2001) and *Lupinus luteus* (Kusnetsov *et al.*, 1998) and that abscisic acid inhibits *Por* gene expression in *L. luteus* (Kusnetsov *et al.*, 1998).

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# Chapter 16

# Carotenoids

Abby J. Cuttriss[1], Joanna L. Mimica[1], Crispin A. Howitt[2] and Barry J. Pogson[1,*]

[1] *ARC Centre of Excellence in Plant Energy Biology, School of Biochemistry and Molecular Biology, The Australian National University, Acton ACT 0200, Australia*

[2] *CSIRO Plant Industry, GPO Box 1600, Canberra ACT 2601, Australia*

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## Summary

Carotenoid pigments provide fruit and flowers with distinctive red, orange and yellow colors and a number of aromas, which make them commercially important in agriculture, food manufacturing and the cosmetic industry. However, it is their roles in photosynthesis and nutrition that explain the absolute requirement for carotenoids in the survival of plants and animals alike. Carotenoids comprise a large family of over 600 members of isoprenoids (Britton *et al*., 2004). Most are $C_{40}$ polyenes and all are derived from phytoene. The carotenoid backbone is either linear or contains cyclic end-groups. The most abundant end-group is the β-ionone ring of β-carotene and its derivatives.

*Author for correspondence, email: barry.pogson@anu.edu.au

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Carotenoids with unmodified β-ionone groups serve as precursors for vitamin A and are therefore essential dietary components. Other cyclic end-groups are the ε-ring found in α-carotene and lutein and the unusual cyclopentane ring of capsanthin and capsorubin that impart the distinct red color to peppers. Non-oxygenated carotenoids are referred to as carotenes, whereas their oxygenated derivatives are designated as xanthophylls. The most commonly occurring carotenes are β-carotene in chloroplasts and lycopene, which accumulates in chromoplasts of flowers and some fruits, e.g., tomatoes. The most abundant xanthophylls, lutein, violaxanthin and neoxanthin, are key components of the light-harvesting complexes of leaves and impart the yellow color of autumn leaves that is normally masked by the green chlorophylls (xanthos = yellow and phyll = leaf).

## I. Introduction

Carotenoid research has a long and distinguished history, with insights into carotenoid chemistry that earned Nobel prizes for Paul Karrer and Richard Kuhn in 1937 and 1938, respectively. Key discoveries have continued to be made to this day. The history of carotenoid research is comprehensively discussed by Govindjee (1999) and there have been a number of significant reviews in recent years (Niyogi, 1999; Hirschberg, 2001; Cunningham, 2002; Sandmann, 2002). Carotenoids are a ubiquitous component of all photosynthetic organisms, because they are required for assembly and function of the photosynthetic apparatus.

Likewise, carotenoids are a vital part of mammalian diets as antioxidants and precursors to vitamin A, and their dietary uptake can pigment the tissues of animals such as fish, crustaceans and birds (see Fig. 1). Vitamin A-deficiency is responsible for a number of disorders that range from impaired iron mobilization, growth retardation and blindness, to depressed immune response and increased susceptibility to infectious disease (Sommer and Davidson, 2002). More than 100 million children are vitamin A-deficient; 250,000 to 500,000 become blind every year, and half of these die within 12 months after losing their sight (http://www.who.int/nut/vad.htm). Simply improving the vitamin A status of children by increasing the uptake of provitamin A, e.g., β- and α-carotene, which can be cleaved to form 11-*cis*-retinal (vitamin A), can reduce overall child mortality by 25% (http://www.unicef.org/immunization/facts_vitamina.html).

The distinctive colors, typically yellow and red, are largely determined by the number of conjugated double bonds in the backbone (Table 1). However, varying structural and oxygenic modifications can impart different spectral properties. For example, astaxanthin is derived from β-carotene, with the oxygenic modifications increasing conjugation and thus shifting the color from yellow to red. Astaxanthin's spectral properties can be further modified by binding to the protein crustacyanin from shellfish, which results in blue pigmentation (Britton *et al*., 1997).

## II. Carotenoid Biosynthesis

### A. Isoprenoid Precursors

Carotenoids are derived from the two isoprene isomers, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the so-called "building blocks" for carotenoids. The same precursors are used to make a range of compounds that include tocopherols, chlorophylls, phylloquinone, gibberellins, abscisic acid, monoterpenes and plastoquinone. The biosynthesis of isoprenoid precursors is covered in detail elsewhere (Lichtenthaler, 1999; Cunningham, 2002; Lange and Ghassemian, 2003), so just a summary follows.

Two distinct pathways exist for IPP production; the mevalonic acid (MVA) pathway and the mevalonate-independent, methylerythritol 4-phosphate (MEP)

*Abbreviations:* ABA – abscisic acid; βLYC – lycopene β-cyclase; βOH – β-hydroxylase; CCD – carotenoid cleavage dioxygenases; CsZCD – crocus zeaxanthin 7,8(7′,8′)-cleavage dioxygenase; CPTA – *N,N*-diethyl-*N*-[2-(4-chlorophenylthio) ethyl]amine; CRTISO – carotenoid isomerase; DMAPP – dimethylallyl diphosphate; DOX – 1-deoxy-D-xylulose; DXP – deoxy-D-xylulose 5-phosphate; DXS – deoxy-D-xylulose 5-phosphate synthase; εLYC – lycopene ε-cyclase; εOH – ε-hydroxylase; GAP – glyceraldehyde-3-phosphate; GGPP – geranylgeranyl diphosphate; HDR – hydroxymethylbutenyl diphosphate reductase; IPP – isopentenyl diphosphate; LHC – light-harvesting complex; LHCP – light-harvesting complex protein; MEP – methylerythritol 4-phosphate; MVA – mevalonic acid; NCED – 9-*cis*-epoxycarotenoid dioxygenase; NPQ – non-photochemical quenching; NXS – neoxanthin synthase; PDS – phytoene desaturase; PSY – phytoene synthase; PTOX – plastid terminal oxidase; RCD – related to carotenoid dioxygenase; VDE – violaxanthin de-epoxidase ZDS – ζ-carotene desaturase; ZE – zeaxanthin epoxidase.

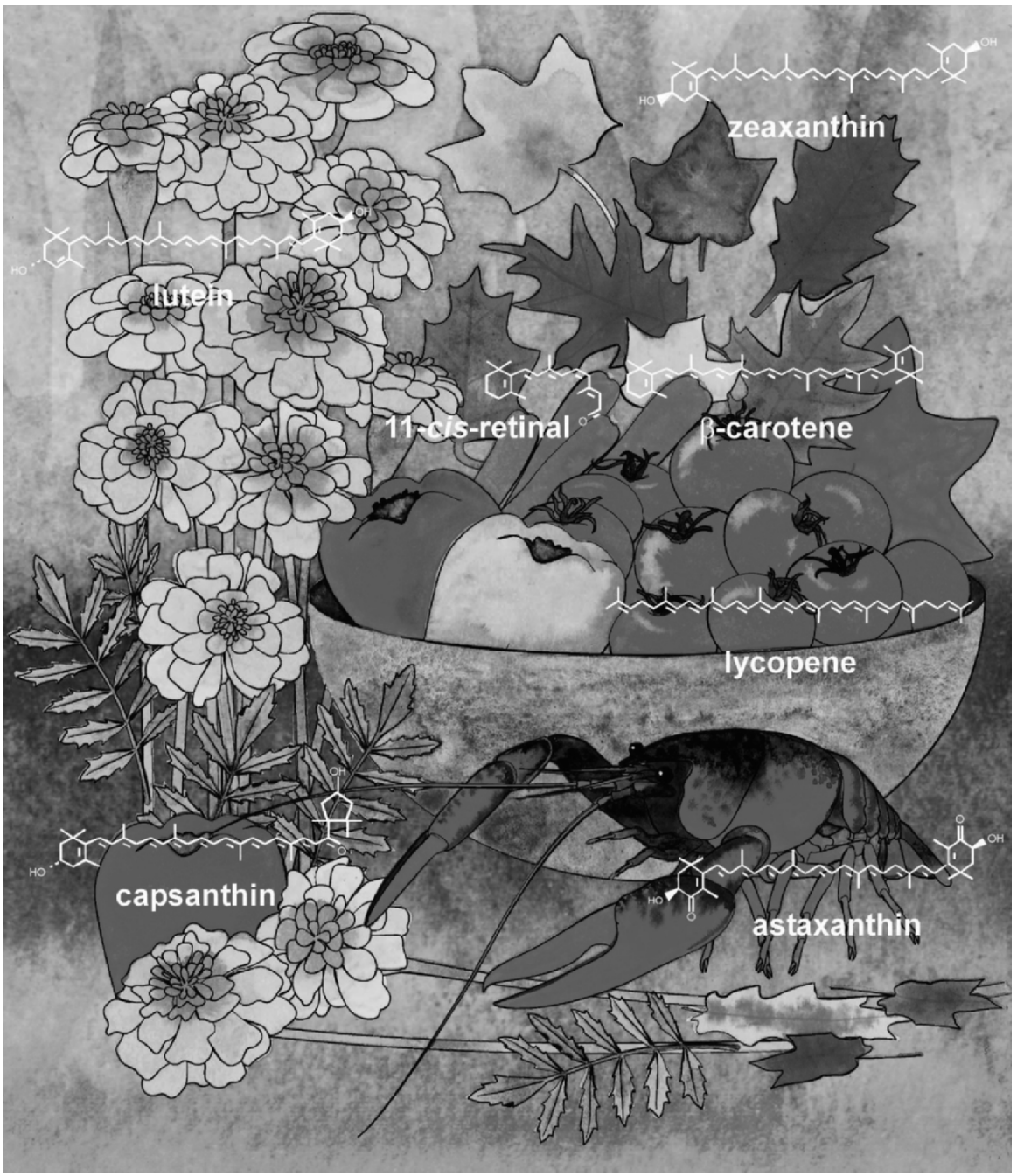


*Fig. 1.* (See also Color Plate 5, p. xxxviii.) Carotenoid diversity. Carotenoids are highly diverse in terms of color and structure. Likewise, they pigment a wide range of bacteria, fungi and plant tissues. The xanthophylls and β-carotene are essential for assembly and function of the photosynthetic apparatus. These yellow pigments, including lutein and zeaxanthin, become apparent in autumn leaves once the green chlorophyll has been degraded. Lutein is the major pigment in marigold flowers, which can range from white to dark-orange because of differences in lutein content. Astaxanthin, complexed with crustacyanin, is responsible for the blue color of lobsters, which shifts to red when the protein is denatured. Capsanthin and capsorubin have unusual cyclopentane rings and are the predominant pigments in red capsicum. Carrot is a source of β- and α-carotene, both of which can be cleaved to form vitamin A (11-*cis*-retinal), and the distinctive red color of ripe tomatoes is due to accumulation of lycopene.

*Table 1.* Semi-systematic names of carotenoids.

| Trivial name[1] | Semi-systematic name[2] | CDBs[3] | $\lambda_{max}$ (nm)[4] | Color |
|---|---|---|---|---|
| phytoene | 7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene | 3 | 276 286 297[a] | colourless |
| phytofluene | 7,8,11,12,7′,8′-hexahydro-ψ,ψ–carotene | 5 | 331 348 367[a] | colourless |
| ζ-carotene | 7,8,7′,8′-tetrahydro-ψ,ψ–carotene | 7 | 378 400 425[a] | pale yellow |
| neurosporene | 7,8-dihydro-ψ,ψ-carotene | 9 | 415 440 468[a] | yellow |
| pro-lycopene | 7Z,9Z,7′Z,9′Z-tetra-*cis*-ψ,ψ-carotene | 11 | 424 442 464 | orange |
| lycopene | ψ,ψ-carotene | 11 | 447 473 505 | pink/red |
| γ-carotene | β,ψ-carotene | 11 | 435 461 490[a] | pink |
| δ-carotene | ε,ψ-carotene | 10 | 421 456 489[a] | yellow/orange |
| α-carotene | β,ε-carotene | 10 | 421 445 473[a] | yellow |
| zeinoxanthin | β,ε-carotene-3,-ol | 10 | 424 446 476 | yellow |
| lutein | β,ε-carotene-3,3′-diol | 10 | 426 447 474 | yellow |
| β-carotene | 7,8-dihydro-β,β-carotene | 11 | 432 454 480 | orange |
| β-cryptoxanthin | β,β-caroten-3-ol | 11 | 425 449 476 | yellow/orange |
| zeaxanthin | β,β-carotene-3,3′-diol | 11 | 432 454 480 | orange |
| antheraxanthin | 5,6-epoxy-5,6-dihydro-β,β-carotene 3,3′-diol | 10 | 424 448 475 | yellow |
| violaxanthin | 5,6:5′6′-diepoxy-5,6,5′6′-tetrahydro β,β-carotene-3,3′-diol | 9 | 417 440 470 | yellow |
| neoxanthin | 5′,6′-epoxy-6,7-didehydro-5,6,5′,6′ tetrahydro-β,β-carotene-3,5,3′-triol | 8 | 414 437 465 | yellow |
| astaxanthin | 3,3′-dihydroxy-β,β-carotene-4,4′-dione | 13 | 478[a] | red |
| capsanthin | 3,3′-dihydroxy-β,κ-caroten-6′-one | | 460 483 518[b] | red |
| capsorubin | 3,3′-dihydroxy-β,κ-carotene-6,6′-dione | | 460 489 523[b] | red |

[1,2] Trivial and semi-systematic names of carotenoids discussed in this chapter.
[3] Number of conjugated double bonds (CDBs)
[4] Absorption maxima ($\lambda_{max}$) and color. Absorption maxima are given in an acetonitrile/ethyl acetate mixture measured in-line by a diode array detector (Pogson *et al*., 1996) unless otherwise indicated: [a]hexane, [b]benzene (Britton *et al*., 1995).

pathway (Lange *et al.*, 2000). The MVA pathway is localized in the cytosol and produces IPP from acetyl-CoA, which can then be converted reversibly to DMAPP by the IPP isomerase. The MEP pathway is in plastids and combines glyceraldehyde-3-phosphate (GAP) and pyruvate to form deoxy-D-xylulose 5-phosphate (DXP), a reaction catalyzed by DXP synthase (DXS). A number of steps are then required to form IPP and DMAPP (Lichtenthaler *et al*., 1997), which are subjected to a sequential series of condensation reactions to form geranylgeranyl diphosphate (GGPP) (Cunningham and Gantt, 2000).

The MEP pathway provides substrates for carotenoids, as indicated by labeling studies (Lichtenthaler *et al*., 1997), the plastidallocalization of carotenoids and their respective biosynthetic enzymes, and inhibitor studies in tomatoes (Zeidler *et al*., 1998). Likewise, the *Arabidopsis Cla1* mutant, in which the *DXS* gene of the MEP pathway is disrupted, is photobleached because of the absence of protective carotenoids, unless the substrate 1-deoxy-D-xylulose (DOX) is applied (Araki *et al*., 2000; Estevez *et al*., 2000). In contrast, the MVA inhibitor, lovastatin, did not affect carotenoid accumulation (Rodriguez-Concepcion and Gruissem, 1999).

Flux through the MEP pathway appears to regulate carotenoid content. Increased expression of IPP isomerase increased carotenoid content (Kajiwara *et al*., 1997) as did over-expression of a plastid-targeted tomato hydroxymethylbutenyl diphosphate reductase (HDR) cDNA in *Arabidopsis*, but the same trend was not observed in etioplasts (Botella-Pavia *et al*., 2004). Assays with GeneChip (Affymetrix) microarrays failed to note concomitant changes in the expression levels of biosynthetic genes of these pathways (Laule *et al*., 2003). In a separate study, DXS was shown to increase during tomato fruit ripening concomitantly with phytoene synthase, the first step in carotenoid biosynthesis (Lois *et al*., 2000). Laule and colleagues suggested that posttranscriptional processes are involved in regulating metabolite flux (Laule *et al*., 2003). There is little doubt that further research will reveal that regulation of the pathway is a combination of transcriptional, posttranscriptional and feedback control, each with differing degrees of influence depending upon the enzyme, tissue, developmental stage and species.

## B. Carotene Synthesis

Carotenoid enzymes are notoriously labile *in vitro*, confounding many classical biochemical approaches to understand carotenoid biosynthesis. Thus, elucidation of the biosynthetic pathway has relied heavily on molecular genetics and only since the 1990s has carotenoid biosynthesis been described at the molecular level (Fig. 2) (Armstrong, 1997; Britton *et al*., 1998; Cunningham and Gantt, 1998; Hirschberg, 1998).

### 1. Phytoene Synthase (PSY)

The first committed step is the condensation of two molecules of GGPP to produce phytoene. This reaction is catalyzed by phytoene synthase (PSY) in higher plants and bacteria (CrtB) (Armstrong, 1994). In *Narcissus pseudonarcissus,* PSY was found to be inactive in its soluble, stromal form and active when lipid-associated (Schledz *et al*., 1996), although there was no evidence of a lipid requirement in a study of PSY from tomato (Fraser *et al*., 2000). This finding, together with reports of a soluble PSY complex, suggested that post-translational regulation may be just as important as transcriptional regulation (Camara, 1993; Fraser *et al*., 2000). Kinetic analyses of PSY partially purified from tomato chloroplasts identified $Mn^{2+}$ and ATP as essential cofactors. In contrast, the chromoplast version was essentially inactive with $Mn^{2+}$ but fully active with $Mg^{2+}$ as a cofactor, had a $K_M$ of 10 μM for GGPP, as compared with 5 μM for the enzyme from chloroplasts, and had a more alkaline pH optimal (pH 7.5 vs. pH 6.5 for the enzyme from chloroplasts) (Fraser *et al*., 2000). Activity of the partially purified PSY from tomato chloroplasts was reduced *in vitro* by β-carotene and chlorophyll, whereas phytoene, ζ-carotene and lycopene did not alter activity (Fraser *et al*., 2000).

PSY is a rate-limiting step and transcript levels are up-regulated in response to light and various other stimuli. The light response is mediated by phytochrome, with both red and far-red light treatments enhancing accumulation of *PSY* mRNA, a trend that is abolished in the *phyA* mutant of *Arabidopsis* (von Lintig *et al*., 1997; Welsch *et al*., 2000). Light quality also has an effect on transcription levels, as different light qualities differentially increased expression of a *PSY* promoter::luciferase reporter transgene. Treatment with norflurazon and gabaculine, inhibitors of carotenoid and chlorophyllide synthesis, respectively, failed to have an effect on expression of the transgene, while the cyclase-blocking herbicide, *N,N*-diethyl-*N*-[2-(4-chlorophenylthio)ethyl]amine (CPTA), appeared to abolish light induction (Welsch *et al*., 2003). In a similar study, light/dark regulation was investigated in pepper (*Capsicum annuum*) leaves. In norflurazon-treated plants, carotenoid biosynthesis was stalled in the dark but up-regulated in the light, mainly due to an increase in *PSY* transcript levels (Simkin *et al*., 2003). As yet, no regulatory or signaling elements have been identified for any step of the biosynthetic pathway. The effect of light on PSY activity, however, extends beyond transcriptional regulation; PSY is relatively inactive when associated with the prolamellar body of dark-grown etioplasts (Welsch *et al*., 2000). White-light induction of photomorphogenesis results in relocation of the enzyme to the newly developing thylakoid membranes, which results in greater activity (Welsch *et al*., 2000).

Seed-specific over-expression of the endogenous *Arabidopsis PSY* gene increased carotenoid and chlorophyll content but germination was delayed, probably due to higher levels of the carotenoid-derived, dormancy hormone, ABA (Lindgren *et al*., 2003). Seeds had a >40% increase in β-carotene and some increase in lutein, violaxanthin, lycopene and α-carotene, with little change in zeaxanthin (Lindgren *et al*., 2003). Over-expression of *PSY* in tobacco (*Nicotiana tabacum*) resulted in dwarfism, altered leaf morphology and pigmentation (Busch *et al*., 2002). Over-expressing the *crtB* phytoene synthase gene from the bacterium *Erwinia uredovora* in a fruit-specific manner resulted in a 2- to 4-fold increase in tomato fruit carotenoids. Other enzymes were not significantly altered and it appears that introduction of the bacterial gene uncoupled normal regulatory restrictions on carotenoid accumulation (Fraser *et al*., 2002).

### 2. Desaturases (PDS and ZDS)

Phytoene is produced as a 15-*cis* isomer, which is subsequently converted to all-*trans* isomer derivatives (Beyer, 1989; Beyer *et al*., 1989; Beyer and Kleinig, 1991). Two desaturases, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), catalyze similar dehydrogenation reactions by introducing four double-bonds to form lycopene. Desaturation is linked to a plastidic respiratory redox chain (Nievelstein *et al*., 1995) and evidence for a quinone requirement was demonstrated in daffodil and *Arabidopsis* (Beyer, 1989; Norris *et al*., 1995). The molecular basis for this phenomenon was elucidated with the cloning of *IMMUTANS*, encoding a plastid terminal oxidase (PTOX)

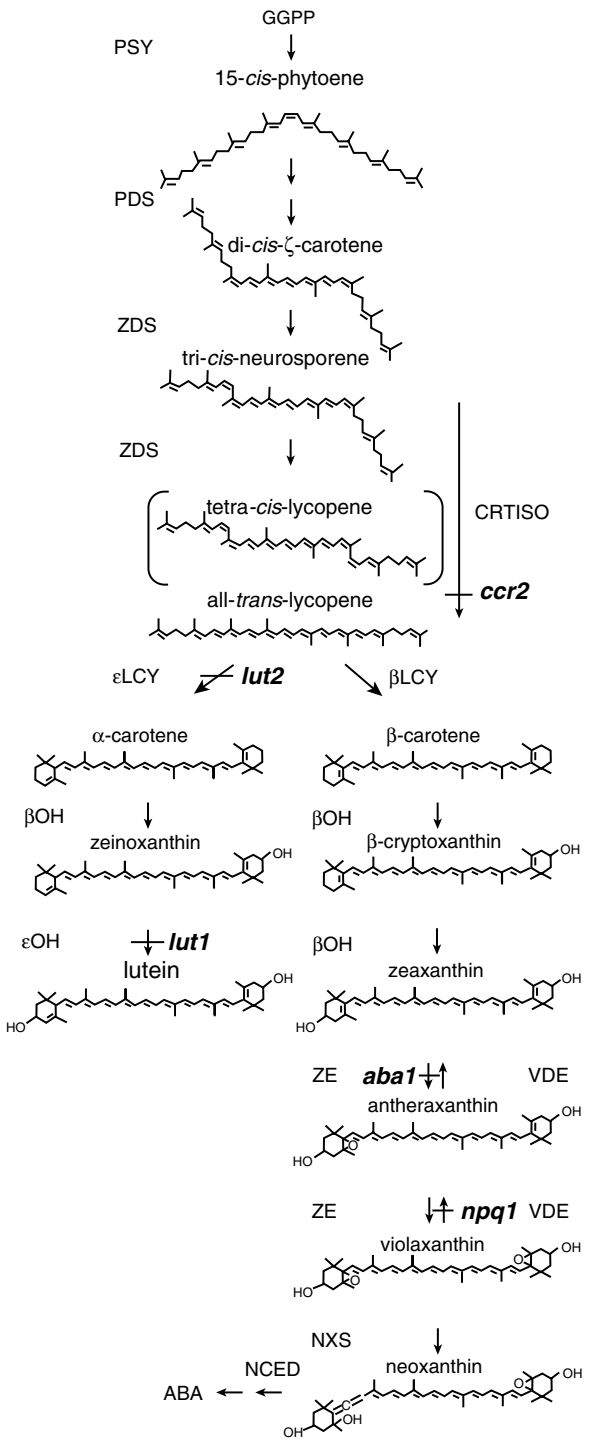


*Fig. 2.* Carotenoid biosynthetic pathway in higher plants. The pathway shows the primary steps found in nearly all plant species. The desaturases introduce a series of four double bonds in a *cis*-configuration, which are isomerized to the all-*trans*-conformations by the carotenoid isomerase. CRTISO appears to act in concert with the ζ-carotene desaturase, resulting in a complex mix of isomers, with tetra-*cis*-lycopene as the main product in the CRTISO mutant (*ccr2*). Additional *Arabidopsis* mutations are shown in italics. ABA, abscisic acid; βLCY, β-cyclase; βOH, β-hydroxylase; CRTISO, carotenoid isomerase; εLCY, ε-cyclase; εOH, ε-hydroxylase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZE, zeaxanthin epoxidase.

required for PDS function, which links desaturation to chloroplast respiratory activity (Carol *et al*., 1999; Wu *et al*., 1999; Josse *et al*., 2000).

While expression of *PSY* is rate-limiting and phytochrome-mediated, the evidence for *PDS* transcriptional regulation is more variable, depending upon the species, tissue and developmental process (Corona *et al*., 1996; Wetzel and Rodermel, 1998; Ronen *et al*., 1999). Transformation of tobacco with an antisense *PDS* transgene resulted in elevated levels of phytoene, which suggested an increased metabolic flux (Busch *et al*., 2002), as was observed upon treatment with norflurazon in the light (Simkin *et al*., 2003). Constitutive expression of the bacterial phytoene desaturase from *Erwinia urdovora*, CrtI, in tobacco (Misawa *et al*., 1994) resulted in norflurazon-resistance, as expected. However, the total carotenoid pool was not altered and lutein decreased as compensation for an increase in β-carotene and its derivatives. In tomato transgenics, expression of CrtI caused β-carotene accumulation but an overall decrease in carotenoid content (Romer *et al*., 2000; Fraser *et al*., 2001).

### 3. Carotenoid Isomerase (CRTISO)

Until recently, the higher plant desaturases were commonly viewed as sufficient for the production of all-*trans*-lycopene. This conclusion was reached despite several contrary observations such as the production of 7,9,7′,9′-tetra-*cis*-lycopene when PDS and ZDS were expressed in *Escherichia coli* (Bartley *et al*., 1999) and isolation of the tetra-*cis*-lycopene-accumulating, *tangerine* tomato mutant (Tomes *et al*., 1953; Isaacson *et al*., 2002) and similar algal mutants (Powls and Britton, 1977; Cunningham and Schiff, 1985; Ernst and Sandmann, 1988). This isomer anomaly suggested a higher plant requirement for a specific isomerase enzyme (Beyer *et al*., 1991), but the isomerase was not identified in plants until recently (Isaacson *et al*., 2002; Park *et al*., 2002). Intriguingly, the carotenoid isomerase gene, *CRTISO,* shows 20 to 30% identity to the bacterial carotenoid desaturases, *crtN* and *crtI*, with regions of higher identity that represent conserved motifs, such as the dinucleotide flavin-binding domain. However, CRTISO has no desaturase activity in an *E. coli* expression system (Park *et al*., 2002).

The carotene biosynthetic pathway thus requires three genes for the production of lycopene from phytoene, *PDS*, *ZDS* and *CRTISO*, in contrast to the single *crtI* gene in bacteria. The PDS and ZDS enzymes introduce a *cis*-isomerization of carbon–carbon double-bonds during each of the four dehydrogenation reactions (Bartley *et al*., 1999). Lesions in *crtISO* in dark-grown plants result in accumulation of *cis* isomers, predominantly tetra-*cis*-lycopene (Park *et al*., 2002). Recent biochemical evidence confirms that the biosynthetic pathway in plants proceeds via various *cis* intermediates, including 9,9′-di-*cis*-ζ-carotene and 7,9,9′-tri-*cis*-neurosporene (Fig. 2) (Isaacson *et al*., 2004; Breitenbach and Sandmann, 2005). Thus, all-*trans*-lycopene, the preferred substrate for the cyclases, is produced by the desaturases in concert with the carotenoid isomerase (Beyer *et al*., 1994; Schnurr *et al*., 1996; Park *et al*., 2002).

In the *Arabidopsis crtISO* mutant (*ccr2*), tetra-*cis*-lycopene is photoisomerised in the light, which raises questions about the necessity of CRTISO in plants and why there are three genes required for the synthesis of lycopene in plants but only one in bacteria. Answers may be found among the following observations. First, in chromoplasts, CRTISO activity is needed for all-*trans*-lycopene accumulation, regardless of the light regime, because the *tangerine* mutant accumulates tetra-*cis*-lycopene in the light (Isaacson *et al*., 2002). Carotenoids are deposited in a crystalline form in tomato chromoplasts and these may be more resistant to photoisomerisation. Second, herbicide treatments that force accumulation of lycopene in daffodil (*Narcissus pseudonarcissus*) increase *PSY* transcript abundance (Al-Babili *et al*., 1999). Third, although the biosynthetic pathway proceeds in chloroplasts, a delayed greening and substantial reduction in lutein occurs in mutants defective in CRTISO (Park *et al*., 2002). Fourth, expression of the bacterial *crtI* (phytoene desaturase) in tobacco plants, which bypasses the isomerization steps that occur during phytoene desaturation in plants, also resulted in a reduction in lutein levels (Misawa *et al*., 1994). The latter results suggest intermediate isomers may mediate transcript levels of other steps in the pathway. Also, CPTA treatment of daffodil flowers resulted in lycopene accumulation, a two-fold increase in carotenoid content, and an increase in *PSY*, *PDS* and *βLYC* transcript and protein abundance (Al-Babili *et al*., 1999). End-product regulation via an effector, possibly originating from a β-carotene derivative, was suggested as a possible mechanism. Another alternate regulatory molecule is lycopene. Thus, CRTISO is required for optimal carotenoid synthesis in etioplasts, chromoplasts and chloroplasts. The enzyme itself, or different lycopene isomers, may contribute also to regulation of the pathway.

It is important to note that control of pigment accumulation in chloroplasts is under very different pressures as compared to chromoplasts, where the

carotenoids are playing quite different roles. This is exemplified in tobacco, where over-expression of *CrtO*, a bacterial β-carotene ketolase gene under the control of a tomato *PDS* promoter, produced only trace amounts of astaxanthin accumulation in chloroplast-containing green tissue but a 170% increase in total carotenoids in the chromoplast-containing nectary tissue. The most predominant carotenoid in these lines was the algal/bacterial pigment astaxanthin (Mann *et al*., 2000).

### 4. Cyclases

After lycopene, the carotenoid biosynthetic pathway divides into two branches, distinguished by different cyclic end-groups, namely beta or epsilon. Two beta rings form the β,β branch (β-carotene and its derivatives) with one beta and one epsilon forming the β,ε branch (α-carotene and its derivatives).

Lycopene β-cyclase (βLCY) introduces a β-ionone ring to either end of all-*trans*-lycopene to produce β-carotene (Cunningham *et al*., 1993; Cunningham *et al*., 1994). Heterodimeric β-cyclases were observed in Gram positive bacteria (Krubasik and Sandmann, 2000), which fueled speculation that higher plant carotenoid enzymes, such as the cyclases, may act as part of a complex (Hirschberg, 2001; Cunningham, 2002). While there is a single *βLCY* gene in *Arabidopsis*, a second lycopene β-cyclase was identified in tomato, whose deduced amino acid sequence is 53% identical with the classical *βLCY*-encoded protein and is 86% identical to the enzyme capsanthin-capsorubin synthase from pepper (*Capsicum annum*). Expression of the alleles of this gene, *Beta* and *old-gold*, showed that developmentally regulated transcription was important in control of pigment accumulation (Ronen *et al*., 2000).

Both the β-cyclase and ε-cyclase enzymes are required to form α-carotene. The *lut2* mutation in the gene encoding the ε-cyclase (εLCY) resulted in the complete absence of α-carotene and its derivatives (Pogson *et al*., 1996; Pogson and Rissler, 2000). The *Arabidopsis* εLCY can only catalyze cyclization of one end-group, demonstrated by a mutation in the *βLCY* gene that accumulated monocyclic ε,ψ-carotene (B.J. Pogson and D. DellaPenna, unpublished results). Lettuce (*Lactuca sativa*) appears to be unique among higher plants in that its εLCY enzyme can catalyze formation of bicyclic ε,ε-carotene and its hydroxylated derivative lactucaxanthin (Phillip and Young, 1995; Cunningham and Gantt, 2001). A detailed study with recombinant cDNA demonstrated that a single amino acid was responsible for bicyclic ring formation. In lettuce, the *εLCY* H457L mutation generated monocyclic rather than bicyclic products, whereas in the *Arabidopsis εLCY*, the converse L448H mutation leads to bicyclic instead of monocyclic products (Cunningham and Gantt, 2001). Another interesting organism is the marine cyanobacterium *Prochlorococcus marinus* MED4, which contains a standard *βLCY* gene and an additional novel cyclase that is capable of forming both β- and ε-end-groups (Stickforth *et al*., 2003).

The semi-dominance of the *lut2* mutant in *Arabidopsis* suggests that εLCY is a rate-limiting step in lutein production (Pogson *et al*., 1996). Over-expression of *εLCY* and co-suppression lines resulted in lutein levels ranging from 10 to 180%, demonstrating that flux down the two branches can be controlled at the level of mRNA for the ε-cyclase (Pogson and Rissler, 2000). Conversely, over-expression of *βLCY* increased β-carotene and total carotenoid content (Rosati *et al*., 2000).

## C. Xanthophyll Synthesis

Xanthophylls are oxygenated derivatives of carotenes and play important roles in photoprotection and light-harvesting antennae formation (Niyogi, 1999).

### 1. Hydroxylases

Nearly all xanthophylls in higher plants have hydroxyl moieties on the 3-carbon in the β- or α-carotene rings to form zeaxanthin and lutein, respectively. There are two distinct hydroxylation reactions of the ε- and β-rings, confirmed by the identification of the ε-hydroxylase (εOH) locus, *lut1* (Pogson *et al*., 1996), and the β-hydroxylase (*βOH*) genes in higher plants (Milborrow *et al*., 1982; Z.R. Sun *et al*., 1996; Hirschberg, 2001; Tian and DellaPenna, 2001). While most enzymes in the *biosynthetic* pathway are each encoded by a single gene in *Arabidopsis*, multiple *hydroxylase* genes occur in *Arabidopsis* and tomato with distinct evolutionary backgrounds. The βOHs of plants share significant identity with those from various bacterial systems (Perry *et al*., 1986; Misawa *et al*., 1990; Misawa *et al*., 1995a; Misawa *et al*., 1995b). βOH enzymes are ferredoxin-dependent and contain an iron-coordinating histidine cluster that is required for activity (Bouvier *et al*., 1998). The authors proposed that the βOH enzymes break the C-H bond using iron-activated oxygen, which facilitates oxygen insertion (Bouvier *et al*., 1998). In contrast, the recently identified εOH is a plastid-targeted cytochrome P450-type monooxygenase, which reveals the εOH as a member

of a different gene family with a distinctly different enzymatic mechanism from the βOHs (Tian *et al*., 2004).

The *Arabidopsis βOHs* are expressed in all tissues, albeit at different levels (Tian and DellaPenna, 2001), whereas in tomato one is expressed in chloroplasts and the other in flowers (Hirschberg, 2001). Expression levels of *βOH* genes were modulated by different intensities of white light during tobacco de-etiolation (Woitsch and Römer, 2003) and were strongly induced by excess light in *Arabidopsis* leaves (Rossel *et al*., 2002). Involvement of a redox component in transcriptional regulation was also inferred, as *βOH* and zeaxanthin epoxidase transcript levels were similarly regulated and found to be sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) treatment (Woitsch and Römer, 2003).

Mutants that lacked activity of all three genes encoding major hydroxylases, the β-ring hydroxylases 1 and 2 and the ε-ring hydroxylase encoded by *lut1*, still maintained at least 50% of the wild-type hydroxylated β-rings despite the absence of both βOHs (Tian *et al*., 2003). The possibility of functional redundancy is consistent with results from βOH antisense and bacterial expression studies (Z.R. Sun *et al*., 1996). One possible explanation for this result is that the εOH enzyme has βOH activity, although this is unlikely because null *lut1* mutants do not alter βOH activity (Tian *et al*., 2004). A possible candidate for the residual βOH activity is CYP97A3, a putative cytochrome P450 with 50% identity to the εOH (Tian *et al*., 2003, 2004).

Two novel enzymes were recently identified in the astaxanthin-accumulating plant, *Adonis aestavalis*, based on their sequence similarity to βOH. Despite greater than 60% similarity to the catalytic portion of *Arabidopsis* βOH1, neither enzyme displayed 3-hydroxylase activity, preferring to desaturate the 3,4-position of the β-ring and hydroxylate the fourth carbon, resulting in the 4-keto-β-ring characteristic of astaxanthin and other ketocarotenoids (Cunningham and Gantt, 2005).

### 2. Zeaxanthin Epoxidase (ZE) and Violaxanthin De-Epoxidase (VDE)

An epoxide group is introduced into both rings of zeaxanthin by zeaxanthin epoxidase (ZE) to form violaxanthin. Under high light stress, the reverse reaction is rapidly undertaken by the violaxanthin de-epoxidase (VDE). While the number of identified species in which lutein 5,6-epoxide accumulates is increasing, the ε,β-cyclic branch typically terminates with lutein (Bungard *et al*., 1999; Matsubara *et al*., 2003). As discussed below, light plays a critical role in modulating the interconversion of zeaxanthin and violaxanthin. Under normal light conditions, when the incident light can be safely utilized for photosynthetic electron transport, zeaxanthin epoxidase (ZE) converts zeaxanthin to violaxanthin by introducing 5,6-epoxy groups to the 3-hydroxy-β-rings. Ferredoxin is required for this reaction (Bouvier *et al*., 1996). However, when incident light is in excess, VDE, whose active site is located in the lumen of thylakoid membranes, becomes activated by an acidification of the lumen, docks onto the thylakoid membrane in a process that requires monogalactosyl-diglyceride (Siefermann and Yamamoto, 1975), and de-epoxidases a substantial pool of violaxanthin to zeaxanthin (Pfundel and Bilger, 1994).

VDE and ZE were the first identified plant lipocalins, a class of β-barrel proteins that bind small hydrophobic molecules but are not usually catalytic (Bugos *et al*., 1998). VDE is active below pH 6.5 and requires ascorbate as a reductant (Hager, 1969; Hager and Holocher, 1994). A number of mutations in each enzyme have been identified in *Arabidopsis*, *Chlamydomonas*, pepper and tobacco (Koornneef *et al*., 1982; Niyogi *et al*., 1998).

### 3. Neoxanthin Synthase (NXS)

Conversion of violaxanthin to neoxanthin is performed by the enzyme neoxanthin synthase (NXS). Genes that encode enzymes with limited NXS activity were identified in tomato (Bouvier *et al*., 2000) and potato (Al-Babili *et al*., 2000). Both enzymes have high similarity to the second βLCY or CCS from tomato and capsicum, respectively. Whether this gene encodes the primary NXS *in vivo* is a matter of debate, especially considering that *Arabidopsis* lacks this gene but has NXS activity. Recently, mutants that lack neoxanthin were identified in both *Arabidopsis* (*Ataba4*) (A. Marion-Poll, Institut National de la Recherche Agronomique, Versailles, France, personal communication) and tomato (J. Hirschberg, The Hebrew University of Jerusalem, Jerusalem, Israel, personal communication). Molecular characterization of these putative NXS mutations should reveal the identity of the neoxanthin synthase enzyme.

## D. Cleavage Products

Characterization of the carotenoid-cleavage gene family has been rapid in recent years. The enzyme products

are varyingly referred to as carotenoid cleavage dioxygenases (CCD), related to carotenoid dioxygenase (RCD), or originally as 9-*cis*-epoxycarotenoid dioxygenases (NCED), reflecting the first characterized member of this gene family (Schwartz *et al*., 1997; Tan *et al*., 1997). There are nine members of the gene family in *Arabidopsis* (Tan *et al*., 2003). The CCD gene family is responsible for the formation of abscisic acid (ABA), vitamin A, volatiles used in the perfume industry such as β-ionone, colored food additives such as saffron and bixin, and potentially a novel class of plant hormones.

The expression and subcellular localization of five of the *Arabidopsis* CCDs were studied (Tan *et al*., 2003). All were targeted to the plastid, but their locations within the plastid differ. One, designated AtNCED5, bound the thylakoid exclusively. Others were both thylakoid-bound and free in the stroma (AtNCED2, 3 and 6), while AtNCED9 remained soluble in the stroma (Tan *et al*., 2003). All members of the CCD family tested to date act on carotenoids, but do show different substrate specificity (Schwartz *et al*., 2001, 2003) and differences in tissue distribution (Tan *et al*., 2003).

## 1. Abscisic Acid

The plant hormone abscisic acid (ABA) is involved primarily in plant stress responses, seed development and dormancy (Seo and Koshiba, 2002). ABA is a cleavage product of 9-*cis*-violaxanthin and/or 9′-*cis*-neoxanthin, an idea that was first proposed by Taylor and Smith (1967). Cleavage of 9′-*cis*-neoxanthin by NCED produces xanthoxin and was first identified in the maize *viviparous14* (*vp14*) mutant (Schwartz *et al*., 1997; Tan *et al*., 1997). Xanthoxin is followed in the pathway by a number of further modified products that are required to produce ABA (Seo and Koshiba, 2002).

## 2. Vitamin A

Vitamin A is a $C_{20}$ cleavage product of carotenoids, which, in addition to its retinoid derivatives, is essential for animal survival. Although cleavage of β-carotene has long been postulated as an important step in the formation on vitamin A, it was not until 2000 that a β-carotene 15,15′-dioxygenase was cloned from *Drosophila melanogaster* (von Lintig and Vogt, 2000) and chicken (Wyss *et al*., 2000). The deduced amino acid sequence showed homology to the plant carotenoid dioxygenase, VP14, involved in the synthesis of ABA. Any carotenoid containing an unmodified β-ionone ring has provitamin A activity, although β-carotene is one of the most active because a single β-carotene molecule is cleaved to form two retinal (vitamin A aldehyde) molecules. Retinal and its derivatives act as chromophores of the various visual pigments in animals (Wald, 1968). Vision aside, retinoic acid, the product of oxidation of vitamin A aldehyde to the carboxylic acid form, exerts most of the physiological effects of vitamin A (Dowling and Wald, 1960).

## 3. Novel Cleavage Products

The presence of other different carotenoid cleavage products (Milborrow *et al*., 1988) raises interesting questions about their function in plant development. Intriguing examples are the *Arabidopsis MAX4* and pea *RMS1* mutants (Sorefan *et al*., 2003). These mutants show increased branching and are disrupted in orthologous CCDs, which were shown to be inducible by the hormone auxin. This led the authors to postulate that MAX4 is involved in the synthesis of a novel carotenoid-derived hormone that acts downstream of auxin, inhibiting shoot branching (Sorefan *et al*., 2003). Indeed, recent results confirm that MAX3 (CCD7) (Booker *et al*., 2004) and MAX4 (CCD8) can sequentially cleave β-carotene to form the $C_{18}$ compound 13-apo-carotenone (Schwartz *et al*., 2004), although further modifications are presumably required to produce an active compound.

As demonstrated above, carotenoid cleavage metabolites are vital for plants and animals. They are also highly prized in the food and cosmetic industries. Bixin (annatto) is a red-colored, di-carboxylic monomethyl ester apocarotenoid, traditionally derived from the plant *Bixa orellana.* Bouvier and colleagues (Bouvier *et al*., 2003a) identified the lycopene cleavage dioxygenase, bixin aldehyde dehydrogenase, and norbixin carboxyl methyltransferase that are required to produce bixin from lycopene. Co-transforming the appropriate constructs into *E. coli*, engineered to produce lycopene, resulted in bixin production at a level of 5 mg/g dry weight (Bouvier *et al*., 2003a).

Saffron, another commercially important colored compound, can attribute the majority of its characteristic color, flavor and aroma to the accumulation of carotenoid derivatives. A crocus zeaxanthin 7,8(7′,8′)-cleavage dioxygenase (CsZCD) was cloned and found to be targeted to the chromoplast and initiated the production of the cleavage products. Another enzyme, 9,10(9′,10′)-cleavage dioxygenase was also cloned and

found to be a less specific cleavage enzyme (Bouvier *et al*., 2003b).

Carotenoid products are also important in the fragrance industry. Enzymatic and photo-oxidative derivatives of various carotenoids are commonly used. While other aroma constituents such as esters, terpenes, and pyrazines are usually also present, these $C_9$ to $C_{13}$ compounds often are essential to the odor profile (Wahlberg and Eklund, 1998).

## III. Regulation of Carotenoid Biosynthesis

As a consequence of the location of the enzymes in the plastid and the genes in the nucleus, carotenoid transcriptional regulation is responsive to environmental stimuli, oxidative stress, redox poise and metabolite feedback regulation. In general, increases in carotenoid accumulation, be it during fruit ripening, flower development or production of stress-induced carotenoids in algae, coincide with increased transcript abundance of some key (but not all) steps in the pathway (von Lintig *et al*., 1997; Cunningham and Gantt, 1998; Grunewald *et al*., 2000; Welsch *et al*., 2000; Hirschberg, 2001). Changes in transcript abundance are particularly evident during morphogenic changes from etioplast to chloroplast or chloroplast to chromoplast (von Lintig *et al*., 1997; Welsch *et al*., 2000; Bramley, 2002). For example, a 100-fold difference exists in carotenoid levels between flowers of marigold cultivars, which emerges during differentiation of the chromoplast. Differences in transcript levels of almost all carotenoid genes suggested that mRNA abundance was responsible for the dramatic differences in carotenoid accumulation between cultivars (Moehs *et al*., 2001).

There is a relative paucity of information about the regulation of carotenoid accumulation in photosynthetic tissues and germinating seedlings (Cunningham, 1998, 2002). In order to avoid extensive photo-oxidative damage, the synthesis of carotenoids and chlorophylls and their subsequent binding to pigment-binding proteins must be precisely balanced to meet the appropriate photosynthetic demands on a daily and seasonal basis (Herrin *et al*., 1992; Anderson *et al*., 1995). To this end, the proportion of different xanthophylls in a developing and mature chloroplast greatly affect plant viability and photoprotection (Pogson *et al*., 1998). Although the pathway is tightly regulated during development and in response to environmental stimuli (Young, 1993; Pogson *et al*., 1996; Welsch *et al*., 2000), not a single component of a signaling pathway has been identified. PSY and PDS are upregulated during photomorphogenesis via a phytochrome-mediated pathway from basal levels in etioplasts (Welsch *et al*., 2000). Furthermore, carotenoid biosynthetic genes are redox sensitive in the green alga *Haematococcus pluvialis* (Steinbrenner and Linden, 2003), which produces the high-value carotenoid astaxanthin. Transcript abundance of *βLCY*, *PSY*, *PDS* and *βOH* increased in response to increased light intensities, a trend that was eradicated by application of DCMU, which caused the plastoquinone pool to become more oxidized, and exacerbated by DBMIB treatment, which caused the plastoquinone pool to become reduced (Steinbrenner and Linden, 2003).

Evidence is also accumulating for metabolite feedback regulation as a mechanism to control carotenoid accumulation (Corona *et al*., 1996; Fraser *et al*., 2000; Welsch *et al*., 2003). Alternatives to metabolite feedback range from a signaling protein bound to specific carotenoids or their isomers to possible soluble or volatile carotenoid cleavage products that could participate in signaling. The accumulation of magnesium protoporphyrin IX, a chlorophyll precursor, is sufficient to regulate the expression of many photosynthetic nuclear genes in response to norflurazon-induced photooxidative stress (Larkin *et al*., 2003; Strand *et al*., 2003; see Chapter 9 and 15). There are compelling reasons why a chlorophyll precursor could control carotenoid gene transcription. Disrupting one pathway usually leads to a parallel reduction in the other pathway (Jilani *et al*., 1996) and damage from toxic chlorophylls may be limited by nascent carotenoids prior to and during photosystem assembly (Hoober and Eggink, 1999). However, the magnesium protoporphyrin IX signal is thought to communicate overall viability of the plastid and does not modulate phytochrome-mediated changes or the other environmental and developmental stimuli that regulate carotenoid gene expression (Cunningham, 2002). For now, the nature of the different forms of regulation remains the subject of current research.

## IV. Carotenoid Function

Carotenoids have a variety of crucial roles in all photosynthetic organisms. Carotenoids are involved in photosystem assembly and they contribute to light-harvesting by absorbing a broader range of wavelengths in the blue region of the visible spectrum than chlorophyll and then transfer the energy to chlorophyll. Finally, carotenoids provide protection from excess light

via energy dissipation and free radical detoxification and thus limit damage to membranes.

### A. Non-Green Plastids

Carotenoid accumulation relies on the presence of structures capable of storing and retaining carotenoids. Chromoplasts (see Chapter 21) accumulate carotenoids in lipoprotein structures (Bartley and Scolnik, 1995; Vishnevetsky *et al*., 1999) that are sequestered as crystals. For example, in a novel cauliflower mutant with orange curd, β-carotene accumulates in the plastids of the pith and curd as sheets, ribbons and crystals (Li *et al*., 2001). During the chloroplast to chromoplast transformation process, carotenoids become localised in plastoglobuli before incorporation into the chromoplast (Tevini and Steinmuller, 1985). Carotenoids within plastoglobuli exhibit much higher light stability than carotenoids within chloroplast membranes, indicating that pigments are better protected from light destruction in these structures (Merzlyak and Solovchenko, 2002). Pigment stability in fruits is important for protection of triacylglycerols, unsaturated lipids and phenol quinones from photooxidation as well as for protection of other light-sensitive constituents of plant tissues such as enzymes and membrane systems (Merzlyak and Solovchenko, 2002).

Most carotenoid studies concentrated on chloroplasts and chromoplasts, although there are many other plastid types (see Chapter 1), some of which are capable of storing carotenoids. Amyloplasts are "colorless" plastids that are specialized for storage of starch granules (Kirk and Tilney-Bassett, 1978). Lutein is the predominant carotenoid present in many seed amyloplasts, including maize (Janick-Buckner *et al*., 1999) and wheat (Hentschel *et al*., 2002). The antioxidant properties of carotenoids combat the process of seed ageing (Pinzino *et al*., 1999; Calucci *et al*., 2004). In wheat amyloplasts, the loss of lutein is subsequently accompanied by an increase in radical and other reactive oxygen species (Galleschi *et al*., 2002). Other plastid types include the pluripotent proplastid, which is found in undifferentiated meristem tissue. Proplastids are colorless but have the potential to form plastids capable of storing chlorophylls and carotenoids (Kirk and Tilney-Bassett, 1978). Leucoplasts are similar to proplastids, in the sense that they are colorless, but they are characteristic of mature root cells and do not develop further. Leucoplasts of the roots of *Arabidopsis*, pea and tobacco accumulate trace levels of neoxanthin and violaxanthin, which amounts to only 0.03 to 0.07% of the levels in light-grown leaves (Parry and Horgan, 1992).

Elaioplasts, which are specialized lipid-storing plastids, provide an ideal hydrophobic sink for accumulation of carotenoids. Perhaps the most dramatic enhancement of carotenoid accumulation has been achieved in the oil seeds of canola and *Arabidopsis*. Wild-type canola seeds contain predominantly lutein. Over-expression of PSY, in a seed-specific manner, produced transgenic plants with a 50-fold increase in total carotenoid content, in particular, α- and β-carotene (Shewmaker *et al*., 1999). A similar approach in *Arabidopsis* resulted in a 43-fold increase in β-carotene and concomitant increases in other carotenoids and chlorophyll (Lindgren *et al*., 2003).

Carotenoids are also important for photomorphogenesis. The dark-grown etioplast is distinguished by the prolamellar body, a uniformly curved lattice of tubular membranes, which contains several of the biochemical building blocks required for the chloroplast (Gunning and Jagoe, 1967) including the xanthophylls lutein and violaxanthin (Joyard *et al*., 1998). The *Arabidopsis crtISO* mutant accumulates tetra-*cis*-lycopene and lacks a prolamellar body. Thus, a mutation in carotenoid biosynthesis apparently disrupts membrane curvature and stabilization of the prolamellar body (Park *et al*., 2002). The absence of this structure in *crtISO* mutants suggests that different carotenoids either directly or indirectly impede formation of the membrane lattices, which results in a delay in plastid development and greening on exposure to light. These data demonstrate an essential role for carotenoids in plastid differentiation (Park *et al*., 2002).

In addition to their roles in photoprotection within photosystems (see below), xanthophylls have also been implicated in photoprotection outside of the photosynthetic apparatus, including protection against lipid peroxidation and maintenance of membrane fluidity and thermostability (Tardy and Havaux, 1997; Gruszecki *et al*., 1999). Xanthophylls are localized in membranes of the chloroplast envelope and in thylakoid membranes, potentially spanning the lipid bilayer (Havaux, 1998), and are bound to the photosystem apoproteins. In fact, certain xanthophylls were shown to increase membrane thermostability and reduce susceptibility to lipid peroxidation (Tardy and Havaux, 1997). Zeaxanthin-deficient mutants of *Arabidopsis*, *npq1*, exhibit enhanced lipid peroxidation and leaf necrosis upon exposure to light or chilling stress (Havaux and Niyogi, 1999). This effect can not be attributed to the lack of non-photochemical quenching (NPQ, see below), because the *npq4* mutant that exhibits a loss of NPQ, without a change in xanthophyll cycle pool size, is more tolerant to lipid peroxidation than *npq1*

(Havaux and Niyogi, 1999). Therefore, zeaxanthin appears to have a specific photo-protective role outside of the light-harvesting antennae.

### B. Photosystem Assembly

PSII consists of an antenna surrounding a core complex comprising D1, D2 and two inner antenna proteins, CP43 and CP47. Xanthophylls are bound to the antenna and are essential for both folding and stability of the antenna light-harvesting complex proteins (LHCPs) and thus their biosynthesis is tightly coordinated with LHCP synthesis (Plumley and Schmidt, 1987). The chloroplast envelope may be the initial site of LHC assembly (Plumley and Schmidt, 1987; Hoober and Eggink, 1999). Free carotenoids in the chloroplast envelope have been suggested to facilitate folding of LHC apoproteins by modifying properties of the lipid bilayer (Paulsen, 1999). Blocking carotenoid biosynthesis with the herbicide norflurazon reduced membrane LHCII complex accumulation and increased recovery of Lhcb in the vacuole of *Chlamydomonas* (Hoober and Eggink, 1999).

The light-harvesting antenna is composed of major and minor LHC apoproteins that are encoded by a large family of homologous genes (Bassi *et al*., 1993; Jansson, 1994; Green and Durnford, 1996; Jansson, 1999). The structure for LHCIIb, the major LHC of PSII, was initially resolved to 3.4 Å and found to contain 12 chlorophyll molecules, tentatively assigned as 7 chlorophyll *a* molecules, 5 chlorophyll *b* molecules and 2 xanthophylls embedded in three transmembrane spanning helices (Kuhlbrandt *et al*., 1994). The structure of the complex was recently resolved to 2.72 Å by X-ray crystallography, which revealed unambiguously 8 chlorophyll *a* molecules and 6 chlorophyll *b* molecules (Liu *et al*., 2004). The carotenoid binding sites have been elucidated by an elegant and thorough combination of structural studies, *in vitro* reconstitution of recombinant proteins, and analyses of pigment binding to native LHCs (Bassi and Caffarri, 2000). These studies identified up to four distinct carotenoid binding sites in LHCs (L1, L2, N1, V1), although only three of these binding sites are occupied in plants grown at moderate light intensities (Bassi and Caffarri, 2000). The L1 and L2 sites bind lutein, although either violaxanthin or zeaxanthin can be accommodated, whereas the N1 site is specific for neoxanthin. The V1 site is proposed to loosely bind violaxanthin under conditions of high-light stress, aiding in accessibility for de-epoxidation to zeaxanthin, which subsequently fills the V1 site (Verhoeven *et al*., 1999; Bassi and Caffarri, 2000). Furthermore, pigments bound to the V1 site do not contribute to light-harvesting and are presumably involved only in xanthophyll cycle activity, which is induced by high-light stress (Yamamoto, 1979; Caffarri *et al*., 2001). Finally, carotenoids are required for correct assembly of the LHC holocomplex, because the absence of lutein results in a loss of LHC trimers (Rissler and Pogson, 2001; Lokstein *et al*., 2002).

The minor LHC antennae proteins, Lhcb 4, 5 and 6 (also known as CP29, CP26 and CP24, respectively), typically bind 8 chlorophyll *a* molecules, 2 chlorophyll *b* molecules, and 2 xanthophyll molecules (Bassi and Caffarri, 2000). In Lhcb 4, the L1 site is occupied by lutein and L2 by violaxanthin or neoxanthin (Bassi *et al*., 1999; Ruban *et al*., 1999). The core complex of PSII binds β-carotene and chlorophyll *a*, and possibly small amounts of lutein (Bassi *et al*., 1993; Satoh, 1993; Seibert, 1993; Alfonso *et al*., 1994). β-Carotene is typically excluded from LHCs, although β-carotene is found associated with Lhca1, the major protein in LHCI of PSI (Schmid *et al*., 1997).

### C. Photoprotection and Plant Fitness

Plants must maintain a balance between absorbing sufficient light for photosynthesis while avoiding oxidative damage caused by too much light. In the absence of cyclic carotenoids, plastids photo-bleach as the result of generation of reactive oxygen species that attack lipids and proteins (Knox and Dodge, 1985). Preventative and acclimatory measures taken by plants include changing orientation of leaves, the orientation of chloroplasts within the leaves, or reducing light-harvesting antennae size. Mechanisms to minimize damage include complementary photoprotective mechanisms such as (1) the harmless dissipation of excess energy via NPQ that is mediated by certain xanthophylls, (2) quenching of triplet chlorophylls by carotenoids, (3) accumulation of antioxidants (ascorbate, tocopherols and carotenoids) and antioxidant enzymes such as ascorbate peroxidase that de-toxify free radicals, and (4) repair of damaged proteins (Anderson *et al*., 1995; Niyogi, 1999).

NPQ-mediated dissipation of excess absorbed energy functions by non-radiative quenching of singlet chlorophyll and requires a functional xanthophyll cycle. This process may limit the formation of chlorophyll triplets and prevent reactive oxygen species that are generated by transfer of excitation energy from chlorophyll to molecular oxygen (Demmig-Adams and Adams, 1992). The xanthophyll cycle, identified by Harry Yamamoto in 1962, is a key component

of the plant's high-light defense strategy (Yamamoto *et al*., 1962). Under excess light, violaxanthin is de-epoxidized (removal of the two oxygen functions, or epoxy groups) to zeaxanthin via antheraxanthin. In the reverse reaction, under low or no light, zeaxanthin is epoxidized to form violaxanthin. The process is pH dependent; reduced lumenal pH activates the de-epoxidase enzyme and facilitates energy dissipation within the antennae via protonation-induced conformational changes. Another essential component of the energy dissipation mechanism is the PsbS protein of PSII (Li *et al*., 2000).

Mutants with altered carotenoid profiles are often affected in terms of NPQ (Niyogi *et al*., 1998; Pogson *et al*., 1998; Pogson and Rissler, 2000; Lokstein *et al*., 2002). The *npq1* mutations of *Arabidopsis* and *Chlamydomonas*, which have markedly reduced levels of NPQ, have lesions in the violaxanthin de-epoxidase (VDE), which prevent the high-light-induced accumulation of zeaxanthin in these and antisense VDE lines (Niyogi *et al*., 1997a, 1998; W.H. Sun *et al*., 2001; Verhoeven *et al*., 2001). The residual NPQ in zeaxanthin-deficient lines can be essentially eradicated by eliminating lutein (Niyogi *et al*., 1997a, 2001). Furthermore, an increase in lutein resulted in faster induction of NPQ (Pogson and Rissler, 2000). Regardless of the genetic background, altered lutein levels invariably affect NPQ (Pogson *et al*., 1998), either directly or indirectly via changes to the antenna conformation.

Alterations in the xanthophyll cycle pool size also affect NPQ. Inhibition of the β-carotene hydroxylase (βOH) resulted in reduced amounts of xanthophylls. No obvious alteration to antennae composition and only a 16% decline in NPQ occurred as a result of the inhibition. A stable pool of violaxanthin remained unconverted, even under high-light stress, suggesting a portion of violaxanthin is sequestered to fill structural sites and is therefore unavailable for xanthophyll cycle conversions (Pogson and Rissler, 2000). Increasing the xanthophyll cycle pool (without the usual compensatory reduction in lutein) by over-expressing the bacterial βOH gene (*chyB*) enhanced stress tolerance in *Arabidopsis*. Transformed plants could withstand extreme light and heat, and displayed less leaf necrosis, anthocyanin accumulation and lipid peroxidation (Davison *et al*., 2002). Zeaxanthin was therefore suggested to prevent oxidative damage of membranes. This hypothesis is corroborated by the converse situation, where reduced zeaxanthin increased sensitivity to light stress (Havaux and Niyogi, 1999; Verhoeven *et al*., 2001).

The physiological relevance of xanthophylls is exemplified by the bleaching, delayed greening and semi-lethal phenotypes observed in several carotenoid and NPQ-deficient mutants (Niyogi *et al*., 1997a; Pogson *et al*., 1998). In the absence of zeaxanthin and lutein, *Chlamydomonas* cultures photobleach, and mature *Arabidopsis* leaves senesce and photobleach in high-intensity light (Niyogi *et al*., 1997b, 2001). This effect can not be attributed to the lack of NPQ, since the *npq4* mutant, which exhibits a loss of NPQ without a change in xanthophyll cycle pool size, is more tolerant to lipid peroxidation than *npq1* (Havaux and Niyogi, 1999). Therefore, zeaxanthin appears to have a specific photo-protective role outside of the light-harvesting antennae (as discussed above). Several recent manuscripts demonstrate that NPQ does indeed enhance plant fitness. The first is both elegant and simple in that the authors measured an indicator of plant fitness in *Arabidopsis*, the amount of seed formed under field conditions and in growth chambers with similar fluctuating light regimes (Kulheim *et al*., 2002). Seed set was reduced by 30 to 50 % in the field experiments, because of both a reduction in the number of fruit (siliques) and the number of seed per fruit. This reduction may reflect increased photoinhibition in *npq4* plants (Li *et al*., 2002) and demonstrates the need to undertake appropriate and relevant physiological experiments. In the converse experiment, Horton and colleagues increased the xanthophyll cycle pool, which resulted in increased tolerance to abiotic stress induced by high light and increased temperature (Davison *et al*., 2002).

Carotenes are essential for quenching chlorophyll triplet states in purple bacterial reactions centers, and their loss results in cell death (Griffiths *et al*., 1955; Frank and Cogdell, 1993). In plants, these fundamental roles must be undertaken by the xanthophylls bound to CP43 and CP47 or the antenna, because the electron-exchange mechanism of triplet quenching requires the carotenoid to be in van der Waals contact (approximately 4 Å distant) with the chlorophyll triplets (Krinsky, 1971). Any β-carotene molecule in close proximity to the chlorophyll special pair, P680, would be oxidized due to P680's oxidative potential (Telfer, 2002). Instead, β-carotene may afford photoprotection by other mechanisms. A β-carotene may donate an electron to the oxidized special pair, $P680^+$, generating a carotenoid cationic radical (Hanley *et al*., 1999; Vrettos *et al*., 1999), or more likely, β-carotene could quench singlet oxygen and contribute to the signaling of D1 degradation, which triggers PSII repair (Anderson and Chow, 2002; Telfer, 2002).

## V. Conclusions and Future Directions

The role of carotenoids in human health and plant photoprotection makes them obvious candidates for enhancement and manipulation (Sandmann, 2001). To this end, molecular genetics, in concert with classical biochemistry, has facilitated an advanced understanding of the biosynthetic pathway. We are learning to create new bioactive compounds by novel mechanisms and produce familiar carotenoids in crops that would otherwise be of low nutritional value. However, there are many aspects of carotenoid metabolism that remain to be elucidated. There is limited structural information for any of the carotenoid enzymes, with the possible exception of VDE and ZE models (Bugos *et al*., 1998). Despite numerous transcriptional studies, no regulatory elements have been identified and the hypothesis that carotenoid enzymes are active as multienzyme complexes is yet to be conclusively proven. Finally, we are only beginning to realize the role that carotenoid cleavage products play in plant development.

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# Chapter 17

# Lipid Synthesis, Metabolism and Transport

Peter Dörmann*
*Department of Molecular Physiology, Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany*

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## Summary

Chloroplasts harbor the enzymes of fatty acid de novo synthesis, and contain a unique set of glycerolipids which is different from yeast, animals and from most bacteria. Fatty acid synthesis in plastids depends on acyl carrier protein, and the acyl chains are directly incorporated into plastid lipids via the prokaryotic pathway or, after hydrolysis and export from the plastid, employed to synthesize eukaryotic lipids at the endoplasmic reticulum. In addition to phospholipids (phosphatidylcholine, PC; phosphatidylglycerol, PG) and sulfolipid (sulfoquinovosyldiacylglycerol, SQDG), high amounts of galactolipids (monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG) are present in plastids. Many of the genes involved in chloroplast lipid synthesis have recently been isolated. The availability of *Arabidopsis* mutants affected in chloroplast lipid synthesis has greatly advanced our understanding of the function of lipids in different physiological processes. MGDG, DGDG and PG, were shown to be essential for photosynthesis. Two of the glycolipids, DGDG and SQDG, strongly increase during growth under phosphate

*Author for correspondence, email: Doermann@mpimp-golm.mpg.de

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limiting conditions, thus replacing phospholipids in the membranes. Analysis of transgenic plants and mutants affected in lipid desaturation revealed that the degree of fatty acid unsaturation is critical for growth at low and high temperatures. Acyl groups in plastids also serve as precursors for the synthesis of biologically active compounds. Jasmonic acid, a phytohormone, is derived from α-linolenic acid by a lipoxygenase reaction. Synthesis and accumulation of lipids and lipid precursors is distributed to different intracellular membranes. However, transport of lipid moieties between different organelles is only poorly understood and will be the focus of future research.

## I. Introduction

Chloroplasts of higher plants contain an intricate membrane system, the thylakoids, which harbor the complexes of the photosynthetic light reactions, including photosystem I, photosystem II, light harvesting complex II, the cytochrome $b_6f$ complex and ATP synthase. The proteins of the photosynthetic complexes are embedded into a matrix of polar lipids, and for a long time, it has been assumed that these lipids play an essential role in photosynthesis. The chloroplast membranes of higher plants are rich in glycolipids, i.e., galactolipids (monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG) and sulfolipid (sulfoquinovosyldiacylglycerol, SQDG). Phosphatidylcholine (PC) and phosphatidylglycerol (PG) are the two most abundant phospholipids in chloroplasts. Furthermore, chloroplasts are the major site of fatty acid de novo synthesis in the plant cell and thus provide acyl groups for the assembly of plastidial and extraplastidial membranes.

In addition to providing biosynthetic capacity for fatty acid and glycerolipid synthesis, chloroplasts harbor the 1-deoxy-xylulose-5-phospahte pathway of isoprenoid lipid synthesis, which is different from the mevalonate pathway localized to the cytosol. The plastid pathway of isoprenoid synthesis is essential for the production of carotenoids and other prenyl lipids such as chlorophyll, tocopherol/tocotrienol (vitamin E), plastoquinone and phylloquinone (vitamin K1). These isoprenoid lipids play critical roles in photosynthesis and in oxidative stress and are the focus of Chapter 16 of this volume.

The lipid composition of thylakoid membranes is conserved throughout all green photosynthetic organisms, including higher plants and cyanobacteria, suggesting that this specific set of lipids originated from the cyanobacterial progenitor of chloroplasts. This evolutionary aspect implies that thylakoid lipids are important for photosynthesis and that cyanobacteria and plants might employ common pathways for their synthesis. As a result of the integration of the prokaryotic lipid synthesis pathways into the metabolism of the host plant cell, different routs of lipid transport were established between the chloroplast and extraplastidial membranes. For this reason, lipid trafficking within the plant cell represents an important process in the overall regulation of membrane lipid redistribution to the different organelles.

Chloroplast lipid biosynthesis and function were the focus of excellent reviews (e.g. Ohlrogge *et al.*, 1993; Joyard *et al.*, 1998; Vijayan *et al.*, 1998), and recently it was also the subject of a book chapter in this series (Block *et al.*, 2001). Research in the area of plant lipid biosynthesis has greatly been advanced by the availability of the full genome sequence of the model plant *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000). Sequence data were used to predict numerous yet unidentified genes of plant lipid synthesis, and a complete set of gene entries of enzymes involved in lipid synthesis was established (Beisson *et al.*, 2003; www.plantbiology.msu.edu/lipids/genesurvey/index.htm). The availability of T-DNA insertional mutants and of chemically induced mutants in combination with tools for gene mapping and plant transformation have rendered *Arabidopsis* the model plant of choice for many aspects of plant research, including

*Abbreviations:* Fatty acids are abbreviated as X:Y, with X indicating the number of carbon atoms and Y the number of double bonds; AAPT – aminoalcoholphosphotransferase; ACP – acyl carrier protein; CoA – coenzyme A; CDS – cytidindiphospho-diacylglycerol synthase; DHAP – dihydroxyacetonephosphate; DGD1, DGD2 – digalactosyldiacylglycerol synthase 1 or 2; DGDG – digalactosyldiacylglycerol; FAT – fatty acyl-ACP thioesterase; GGGT – galactolipid:galactolipid galactosyltransferase; GPAT – glycerol-3-phosphate acyltransferase; GPDH – glycerol-3-phosphate dehydrogenase; Gro-3P – glycerol-3-phosphate; LACS – long-chain acyl-CoA synthetase; LPA – lysophosphatidic acid; LPAAT – lysophosphatidic acid acyltransferase; lyso-PC – 1-lysophosphatidylcholine; MGD1, MGD2, MGD3 – monogalactosyldiacylglycerol synthase 1, 2 or 3; MGDG – monogalactosyldiacylglycerol; PA – phosphatidic acid; PAP – phosphatidic acid phosphatase; PC – phosphatidylcholine; PE –phosphatidylethanolamine; PG – phosphatidylglycerol; PGP – phosphatidylglycerol-phosphate; PGP1 – phosphatidylglycerol-phosphate synthase 1; PGPP – phosphatidylglycerol-phosphate phosphatase; SQD1 – UDP-sulfoquinovose synthase; SQD2 – sulfoquinovosyldiacylglycerol synthase; SQDG – sulfoquinovosyldiacylglycerol; TriGDG – trigalactosyldiacylglycerol; UGE – UDP-glucose 4-epimerase.

lipid metabolism. The present chapter focuses on the recent advances in the understanding of the biosynthesis and function of glycerolipids in plants, with an emphasis on the characterization of genes and mutants using *Arabidopsis* as the model organism.

## II. Structure and Distribution of Lipids in Chloroplasts

### A. Glycolipids

The two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) make up about 50 and 25 mol% of thylakoid glycerolipids, respectively (Block *et al.*, 1983). Because thylakoids of chloroplasts represent the most abundant membrane system in leaves, galactolipids constitute more than 60% of total leaf lipids. The membrane lipid compositions in thylakoids and in the inner envelope membrane (49 and 30 mol% of MGDG and DGDG, respectively; Block *et al.*, 1983) are very similar. In the outer envelope, however, DGDG accounts for 29 mol%, and thus is more abundant than MGDG (17 mol%). Galactolipids were first isolated and structurally characterized by Carter *et al.* (1956). The structures of MGDG and DGDG were determined as 1,2-diacyl-3-O-(β-D-galactopyranosyl)-*sn*-glycerol, and 1,2-diacyl-3-O-(β-D-galactopyranosyl-1→6-α-D-galactopyranosyl)-*sn*-glycerol. Therefore, the galactose residues of MGDG and DGDG are linked in β-glycosidic linkage to diacylglycerol, and the second galactose of DGDG is bound via an α-glycosidic linkage (Fig. 1a). Galactolipids are phosphorus-free and do not carry a positive or negative charge.

The sulfolipid sulfoquinovosyldiacylglycerol (SQDG) contains a sulfonic acid derivative of glucose (6-deoxy-6-sulfono-glucose or sulfoquinovose) bound in α-glycosidic linkage to diacylglycerol. The structure of SQDG was first described as 1,2-diacyl-3-O-(6-sulfo α-D-quinovosyl)-*sn*-glycerol by Benson *et al.* (Benson *et al.*, 1959; Benson, 1963; Fig. 1a). SQDG makes up about 5 mol% of glycerolipids in thylakoids and in the envelope membranes (Block *et al.*, 1983). As for the galactolipids, SQDG contains no phosphate, but it carries one negative charge and is believed to serve as a surrogate lipid for the anionic phospholipid phosphatidylglycerol (PG).

Phosphatidylglycerol (PG) carries a phosphoglycerol moiety in its head group, and is the most abundant phospholipid in thylakoid membranes (Fig. 1a). The PG content in thylakoids and in the envelopes is very similar (about 10 mol%; Block *et al.*, 1983), and PG is also present in extraplastidial membranes (mitochondria, ER, tonoplast, plasma membrane). The structure of PG from *Scenedesmus* was identified as 1,2-diacyl-*sn*-glycerol-3-(phospho-1'-*sn*-glycerol) by Benson and Maruo (1958). PG contains one negative charge in its head group.

Another phospholipid, phosphatidylcholine (PC, also named lecithin), is present in the outer envelope membrane where it accounts for about 32 mol% (Block *et al.*, 1983). PC concentration is very low in thylakoids and in the inner envelope. Plastidial PC is believed to originate from the ER, and PC or a PC-derived lipid supposedly serves as a precursor for eukaryotic lipid synthesis in the chloroplast. PC is not restricted to chloroplasts and is the most abundant phospholipid in extraplastidial membranes. The structure of PC was first determined as 1,2-diacyl-*sn*-glycerol-3-phosphocholine by Folch (1942). Because phosphocholine, the head group of PC, contains one negative and one positive charge, this lipid does not carry any net charge, but is zwitterionic (Fig. 1a).

### B. Fatty Acids

The majority of fatty acids in membranes of higher plants contain 16 or 18 carbon atoms (Fig. 1b). Palmitic acid (16:0) is the most abundant saturated fatty acid, while stearic acid (18:0) is only a minor component of glycerolipids. Chloroplast membrane lipids are characterized by a high content of unsaturated fatty acyl groups, such as 16:1Δ3*trans*, 16:3Δ7,10,13*cis*, 18:1Δ9*cis*, 18:2 Δ9,12*cis* and 18:3Δ9,12,15*cis*. Two groups of plants can be distinguished based on the presence of the triunsaturated fatty acid 16:3Δ7,10,13*cis*, i.e., "16:3" plants (containing 16:3 fatty acid) and "18:3" plants (without 16:3, where 18:3 is the most abundant fatty acid) (Roughan and Slack, 1982; Heinz and Roughan, 1983). Some fatty acids are found in specific membrane lipids. For example, 16:1Δ3*trans* is restricted to the *sn*-2 position of PG, and 16:3Δ7,10,13*cis* is abundant at the *sn*-2 position of MGDG.

## III. Biosynthesis of Fatty Acids in Plastids

### A. Origin of Precursors for Fatty Acid Synthesis

Fatty acid de novo synthesis in plants is localized to plastids, with only a small amount of fatty acids synthesized in mitochondria (Ohlrogge *et al.*, 1979). In contrast to animals and yeast, the cytosol of plant cells

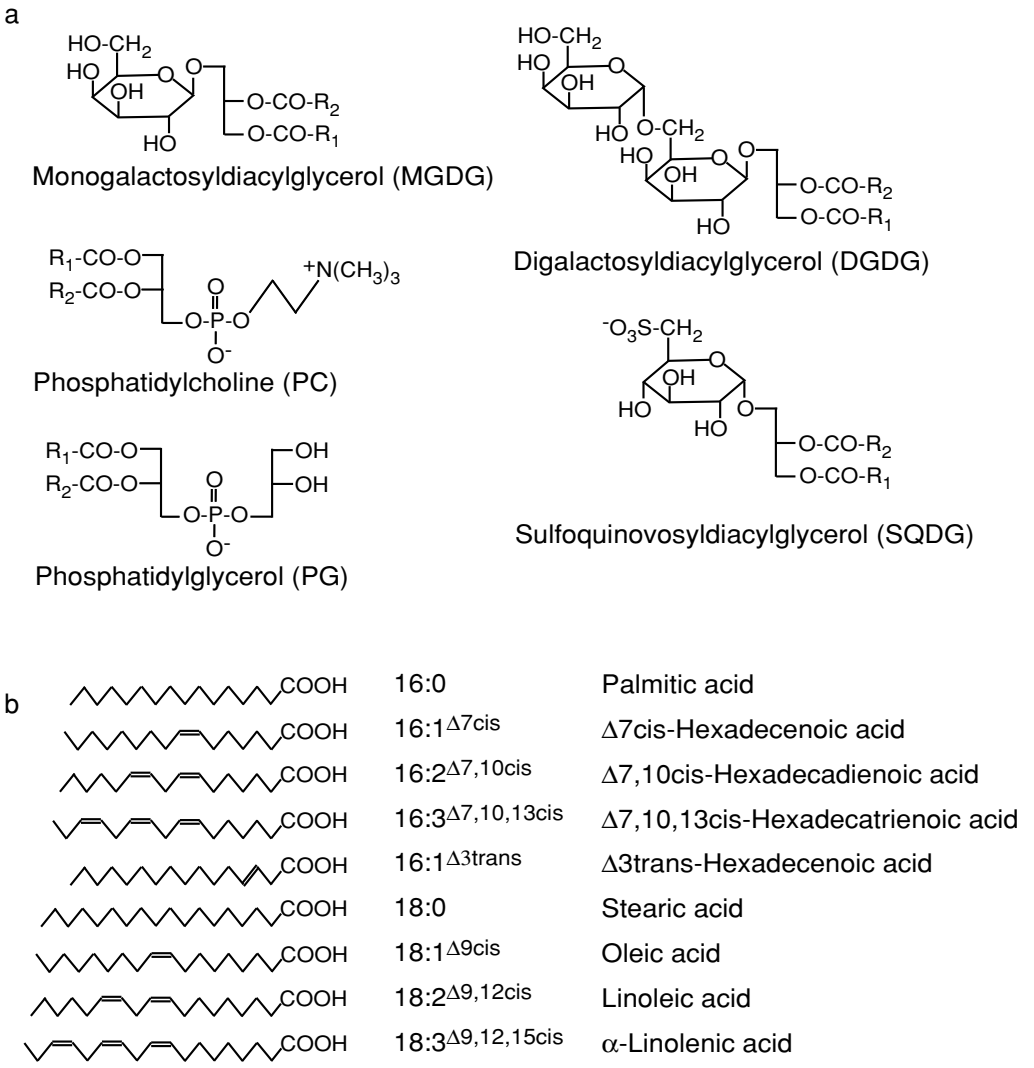


*Fig. 1.* Structures of glycerolipids and fatty acids in chloroplasts. (a) Galactolipids (MGDG, DGDG) are the predominant lipid classes of chloroplasts, whereas sulfolipid (SQDG) and phosphatidylglycerol (PG) are of less abundance. Phosphatidylcholine (PC) is enriched in the outer membrane of the envelope of chloroplasts. (b) Palmitic acid, oleic acid, linoleic acid and α-linolenic acid are abundant constituents of chloroplast glycerolipids. The occurrence of Δ3*trans*-hexadecenoic acid is restricted to PG, and Δ7,10,13all-*cis*-hexadecatrienoic acid is abundant in MGDG of some plant species ("16:3" plants).

is not involved in de novo synthesis of fatty acids, but it harbors an acyl chain elongation system that is capable of adding C2 units onto C16 and C18 acyl chains, thereby producing very long-chain fatty acids for wax and storage lipid synthesis. The enzymes of fatty acid synthesis in the stroma of plastids are encoded by single genes and are of prokaryotic origin, thus resembling the enzymes of bacteria. The bacterial/plant type fatty acid synthesis (FAS II type) is distinct from yeast and animals, where the activities of fatty acid de novo synthesis reside on large multifunctional polypeptides, and where the acyl chain is bound to a central carrier domain on the polypeptide (FAS I type). In FASII fatty acid synthase, a small protein (acyl carrier protein, ACP) serves as carrier for the growing acyl chain. *Arabidopsis* contains several genes encoding ACP, five isoforms predicted to be localized to the chloroplast and one to the mitochondrion (Shintani and Ohlrogge, 1994; Beisson *et al.*, 2003). The importance of ACP during fatty acid synthesis was demonstrated by antisense inhibition of the major leaf isoform ACP4; reduction in ACP4 expression strongly affected synthesis of fatty acids and of plastidial membrane lipids (Branen *et al.*, 2003).

Acetyl-CoA is the precursor for chain elongation during fatty acid de novo synthesis in all organisms.

Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, and the malonyl moiety is employed for the stepwise elongation of the acyl chain (Fig. 2). The origin of acetyl-CoA for fatty acid synthesis in plastids has been the matter of intense studies (Ohlrogge *et al.*, 1993; Bao *et al.*, 2000). Two compounds, plastidial pyruvate derived from glycolysis or free acetate, possibly originating from the mitochondria, were suggested to serve as precursors for acetyl-CoA synthesis. Analysis of spatial and temporal

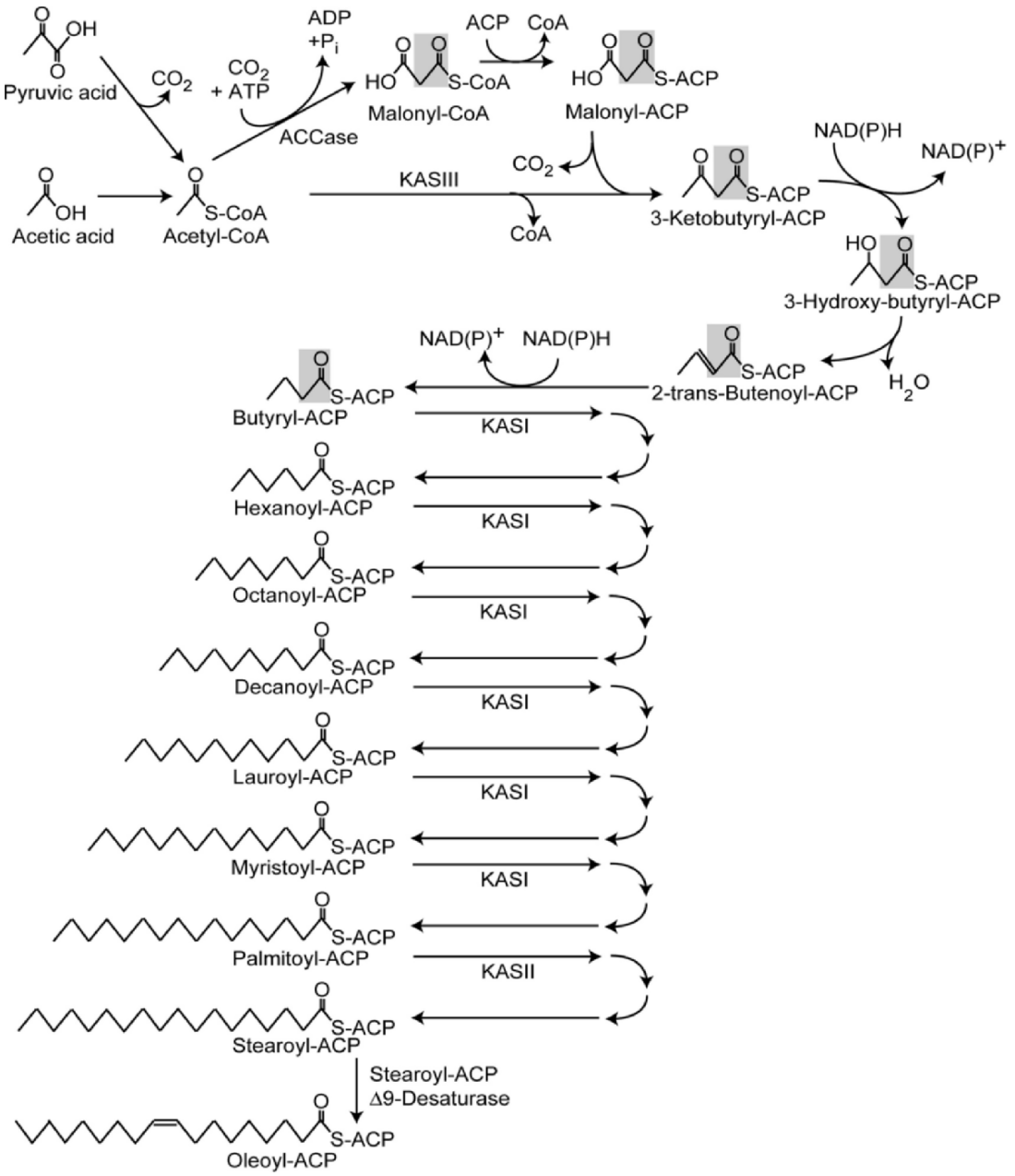


*Fig. 2.* Fatty acid de novo synthesis in plastids. Pyruvate is believed to be the predominant precursor for acetyl-CoA synthesis in plastids. Acetyl-CoA is converted to malonyl-CoA and subsequently to malonyl-ACP. Malonyl-ACP represents the elongation factor for the acyl-chain, which is always bound to acyl carrier protein (ACP). The two carbon atoms derived from malonyl-ACP that give rise to chain elongation are shaded in grey. The initial condensation step between acetyl-CoA and malonyl-ACP is catalyzed by KASIII, whereas all further condensation reactions are acyl-ACP dependent and are catalyzed by KASI (C4 to C16) and KASII (C16). Desaturation of stearoyl-ACP by the stroma localized stearoyl-ACP Δ9-desaturase yields oleoyl-ACP.

gene expression patterns suggested that the plastidial pyruvate dehydrogenase complex, rather than acetyl-CoA synthetase represents the most likely pathway for acetyl-CoA production in plastids (Ke *et al.*, 2000b).

Two structural types of ACCase exist in higher plants, a heteromeric form composed of different subunits found in plastids of all dicotyledons and some monocotyledons (excluding *Gramineae*), and a homomeric form composed of a single polypeptide that is found in the cytosol and in plastids (Sasaki *et al.*, 1995; Konishi *et al.*, 1996). Acetyl-CoA carboxylase in plastids of dicotyledonous plants, e.g., *Arabidopsis*, is composed of four subunits encoded by different genes, i.e., biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyltransferase (two subunits). Interestingly, the α subunit of carboxyltransferase is encoded in the plastid genome (*accD*; Ke *et al.*, 2000a), whereas the β subunit (CAC3) as well as the biotin carboxylase gene and the two BCCP genes are nuclear encoded (Thelen *et al.*, 2001). Two genes encoding the homomeric ACCase are present in the *Arabidopsis* genome, *ACC1* and *ACC2*. The *ACC1* gene encodes a cytosolic form of ACCase (Roesler *et al.*, 1997). The second gene, *ACC2*, is similar to an ACCase isoform from rapeseed, which was shown to be plastid localized (Schulte *et al.*, 1997).

In plastids, the malonyl moiety from malonyl-CoA is transferred from the thiol group of CoA onto a thiol group on ACP by malonyl-CoA:ACP malonyltransferase. One gene with sequence similarity to bacterial malonyl-CoA:ACP malonyltransferases is present in *Arabidopsis*, but there is no report on the characterization of this enzyme on a molecular level (Beisson *et al.*, 2003).

Fatty acid synthesis requires high amounts of ATP and reducing equivalents (NADH, NADPH). In photosynthetically active tissues, such as the mesophyll cells of leaves that are exposed to the light, ATP and NAD(P)H are supposedly derived from the electron transport chain of photosynthesis. In the dark, as well as in non-photosynthetic tissues, NAD(P)H is thought to be produced by the reactions of the oxidative pentose phosphate cycle (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase). However, import of reducing equivalents from the cytosol into the plastid might contribute to accumulation of NAD(P)H in plastids. An alternative source of ATP for plastid fatty acid synthesis is the import from the cytosol via the envelope-localized ADP/ATP transporter (Möhlmann *et al.*, 1998). Overexpression of the ADP/ATP transporter in potato tubers resulted in an increase in starch, but had no effect on lipid accumulation (Tjaden *et al.*, 1998; Klaus *et al.*, 2004). Thus, the relative contribution of various pathways to the plastidial ATP and NAD(P)H synthesis in different plant tissues is not clear.

### *B. Condensation of the Acyl Chain With Malonyl-ACP*

The chain elongation of fatty acid synthesis in plastids is catalyzed by successive condensation reactions of an acyl-group with malonyl-ACP, resulting in the attachment of two carbon atoms in each elongation cycle. One molecule of carbon dioxide is released during the condensation reaction with malonyl-ACP. The initial condensation reaction is catalyzed by β-ketoacyl-ACP synthase III (KASIII) which uses acetyl-CoA as primer. In contrast to the KASIII reaction, all subsequent condensation reactions are specific for acyl-ACPs (Fig. 2; Tai and Jaworski, 1993). A different β-ketoacyl-ACP synthase, KASI, is specific for condensations with acyl-ACP esters ranging in size from C4 to C16. The final elongation in plastids is catalyzed by KASII which converts C16-ACP to C18-ACP. A mutant in the *KASII* gene of *Arabidopsis* (*fatty acid biosynthesis 1* or *fab1* mutant) contains increased amounts of 16:0 in all membrane lipids, particularly in PG (Wu *et al.*, 1994). This change in fatty acid composition was complemented by transformation with a *Brassica kas2* cDNA (Carlsson *et al.*, 2002).

During each round of fatty acid synthesis, the 3-ketoacyl-ACP ester produced by condensation of an acyl-primer with malonyl-ACP is reduced to the corresponding acyl-ACP thioester, which serves as substrate for the next round of elongation (Fig. 2). The first reaction is the reduction to 3-hydroxy-acyl-ACP by 3-ketoacyl-ACP reductase using NAD(P)H as electron donor. *Arabidopsis* contains five genes encoding 3-keotacyl-ACP reductase (Beisson *et al.*, 2003). Subsequently, dehydration by 3-hydroxyacyl-ACP dehydratase yields 2-*trans*-enoyl-ACP. Finally, 2-*trans*-enoyl-ACP is reduced by enoyl-ACP reductase. A mutation in the *Arabidopsis* enoyl-ACP reductase gene (*mosaic cell death 1* mutant or *mod1*) results in strong inhibition of plastidial fatty acid synthesis, and as a consequence, *mod1* plants show premature cell death (Mou *et al.*, 2000).

### *C. Desaturation of Acyl Groups*

The stroma of plastids contains a soluble, ACP-dependent desaturase, which introduces a *cis* double bond at the C-9 position of stearoyl-ACP. Thus,

stearoyl-ACP Δ9-desaturase produces oleoyl-ACP, the major C18 fatty acid derived from plastidial fatty acid de novo synthesis (Fig. 2 and 3). The two electrons released by the desaturation reaction are transferred onto molecular oxygen, which is thereby reduced to water. Because reduction of oxygen requires four electrons, two additional electrons are derived from reduced ferredoxin in the plastids (Fig. 3). *Arabidopsis* contains seven genes encoding plastidial stearoyl-ACP Δ9-desaturase (Beisson *et al.*, 2003). A mutant in one of these genes, *fab2* (*fatty acid biosynthesis 2*) contains reduced amounts of 18:1 and increased amounts of 18:0 in the leaves (Lightner *et al.*, 1994a, b). Interestingly, this mutant is allelic to *ssi2* which was identified during a screening program for *suppressor of salicylic acid insensitivity* mutants (Kachroo *et al.*, 2001; Shah *et al.*, 2001; see Section V.D).

Plant membranes, particularly thylakoids, are rich in polyunsaturated fatty acids. With the exception of the soluble stearoyl-ACP Δ9-desaturases, the other desaturases are membrane bound and act on glycerolipid substrates rather than acyl-ACPs (for a recent review see Wallis and Browse, 2002). Two enzymes, FAD2 and FAD3, are localized to the ER and introduce a second and third double bond into oleoyl moieties attached to phospholipids, thus producing linoleic acid (18:2) and α-linolenic aicd (18:3), respectively. In contrast to plastidial desaturases, FAD2 and FAD3 employ cytochrome $b_5$ rather than ferredoxin as electron donor for oxygen reduction (Fig. 3). *Arabidopsis* contains five additional plastid-localized, membrane bound desaturases. FAD4 specifically introduces a *trans* double bond into the C3 position of palmitic acid in PG, thereby producing 16:1Δ3*trans*. FAD5 converts 16:0 at the *sn*-2 position of MGDG to 16:1Δ7*cis*, the precursor for 16:3 synthesis. Further desaturation of monounsaturated fatty acids (16:1Δ7*cis*, 18:1Δ9*cis*) in chloroplasts by FAD6, FAD7 and FAD8 results in the production of triunsaturated fatty acids (16:3 and 18:3). *Arabidopsis* mutants of all membrane-bound desaturases have been isolated and used to study the role of lipid unsaturation in different physiological processes (Wallis and Browse, 2002; see Section V.A, D).

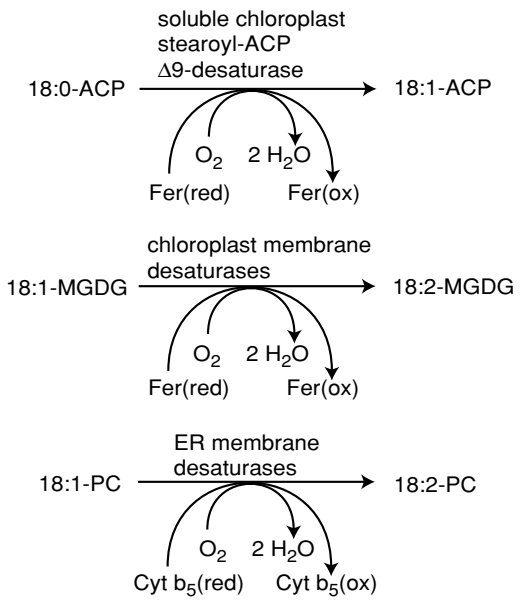


*Fig. 3.* Desaturation of acyl groups in the chloroplast and at the ER. In addition to the soluble, stroma localized stearoyl-ACP Δ9-desaturase, plastids contain membrane-bound desaturases that act on different glycerolipids, i.e., galactolipids, sulfolipid and PG. ER membranes contain membrane-bound desaturases that are active with phospholipids, e.g., PC. Whereas chloroplast desaturases obtain electrons from reduced ferredoxin (Fer), ER desaturases require reduced cytochrome $b_5$ (Cyt $b_5$) as cofactor. In the two compartments, molecular oxygen is the final electron acceptor of the desaturation reaction.

## IV. Glycerolipid Synthesis

### *A. Synthesis of Phosphatidic Acid and Diacylglycerol*

Two subcellular compartments are involved in glycerolipid synthesis, the plastid and the ER. The plastid pathway results in the synthesis of prokaryotic lipids by direct acyl transfer onto glycerol-3-phosphate using acyl-ACPs as substrates (Fig. 4). The glycerol moiety for lipid synthesis originates from dihydroxyacetone phosphate which is derived from glycolysis. Reduction by glycerol-3-phosphate dehydrogenase (GPDH or dihydroxyacetone-phosphate reductase) yields glycerol-3-phosphate. Different isoforms of GPDH are found in plants, some of which were localized to the plastids or mitochondria (Wei *et al.*, 2001; Shen *et al.*, 2003). In the GPDH deficient *gly1* mutant of *Arabidopsis*, plastidial lipid synthesis is reduced resulting in a decrease of 16:3 in galactolipids (Miquel *et al.*, 1998). The recently identified *sfd1* mutation maps very close to the *gly1* locus and shows a lipid composition very similar to *gly1*. This strongly suggests that *sfd1* and *gly1* are allelic (Nandi *et al.*, 2004). The *sfd1* plant carries a point mutation in a gene encoding a GPDH isoform presumed to be localized to plastids. Interestingly, the *sfd1* mutation affects pathogen resistance (Nandi *et al.*, 2004; see Section V.D). The phosphorylation of glycerol by glycerol kinase represents an alternative

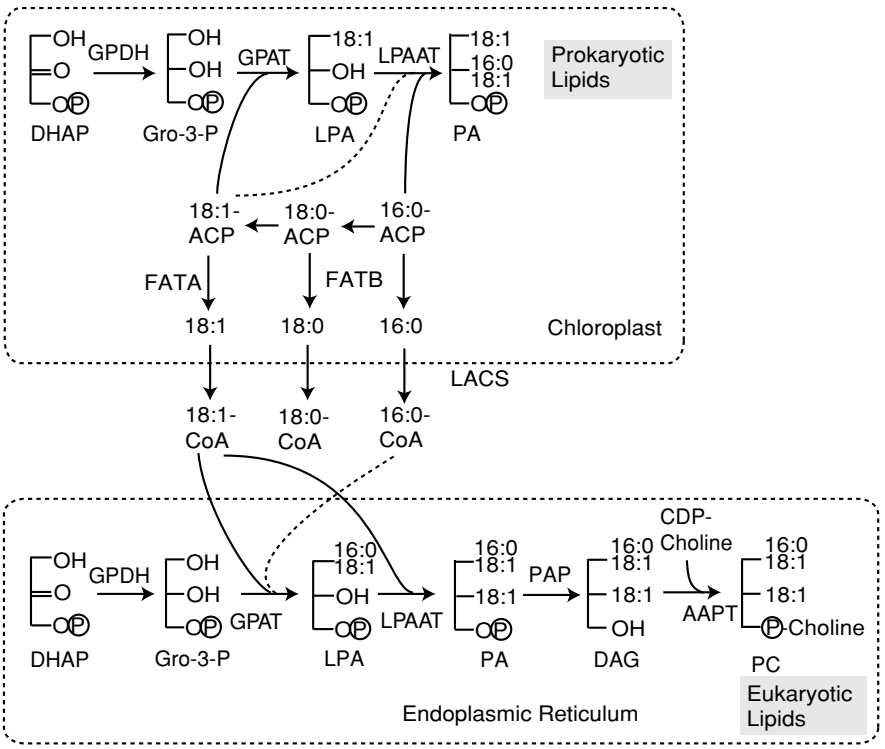


*Fig. 4.* Biosynthesis of phosphatidic acid in chloroplasts and ER. Phosphatidic acid is synthesized from glycerol-3-phosphate by sequential acylation reactions. The differences in substrate specificity of plastid and ER-localized acyltransferases lead to the formation of prokaryotic and eukaryotic lipid structures with different fatty acyl groups attached to the *sn*-1 or *sn*-2 positions of the glycerol backbone. At the ER, diacylglycerol is the precursor for PC synthesis.

pathway for the synthesis of glycerol-3-phosphate, and this reaction was suggested to be operating in germinating seeds, where large amounts of glycerol accumulate from triacylglycerol breakdown (Huang, 1975). *Arabidopsis* contains only one glycerol kinase gene. Interestingly, a mutation in this gene (*gli1*, *glycerol insensitive 1* or *nho1*, *non-host resistance 1*) results in insensitivity to glycerol, accumulation of glycerol during seed storage lipid breakdown and an increased resistance to pathogens (Kang *et al.*, 2003; Eastmond, 2004).

Plastidial glycerol-3-phosphate is the substrate for the first acylation reaction catalyzed by glycerol-3-phosphate acyltransferase (GPAT). The second fatty acid is esterified onto the *sn*-2 position by lysophosphatidic acid acyltransferase (LPAAT). Due to the substrate specificities of the acyltransferases, the product of the two acylation reactions, phosphatidic acid, carries mostly C18 fatty acids at the *sn*-1 and C16 or C18 moieties at the *sn*-2 position (Bertrams and Heinz, 1981; Ishizaki *et al.*, 1988; Frentzen, 1993). The C16 fatty acid at position *sn*-2 of MGDG is further desaturated to yield 16:3, and therefore, plants containing the prokaryotic pathway of lipid synthesis are referred to as "16:3" plants (Browse *et al.*, 1986; Mongrand *et al.*, 1998). However, plants that have lost the plastidial lipid synthesis pathway and therefore are devoid of 16:3, depend entirely on extraplastidial glycerolipid synthesis ("eukaryotic lipids"). These plants accumulate 18:3 as the predominant fatty acid and therefore are called "18:3" plants. A mutation in the gene encoding plastidial GPAT in *Arabidopsis* (*act1*, *acyltransferase mutant 1*) inhibits synthesis of prokaryotic lipids resulting in a reduction of 16:3 fatty acids (Kunst *et al.*, 1988). As a consequence, *act1* plants almost entirely depend on the eukaryotic pathway of lipid synthesis. The *Arabidopsis* genome contains five genes encoding LPAAT, one of them predicted to be plastid localized. In contrast to *act1*, a mutation in the gene encoding plastidial LPAAT is embryo lethal (Kim and Huang, 2004; Yu *et al.*, 2004).

In the eukaryotic pathway of lipid synthesis, acyl groups from acyl-ACPs are hydrolyzed by acyl-ACP thioesterases in the stroma of plastids. Two classes of thioesterases are present in plants, FATA with specificity for oleoyl-ACP and FATB with broad specificity

towards long chain acyl-ACPs (18:0, 16:0; Jones *et al.*, 1995; Dörmann *et al.*, 1995b). Because hydrolysis of acyl-ACP esters is considered to be the prerequisite for fatty acid export from the plastid, a block in one of the thioesterases is expected to result in a reduction in the amount of glycerolipids derived from the ER. Indeed, a T-DNA insertion mutant of *Arabidopsis* deficient in FATB activity contained reduced amounts of 16:0, particularly in extraplastidial lipids (Bonaventure *et al.*, 2003). After crossing the two envelope membranes of the plastid, free fatty acids are believed to be esterified to the thiol group of Coenzyme A. A number of long chain acyl-CoA synthetase genes were detected in *Arabidopsis*, and it is not yet clear which of these enzymes controls export of acyl groups from the plastid (Schnurr *et al.*, 2002; Shockey *et al.*, 2002). The *LACS9* gene of *Arabidopsis* was shown to encode a plastid localized long chain acyl-CoA synthetase implicated in re-esterification of fatty acids exported from the plastid (Schnurr *et al.*, 2002). However, eukaryotic lipid synthesis in the corresponding mutant, *lacs9*, was not affected, indicating that alternative pathways of acyl-CoA synthesis might exist. Similar to plastids, two acylation reactions (GPAT and LPAAT) are involved in the production of PA at the ER. In contrast to the plastid enzymes, the enzymes in the endoplasmic reticulum use acyl-CoA thioesters rather than acyl-ACP thioesters for acylation. Furthermore, because of differences in substrate specificities, the major form of PA synthesized at the endoplasmic reticulum contains C16 and C18 acyl groups at *sn*-1 and C18 groups at the *sn*-2 position (Frentzen, 1993).

PA derived from the plastid or ER pathway of lipid synthesis is dephosphorylated to diacylglycerol by PA phosphatase (PAP). Three PAP-like proteins are present in *Arabidopsis* that are predicted to be localized to the plastid, ER or mitochondrion (Pierrugues *et al.*, 2001; Beisson *et al.*, 2003). PC, the predominant glycerolipid in extraplastidial membranes, is produced from diacylglycerol and CDP-choline by the action of aminoalcoholphosphotransferase (AAPT). Two AAPT proteins are found in *Arabidopsis* (Goode and Dewey, 1999; Beisson *et al.*, 2003). Eukaryotic lipid precursors are not only targeted to different extraplastidial membranes, but are also transported to the chloroplast, where they are incorporated into thylakoid membranes.

### B. Synthesis of Phosphatidylglycerol

Phosphatidylglycerol (PG) is a major phospholipid in chloroplasts and in mitochondria (Block *et al.*, 1983; Griebau and Frentzen, 1994). PG synthesis in chloroplasts starts with the conversion of PA to CDP-diacylglycerol by CDP-diacylglycerol synthase (Fig. 5a; PA cytidylyltransferase, CDS). PG-phosphate is produced from CDP-diacylglycerol and glycerol-3-phosphate by PG-phosphate synthase (PGP1). Two genes (*PGP1*, *PGP2*) encoding PG-phosphate synthase are present in *Arabidopsis* (Müller and Frentzen, 2001). Whereas PGP2 is localized to the ER, PGP1 contains an N-terminal dual targeting sequence for the chloroplast and for the mitochondrion (Müller and Frentzen, 2001; Xu *et al.*, 2002; Babiychuk *et al.*, 2003). A mutation in *PGP1* affects PG synthesis in the plastid and as a consequence, *pgp1* mutants contain reduced amounts of chlorophyll and are incapable of photoautotrophic growth (Hagio *et al.*, 2002; Xu *et al.*, 2002; Babiychuk *et al.*, 2003). Interestingly, the mitochondrial PG content is not compromised in *pgp1*, suggesting that glycerolipid import from the ER can complement lipid deficiency in mitochondria but not in plastids. The final step of PG synthesis is catalyzed by PG-phosphate phosphatase (PGPP), however, the respective gene has not yet been isolated from higher plants.

### C. Synthesis of Sulfoquinovosyldiacylglycerol

The sulfolipid sulfoquinovosyldiacylglycerol (SQDG) is synthesized by transfer of the activated head group, UDP-sulfoquinovose, onto diacylglycerol by SQDG synthase (SQD2; Fig. 5a; Yu *et al.*, 2002). The corresponding gene has been isolated from *Arabidopsis*, and a T-DNA mutant (*sqd2*) was obtained that is totally devoid of SQDG. The *sqd2* mutant is very similar to wild-type under normal growth conditions, and only under phosphate-limiting conditions is growth of *sqd2* retarded. The unusual head group of SQDG is synthesized from UDP-glucose and sulfite in the stroma of plastids by action of UDP-sulfoquinovose synthase (SQD1; Fig. 5b; Essigmann *et al.*, 1998; Sanda *et al.*, 2001). The *SQD1* cDNA, which shows sequence similarity to nucleotide-sugar epimerases/dehydrogenases, was isolated from *Arabidopsis*. Enzyme assays with recombinant protein demonstrated that it catalyzes the formation of a sulfur carbon bond by incorporation of inorganic sulfite into UDP-glucose through a UDP-glucose-5-ene intermediate (Mulichak *et al.*, 1999).

### D. Synthesis of Galactolipids

UDP-galactose, the substrate of galactolipid synthesis, is derived from UDP-glucose via epimerization of the

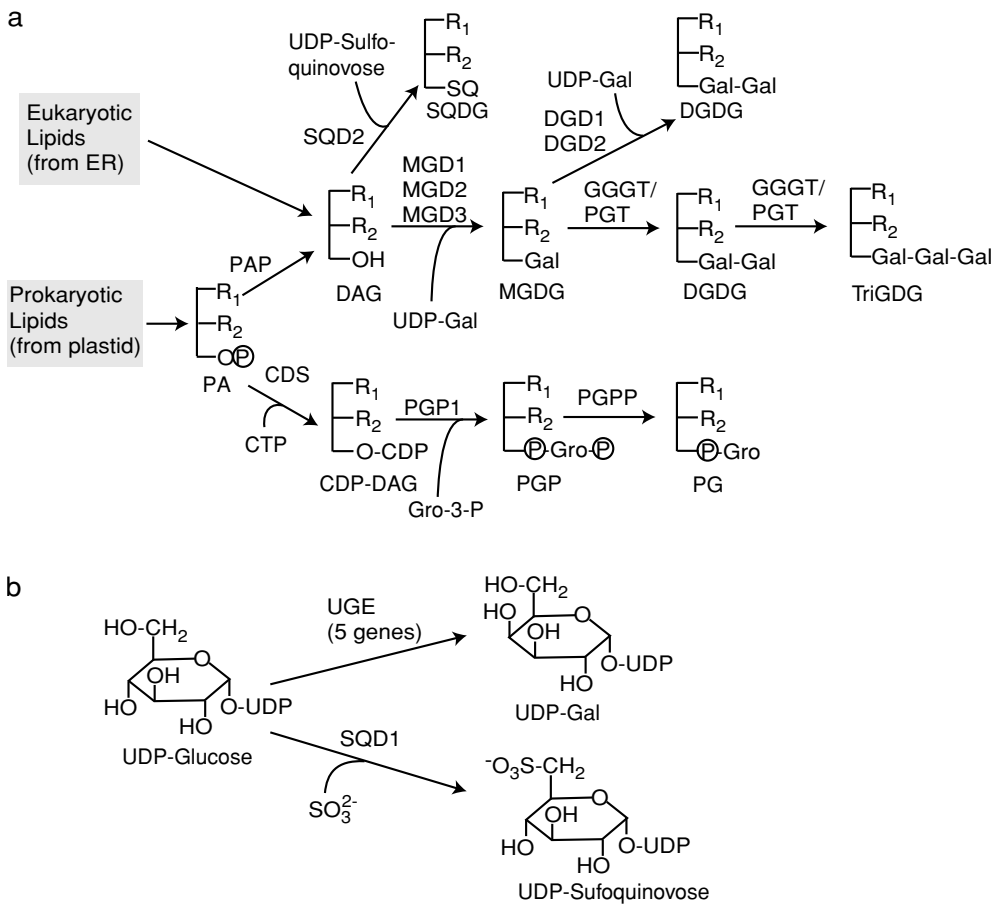


*Fig. 5.* Glycerolipid synthesis in chloroplasts. (a) The glycerolipids, MGDG, DGDG and SQDG, in chloroplasts are synthesized from diacylglycerol originating from the plastid (prokaryotic lipids) or from the ER (eukaryotic lipids). PG is synthesized from CDP-diacylglycerol, which is derived from prokaryotic phosphatidic acid. (b) The activated head groups of galactolipid and sulfolipid, UDP-galactose and UDP-sulfoquinovose, are derived from UDP-glucose.

hydroxyl group at the C4 position (Königs and Heinz, 1974; Dörmann *et al.*, 1998). Five genes encoding UDP-glucose epimerases are known in *Arabidopsis*, but their role in UDP-galactose production for galactolipid or cell wall synthesis is poorly understood (Fig. 5b; Reiter and Vanzin, 2001). Antisense expression of *UGE1* (Dörmann *et al.*, 1998) or a mutation in *uge4* (synonymous: *rhd1*, *root hair deficient1*; Seifert *et al.*, 2002) had no impact on galactolipid content.

MGDG is synthesized by transferring galactose from UDP-galactose, with an α-glycosidic linkage, onto diacylglycerol in a β-glycosidic linkage in the envelope membranes of chloroplasts. Three MGDG synthase genes are present in *Arabidopsis* (Fig. 5a; Awai *et al.*, 2001). MGD1 contains a classical N-terminal targeting sequence for the chloroplast and localizes to the inner envelope (Shimojima *et al.*, 1997; Miège *et al.*, 1999). The *mgd1* mutant of *Arabidopsis* is partially inhibited in MGD1 activity, and shows a 50% reduction in MGDG content accompanied by a decrease in the amount of chlorophyll and thylakoid membranes (Jarvis *et al.*, 2000). MGD2 and MGD3, which have closely related sequences, are localized to the outer site of chloroplast membranes (Awai *et al.*, 2001). These two enzymes are believed to be involved in galactolipid synthesis in specific plant organs or during phosphate deprivation (Kobayashi *et al.*, 2004).

*Arabidopsis* contains two DGDG synthases (DGD1 and DGD2), which are localized to the outer side of chloroplast envelope membranes (Fig. 5a; Dörmann *et al.*, 1999; Kelly *et al.*, 2003). In contrast to DGD2, DGD1 contains an additional N-terminal extension of about 40-kDa of unknown function (Froehlich *et al.*, 2001). Enzyme assays with recombinant proteins demonstrated that the two enzymes use UDP-galactose as galactose donor for galactosylation of

MGDG. During this reaction, an α-glycosidic linkage between the second and the first galactose moiety is formed (Kelly and Dörmann, 2002; Kelly *et al.*, 2003). Analysis of *dgd1* and *dgd2* mutants of *Arabidopsis* revealed that DGD1 mostly produces DGDG for thylakoid membranes, whereas DGD2 is not active under normal growth conditions (Dörmann *et al.*, 1995a; Kelly *et al.*, 2003). However, expression of the two genes is strongly induced during phosphate deprivation resulting in an increase in the amount of DGDG at the expense of phospholipids (see Section V.D). Although the double mutant plant, *dgd1 dgd2,* contains only trace amounts of DGDG, the incorporation of radioactivity from UDP-[$^{14}$C]galactose into galactolipids in *in vitro* assays with *dgd1 dgd2* chloroplasts was not altered (Kelly *et al.*, 2003). This result implies the existence of a third galactolipid synthase which is highly active *in vitro*, but does not contribute to net galactolipid synthesis in the leaves. The corresponding gene is unknown, but it is most likely related to the galactolipid:galactolipid galactosyltransferase (GGGT) that was previously suggested to be involved in galactolipid synthesis in plants (van Besouw and Wintermans, 1978; Heemskerk *et al.*, 1990). In *in vitro* experiments, this enzyme is also capable of producing oligogalactolipids with three or more galactose moieties (Fig. 5a). However, these oligogalactolipids are absent from leaves, and only accumulate in low amounts in some plant species (Fujino and Miyazawa, 1979; Kojima *et al.*, 1990). By screening for *dgd1* suppressor mutants, Xu *et al.* (2003) isolated a mutant (*tgd1, trigalactosyldiacylglycerol1*) that accumulates DGDG and unusual oligogalactolipids in the leaves. This result was explained by the stimulation of an additional galactolipid synthase designated PGT (processive galactolipid synthase), which might be related to the GGGT activity described above. The *TGD1* gene encodes a permease-like protein localized to the outer side of chloroplast envelope membranes (see Section VI.A).

## V. Function of Chloroplast Lipids

### *A. Growth at Non-optimal Temperatures*

The degree of fatty acid unsaturation has a strong impact on the biophysical characteristics of biological membranes, because lipid bilayers containing saturated acyl chains have a more rigid structure, whereas the introduction of *cis* double bonds results in increased membrane fluidity. Double bonds with *cis* configuration confer a bent structure into the acyl chain, and as a consequence, unsaturated fatty acids disturb the acyl bilayer of the membrane resulting in an increase in membrane fluidity. At low temperature, when lipid mobility is decreased, an increase in membrane fluidity can be achieved by increasing the degree of fatty acid unsaturation. Therefore, it is assumed that polyunsaturated fatty acids are particularly important for growth at low temperatures, whereas saturated fatty acids are critical at higher temperatures (Fig. 6). The study of *Arabidopsis* mutants deficient in lipid desaturation has greatly advanced our understanding of the interactions between fatty acid composition and growth at low or high temperatures. From these studies it became clear that adaptation to non-optimal growth temperatures is complex, and that lipid unsaturation is just one of many factors involved in this process (for a review, see Wallis and Browse, 2002). The hypothesis that unsaturated fatty acids are important for growth at low temperatures is in agreement with the observation that the amounts of poylunsaturated fatty acids increase (Graham and Patterson,1982) and that expression of specific desaturase genes is induced at low temperatures (Gibson *et al.*, 1994). Alterations in the amounts of triunsaturated fatty acids as observed in an *Arabidopsis* triple mutant (*fad3 fad7 fad8;* low in16:3, 18:3) or in transgenic tobacco over-expressing *FAD7* (high in 16:3, 18:3) resulted in only minor changes in photosynthetic efficiency and growth at low temperatures (Kodama *et al.*, 1994; McConn and Browse, 1996). However, a decrease in 16:3 and 18:3, e.g., in transgenic tobacco plants that have reduced desaturase activity, or in the *Arabidopsis fad7 fad8* mutant, resulted in enhanced growth and photosynthetic capacity at high temperatures (Murakami *et al.*, 2000). The *Arabidopsis* double mutant *fad2 fad6* contains reduced amounts of polyunsaturated fatty acids (18:2, 18:3, 16:3). Growth of *fad2 fad6* is more severely affected as compared to *fad3 fad7 fad8* mutants, due to the loss of di- and triunsaturated fatty acids. The *Arabidopsis* mutants *fad5* and *fad6* show a general decrease in fatty acid desaturation and a decreased growth rate at low, but increased growth rate at high, temperatures (Kunst *et al.*, 1988; Hugly *et al.*, 1989; Hugly and Somerville, 1992). A block in stearoyl-ACP desaturation as observed in the *fab2* mutant of *Arabidopsis* results in an accumulation of 18:0 accompanied by a reduction in 18:1 (Lightner *et al.*, 1994a, b). These plants are strongly reduced in growth at normal temperatures. However, growth is only slightly affected at high temperatures, suggesting that the elevated 18:0 content results in an increased membrane rigidity.

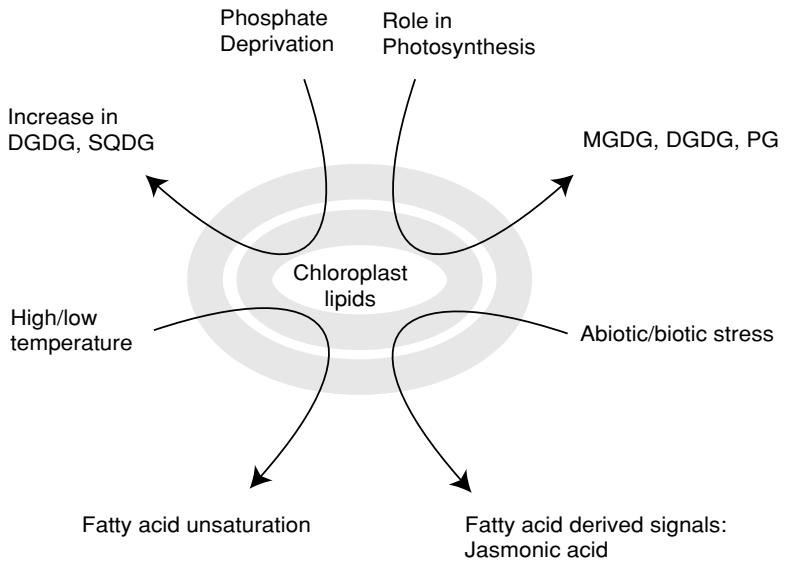


*Fig. 6.* Physiological roles of polar lipids in chloroplasts. The two glycolipids, DGDG and SQDG, replace phospholipids under phosphate-limiting conditions to save phosphate for other cellular processes. Three lipids, MGDG, DGDG and PG, are localized to the complexes of photosynthetic light reactions and are critical to sustain an optimal rate of photosynthesis. The degree of unsaturation of fatty acyl groups of plastidial and extraplastidial membrane lipids is critical for growth at high and low temperatures. α-Linolenic acid derived from plastidial lipids is the precursor for oxylipin/jasmonic acid production via the lipoxygenase pathway.

PG in plants contains high amounts of 16:1Δ3*trans* at the *sn*-2 position. For a long time it was believed that this fatty acid is essential for the assembly and function of chloroplast membranes. However, the isolation of a mutant (*fad4*) devoid of 16:1Δ3*trans* demonstrated that this fatty acid is dispensable for photosynthesis (Browse *et al.*, 1985). The degree of unsaturation of the acyl group attached to the *sn*-1 position of PG was believed to be critical for chilling tolerance, because many chilling-tolerant plants (*Arabidopsis*, spinach) contain 18:2 or 18:3 fatty acids in this position, whereas in chilling-sensitive plants (squash, tobacco) 16:0 is the most abundant fatty acid. Therefore, the substrate specificity of plastidial GPAT was suggested to be critical in determining chilling sensitivity in plants. Indeed, overexpression of the GPAT from squash or *Escherichia coli* in transgenic plants resulted in an increase in saturated fatty acids in PG, accompanied by a reduced growth at low temperatures, whereas overexpression of the *Arabidopsis* GPAT led to increased amounts of unsaturated fatty acids in PG and an improved growth at low temperatures (Murata *et al.*, 1992; Wolter *et al.*, 1992; Moon *et al.*, 1995). The *fab1* mutant of *Arabidopsis* contains increased amounts of 16:0 in PG due to a block in β-ketoacyl-ACP synthase II. However, chilling tolerance of *fab1* plants was not affected, and only after long exposure to low temperature was a reduction of growth rate observed. Therefore, other factors than acyl group unsaturation of PG seem to contribute to chilling sensitivity in plants.

## *B. Photosynthesis*

The occurrence of galactolipids MGDG and DGDG and of SQDG seems to be restricted to photosynthetic organisms, in particular, higher plants and cyanobacteria (Fig. 6). However, there are some exceptions to this general rule, e.g., sulfolipid was found in non-photosynthetic *Rhizobia* (Weissenmayer *et al.*, 2000), and glycolipids with different sugars in their head groups are present in many non-photosynthetic bacteria (e.g. Jorasch *et al.*, 1998). Further evidence for the critical role of glycerolipids in photosynthesis came from recent structural studies on the photosynthetic complexes by X-ray crystallography. Photosystem I from cyanobacteria was shown to contain four lipid molecules, one MGDG and three PG molecules, which are bound to polypeptide chains via hydrogen bonding (Klukas *et al.*, 1999; Jordan *et al.*, 2001). In the crystal structure of the major light-harvesting complex (LHCII) of the dinoflagellate *Amphidinium carterae,* two lipids (PG and DGDG) were detected (Hofmann *et al.*, 1996). PG and DGDG were also shown to be critical for the stabilization of LHCII preparations *in vitro* (Nußberger *et al.*, 1993; Reinsberg *et al.*, 2000) and were detected in the crystal structure of

plant LHCII (Liu *et al.*, 2004). The cytochrome $b_6f$ complex harbors three lipid molecules, one SQDG and two molecules tentatively identified as MGDG (Stroebel *et al.*, 2003). Until now, no lipid molecules were detected in the structure of photosystem II from cyanobacteria or from plants (Rhee *et al.*, 1997; Zouni *et al.*, 2001).

The presence of glycerolipid molecules within the crystal structure strongly suggests that these lipids are indispensable components of the complexes of photosynthesis. Functional studies with *Arabidopsis* mutants deficient in the synthesis of thylakoid glycerolipids confirmed that indeed, these lipids are essential for photosynthetic light reactions.

MGDG synthase activity is reduced in the *Arabidopsis* mutant *mgd1* to about 50% of wild-type level (Jarvis *et al.*, 2000). Thus, chloroplasts of *mgd1* plants contain only half the amount of MGDG, and as a consequence, the total amount of thylakoid membranes and of total chlorophyll is drastically decreased. Given that *mgd1* plants still contain substantial amounts of MGDG, these findings demonstrate that this lipid is essential for an optimal rate of chloroplast development and for normal assembly of the photosynthetic complexes.

Two mutants, *dgd1* and *dgd2*, were isolated for the two DGDG synthase genes of *Arabidopsis*. The *dgd1* mutant contains only 10% of wild-type amounts of DGDG, and the double-mutant *dgd1 dgd2* is almost completely devoid of DGDG (Dörmann *et al.*, 1995a; Kelly *et al.*, 2003). Single-mutant plants (*dgd1*) are strongly reduced in growth and photosynthetic capacity (Dörmann *et al.*, 1995a), and different aspects of photosynthetic light reactions are affected in these plants (Härtel *et al.*, 1997; Reifarth *et al.*, 1997). In *dgd1 dgd2*, photosynthetic capacity is further reduced as compared to *dgd1*, and therefore, double mutant plants are unable to grow photoautotrophically (Kelly *et al.*, 2003). These results emphasize that DGDG plays an essential role in photosynthesis of higher plants.

The *pgp1* mutant of *Arabidopsis* is blocked in the biosynthesis of plastidial PG-3-phosphate synthase resulting in a drastic reduction of PG in plastids (Hagio *et al.*, 2002; Xu *et al.*, 2002; Babiychuk *et al.*, 2003). Mutant plants are pale green, have reduced amounts of thylakoid membranes and loose their capability of photoautotrophic growth. Therefore, the anionic lipid PG indeed plays a central role in thylakoid membrane assembly and is indispensable for photosynthesis.

For a long time, it was assumed that the plant sulfolipid, SQDG, also plays a critical role in photosynthesis. The characterization of the sulfolipid-deficient *sqd2* mutant of *Arabidopsis*, however, demonstrated that SQDG is required for growth under phosphate limiting conditions, whereas photosynthetic efficiency in *sqd2* plants raised under normal phosphate conditions was very similar to wild-type (Yu *et al.*, 2002). In the *sqd2* mutant, the anionic phospholipid PG seems to substitute for the loss of SQDG, and in agreement with this hypothesis, thylakoid structure and photosynthetic efficiency were severely compromised in the double-mutant, *sqd2 pgp1,* deficient in SQDG and PG (Yu and Benning, 2003).

### C. Phosphate Deprivation

During growth under phosphate limiting conditions, the amounts of DGDG and SQDG increase at the expense of phospholipids (PC, PE, PG). Thus, phosphate bound to phospholipids in the membranes is made available for other important cellular processes, e.g., nucleic acid synthesis (for review, see Dörmann and Benning, 2002). This adaptive process for the replacement of phospholipids with glycolipids was first discovered in bacteria (Minnikin *et al.*, 1974). The increase in DGDG and SQDG is mediated via induction of gene expression. After phosphate deprivation, expression of the two genes of sulfolipid biosynthesis (*SQD1* and *SQD2*) is strongly induced (Essigmann *et al.*,1998; Yu *et al.*, 2002). Furthermore, gene expression of enzymes required for DGDG synthesis *(MGD2*, *MGD3*, *DGD1* and *DGD2*) is induced during phosphate deprivation (Awai *et al.*, 2001; Kelly and Dörmann, 2002; Kelly *et al.*, 2003). Interestingly, the amount of DGDG increases both in the chloroplasts and in extraplastidial membranes (Härtel *et al.*, 2000). The plasma membrane was shown to contain high amounts of DGDG (up to 70%) after phosphate deprivation (Andersson *et al.*, 2003). The increase in plastidial DGDG is mediated by DGD1, whereas DGD2 is required for synthesis of DGDG for extraplastidial lipids (Kelly *et al.*, 2003). Because the two DGDG synthases DGD1 and DGD2 are localized to the envelope membranes of chloroplasts, the accumulation of DGDG in thylakoids and in extraplastidic membranes implies the existence of a highly active, intracellular transport system for lipids.

### D. Signaling and Plant Pathogen Interactions

Fatty acids derived from chloroplast lipids play a major role in plant cell signaling and in the response to pathogen attack. Unsaturated fatty acids in

chloroplasts, in particular 18:3, are the precursors for oxylipin synthesis (Fig. 6; Weber, 2002; Farmer *et al.*, 2003). The lipoxygenase reaction represents the committed step of the octadecanoic acid pathway, because it inserts a hydroperoxy group into position 13 of 18:3. Subsequent reactions, catalyzed by allene oxide synthase and allene oxide cyclase, convert the hydroperoxy fatty acid into 12-oxophytodienoic acid (OPDA), an important, functionally-active intermediate of jasmonic acid production. The initial reactions of oxylipin synthesis are localized to plastid membranes. Interestingly, a large fraction of OPDA in chloroplasts was found to be bound to MGDG (Stelmach *et al.*, 2001). Therefore, it is not clear whether the lipoxygenase acts on free fatty acids or on acyl groups bund to galactolipids or phospholipids. Furthermore, 16:3 can also enter the lipoxygenase pathway, resulting in the production of dinor-OPDA, a C16 homolog of OPDA (Weber *et al.*, 1997). After reduction and three cycles of β-oxidation in peroxisomes, OPDA is converted to the phytohormone jasmonic acid. The *Arabidopsis* triple-mutant *fad3 fad7 fad8* is devoid of triunsaturated fatty acids, and thus contains negligible amounts of 16:3 and 18:3 (McConn *et al.*, 1997). As a consequence, the octadecanoic acid pathway is blocked in these plants resulting in a deficiency of jasmonic acid and an increased susceptibility towards pathogen attack.

A mutant in one of the stearoyl-ACP desaturase genes, *ssi2*, shows constitutive over-expression of the pathogen related gene *PR1* and is more resistant to pathogens including *Peronospora parasitica* (Kachroo *et al.*, 2001; Shah *et al.*, 2001). The block in 18:0 desaturation results in an increase of 18:0 with a concomitant decrease in unsaturated fatty acids, particularly 18:1, in the leaves. The effect of the *ssi2* mutation on pathogen resistance was suggested to be mediated via jasmonic acid synthesis. However, the fact that 18:3, the precursor for jasmonic acid production, is only slightly reduced, strongly indicates that jasmonic acid might not directly be the cause for pathogen resistance in *ssi2*. By analyzing additional *Arabidopsis* desaturase mutants, Kachroo *et al.* (2003) concluded that in addition to jasmonic acid, another lipid signal is critical for pathogen responses in *Arabidopsis*. An alternative approach was employed by Nandi *et al.* (2003, 2004), who searched for suppressor mutants of *ssi2*. Interestingly, one of these suppressor plants (*sdf1*, *suppressor of fatty acid desaturase deficiency 1*) was shown to carry a mutation in the gene encoding a plastidial isoform of GPDH, again suggesting a critical role for a chloroplast lipid-derived signal in plant pathogen responses.

## VI. Lipid Trafficking

### *A. Lipid Transport Between Chloroplast and Endoplasmic Reticulum*

Fatty acids produced in plastids are subsequently incorporated into glycerolipids at the ER and (in 16:3 plants) in the plastid. Therefore, a large fraction of fatty acids must be exported from the plastid to the ER for glycerolipid synthesis (Fig. 7). Acyl groups (predominantly 16:0 and 18:1) are hydrolyzed from acyl carrier protein by thioesterases and moved through the envelope membranes by an unknown mechanism. Subsequently, acyl groups are re-esterified to Coenzyme A by acyl-CoA synthetases in the plastid envelope (Schnurr *et al.*, 2002; Shockey *et al.*, 2002). Acyl-CoA thioesters can easily partition into the aqueous compartment of the cytosol, thus traveling to the ER where they are employed for glycerolipid synthesis.

A large fraction of eukaryotic glycerolipids assembled at the ER is transported back to the chloroplast, where they are used as precursors for the synthesis of galactolipids and sulfolipid (Fig. 7). The identity of the molecule employed for transport is still a matter of debate, but it is generally accepted that it is a PC-derived lipid such as PC, lyso-PC, diacylglycerol or PA (Mongrand *et al.*, 2000). Transport of lyso-PC, which lacks one of the fatty acyl group, from the ER to the plastid membranes might be mediated by diffusion, because lyso-PC can readily partition into the aqueous phase of the cytosol. Alternatively, lipid moieties originating from the ER might be transported to the plastids via vesicles or via direct contact sites between the ER membrane and the outer chloroplast envelope (Moreau *et al.*, 1998). Contact sites between the ER and the plastid have been observed by electron microscopy (e.g. Schötz, 1975). Possibly, these membrane sites are employed for lipid trafficking in a similar way as was shown for the contact sites between ER and mitochondria (MAMs, mitochondrial associated membranes) and ER and plasma membrane (PAMs, plasma membrane associated membranes) (Staehelin, 1997). The recent identification of an *Arabidopsis* mutant, *tgd1*, affected in ER-to-plastid lipid trafficking was the basis for the isolation of the corresponding gene, *TGD1*, which encodes a permease-like protein localized to the outer side of plastid envelopes (Xu *et al.*, 2003).

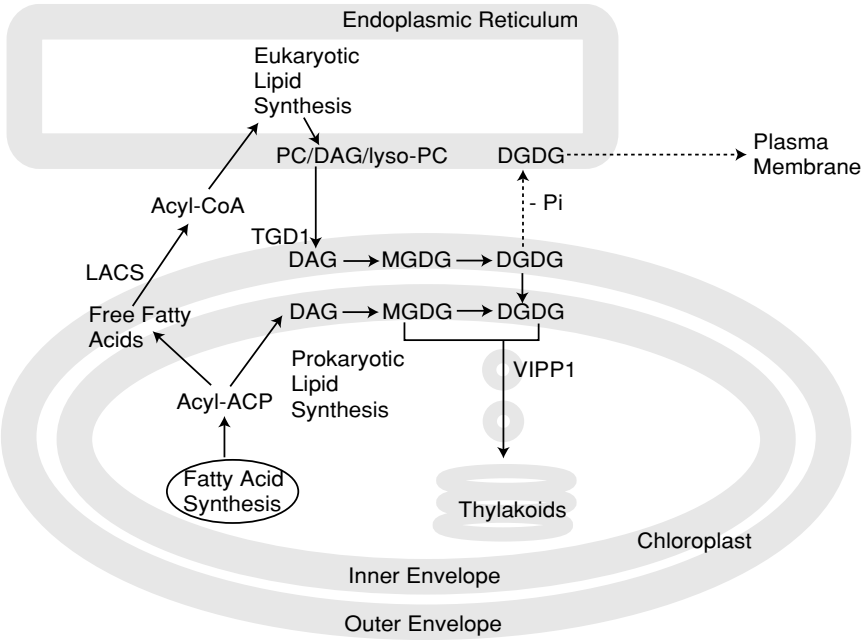


*Fig. 7.* Lipid trafficking. Fatty acids synthesized inside the plastid are exported as free fatty acids and converted into their CoA thioesters by long-chain acyl-CoA synthetases (LACS) before incorporation into eukaryotic lipids at the ER. Eukaryotic lipids are transported back to plastid envelope membranes where they are used for galactolipid and sulfolipid synthesis. The exact structure of the lipid molecule that is transported from the ER to the plastid is unknown, but it is likely derived from PC (PC, lyso-PC, PA or DAG). This lipid transport is probably controlled by protein factors, e.g., TGD1 (see text for details). Prokaryotic lipid synthesis inside the chloroplast adds to the net production of plastid lipids. The mechanism of lipid exchange between the two envelope membranes is unknown. Transport of polar lipids from the inner envelope to the thylakoids is believed to be mediated by vesicles (controlled by VIPP1 protein). Under phosphate-limiting conditions, DGDG synthesized in the outer envelope is transported to the ER and to the plasma membrane via an unknown mechanism (dashed arrows).

TGD1 shows sequence similarity to ATP-binding cassette (ABC) transporters and represents the first protein isolated from plants that was implicated in lipid transport between ER and plastid.

During phosphate-limited growth conditions, large amounts of DGDG accumulate in the plastid, but also in the extraplastidial membranes such as the plasma membrane (Härtel *et al.*, 2000; Andersson *et al.*, 2003). Because the two enzymes involved in DGDG synthesis have been localized to the plastid envelope membranes, lipid trafficking must be involved in the export of DGDG from the plastid to the ER and to other extraplastidial membranes. Similarly to the ER-to-plastid lipid transport, vesicle transport or membrane contact sites ("plastid associated membranes") might be involved in the export of lipids from the plastid. Further transport of lipids from the ER to the plasma membrane might be mediated via contact sites or via the Golgi apparatus, because vesicles derived from the *trans*-Golgi network are known to eventually fuse with the plasma membrane.

### *B. Lipid Transport From Chloroplast Envelopes to Thylakoids*

The enzymes of DGDG synthesis, DGD1 and DGD2, were localized to the outer membrane of the chloroplast envelope. However, a large fraction of DGDG finally accumulates in the thylakoid membranes. The mechanism of DGDG transport from the outer membrane to the inner membrane of the envelope is unknown, but the N-terminal extension of the DGD1 protein was suggested to play a role in this process (Dörmann *et al.*, 1999). Glycerolipids synthesized in the inner envelope (MGDG, SQDG, PG), as well as DGDG derived from the outer envelope, are subsequently transported to the thylakoids via a mechanism involving vesicles derived from the inner envelope, which diffuse through the stroma and subsequently fuse with thylakoid membranes (Fig. 7). Indeed, such vesicles were observed in the stroma by electron microscopy, and it was shown that high numbers of vesicles accumulate when leaves are chilled (Morré *et al.*, 1991). A mutation in the

vesicle-inducing in plastid protein 1, VIPP1 (Kroll *et al.*, 2001), affects thylakoid membrane assembly by decreasing lipid transport to the thylakoids. Therefore, VIPP1 seems to be critical for vesicle formation and transport at the inner envelope membrane.

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# Chapter 18

# Amino Acid Synthesis in Plastids

Muriel Lancien and Peter J. Lea*
*Department of Biological Sciences, Lancaster University, Lancaster, LA1 4YQ, UK*

Ricardo A. Azevedo
*Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, CP 83, CEP 13418-900, Piracicaba, SP, Brasil*

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## Summary

Plants have the capacity to assimilate inorganic nitrogen and synthesize the twenty amino acids required for normal protein synthesis, unlike animals that need at least nine essential amino acids in their diet. The major metabolic reactions involved in the synthesis of essential amino acids, and the enzymes that catalyse them, are located in plastids. In the chloroplast, ATP and reductant required for amino acid synthesis are derived directly from light energy via photosystems I and II. Non-green plastids are also able to synthesize amino acids, using reductant

* Author for correspondence, email: p.lea@lancaster.ac.uk

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derived from the oxidative pentose-phosphate pathway. Ammonia produced either from the reduction of nitrite, or a variety of other reactions including the decarboxylation of glycine during photorespiration, is rapidly assimilated into glutamine by glutamine synthetase (GS) and transferred to the α-amino position of glutamate by glutamate synthase (GOGAT). The amino group can then be used for the synthesis of lysine, threonine, methionine, isoleucine, leucine, valine, tryptophan, phenylalanine, tyrosine and histidine inside the plastid, through strictly regulated pathways. The two major mechanisms by which regulation of amino acid biosynthesis occurs in plastids, (1) feedback inhibition of the enzymes by end-product amino acids and (2) regulation of transcription of the multigene families that encode the protein(s) that make up the individual enzymes, are described in detail.

## I. Introduction

Chloroplasts assimilate $CO_2$ by photosynthesis. However, as will become obvious to the reader of this book, chloroplasts are also able to carry out a large number of other synthetic reactions by making use of the ATP and reductant derived directly from light energy via photosystems I and II. In addition, non-green tissues of plants contain plastids that, although not able to assimilate $CO_2$, are still able to carry out synthesis of amino acids.

Plants take up nitrogen from the soil as nitrate and ammonium ions, and to a lesser extent directly as amino acids. Nitrate is reduced to nitrite outside the chloroplast/plastid, but all further metabolism may take place totally within the plastid. Ammonium ions taken up by the root are normally assimilated directly by glutamine synthetase (GS), which is located in both the plastid and cytosol. In the following sections the metabolism of nitrite to glutamate is covered in detail. The α-amino group can then be transferred to the two other key amino acids, alanine and aspartate, by standard aminotransferase enzymes, isoforms of which are located in plastids (Ireland and Lea, 1999). In this chapter we have concentrated on the aspartate, branched chain and aromatic amino acid biosynthetic pathways. Of the remaining essential amino acid biosynthetic pathways that are also located in plastids, methionine and cysteine are covered by Pilon-Smits and Pilon in Chapter 19 and histidine has been omitted due to lack of space (see Ward and Ohta, 1999, for the most recent review). Of the non-essential amino acids, serine and glycine are metabolised predominantly in the peroxisomes and mitochondria. The synthesis of asparagine and proline takes place in the cytoplasm, while the location of the synthesis of arginine has not been clarified.

*Abbreviations:* AHAS – acetohydroxyacid synthase; AICAR – 5-aminoimidazole-4-carboxiamide ribonucleoside; AK – aspartate kinase; AOAT – alanine:2-oxoglutarate aminotransferase; AS – anthranilate synthase; BCAT – branched-chain amino acid aminotransferase; CM – chorismate mutase; CS – chorismate synthase; DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate; DCT – 2-oxoglutarate/glutamate transporter; DHDPS – dihydrodipicolinate synthase; DHQ – 3-dehydroquinate; DHQase – 3-dehydroquinate dehydratase; DiT – dicarboxylate transporter; EPSP – 5-enolpyruvylshikimate 3-phosphate; Fd – -ferredoxin; FNR – -ferredoxin-$NADP^+$ reductase; GFP – green fluorescent protein; GGAT – glutamate:glyoxylate aminotransferase; Glc6PDH – glucose 6-phosphate dehydrogenase; GOGAT – glutamate synthase; GS – glutamine synthetase; GUS – glucuronidase; HSDH – homoserine dehydrogenase; HSK – homoserine kinase; KARI – ketolacid reductoisomerase; NiR – nitrite reductase; NR – nitrate reductase; OPPP – oxidative pentose-phosphate pathway; PAI – phosphoribosyl-anthranilate isomerase; PAT – phosphoribosyl-anthranilate transferase; SAM – S-adenosyl methionine; SORase – shikimate:$NADP^+$ oxidoreductase; TD – threonine deaminase; TrpS – tryptophan synthetase; TS – threonine synthase.

## II. Synthesis of Glutamine

### A. Nitrite Reductase (NiR)

NiR (EC 1.7.7.1) catalyses the reduction of nitrite to ammonium using reduced ferredoxin (Fd) (Emes and Neuhaus, 1997). NiR is located within the chloroplast in green leaves and within the plastids in roots and heterotrophic tissues. NiR is thought to be a monomeric enzyme of around 61-kDa in mass that contains two prosthetic groups, a [4Fe-4S] cluster and a siroheme, which transfer electrons from Fd to nitrite (Meyer and Caboche, 1998). *Nii* cDNAs or *Nii* genes have been cloned from several species such as spinach, maize, birch, rice, *Arabidopsis thaliana* and tobacco (Meyer and Caboche, 1998).

Light and sugar induce an increase in NiR mRNA level (Vincentz *et al.*, 1993; Sivasankar *et al.*, 1997). Sucrose can relieve the inhibition of NiR by amides (Sivasankar *et al.*, 1997). Photo-oxidative damage to chloroplasts in norflurazon-treated plants was shown to inhibit NiR gene expression in tobacco and sunflower, suggesting that some plastidic factor could be required for NiR expression in the nucleus (Cabello *et al.*, 1998).

Light-induced expression of NiR was shown to be phytochrome-mediated (Becker *et al.*, 1992). NiR activity and protein levels are more stable in darkness in the roots than in the leaves, suggesting a considerable capacity for nitrite reduction under these conditions. Stohr and Mäck (2001) suggested that NiR acts in concert with the cytosolic GS to ensure that nitrite does not accumulate but is further assimilated into organic compounds both during the day and night in the roots.

The NiR mRNA level is increased by nitrate (Vincentz *et al.*, 1993; Cabello *et al.*, 1998; R. Wang *et al.*, 2003) and the effect was shown to occur at the transcriptional level (Truong *et al.*, 1994). Ammonium, glutamine and asparagine inhibit the expression of NiR in detached tobacco leaves and roots (Vincentz *et al.*, 1993; Sivasankar *et al.*, 1997). Promoter-deletion and footprinting analysis of the spinach *Nii* gene revealed GATA elements within the 331-bp promoter sequence that are related to induction by nitrate (Rastogi *et al.*, 1997; Dorbe *et al.*, 1998). Post-transcriptional regulation of NiR expression by nitrogen sources was shown in tobacco and *A. thaliana* expressing NiR under the 35S promoter (Crete *et al.*, 1997). Although the level of NiR mRNA derived from transgene expression was unchanged, NiR activity and protein level were strongly reduced by ammonium but not by nitrate.

## B. Glutamine Synthetase (GS)

GS (EC 6.3.1.2) catalyses the ATP-dependent conversion of glutamate to glutamine, utilizing ammonia as substrate (Fig. 1). Two classes of GS isoenzymes were originally identified by ion-exchange chromatography, cytosolic GS1 and chloroplastic GS2. The chloroplastic form is the major isoenzyme of GS found in photosynthetic tissues (O'Neal and Joy, 1973). The presence of the enzyme in the chloroplast of mesophyll cells has been confirmed by immuno-localization in many species including tomato, soybean, tobacco and potato (Carvalho *et al.*, 1992; Dubois *et al.*, 1996). In maize, the chloroplastic form is localized in both the mesophyll and bundle sheath cells (Yamaya and Oaks 1988; Becker *et al.*, 1993b). However, in the majority of gymnosperms, which include conifers, there is no evidence of GS being present in the chloroplasts, suggesting that ammonia assimilation takes place in the cytoplasm (Avila *et al.*, 2001). The chloroplastic GS has also been detected in the roots of many species (for review see Hirel and Lea, 2003). GS2 is an octameric protein, with a native molecular mass of 350- to 400-kDa and a subunit mass of 43- to 45-kDa (Lea

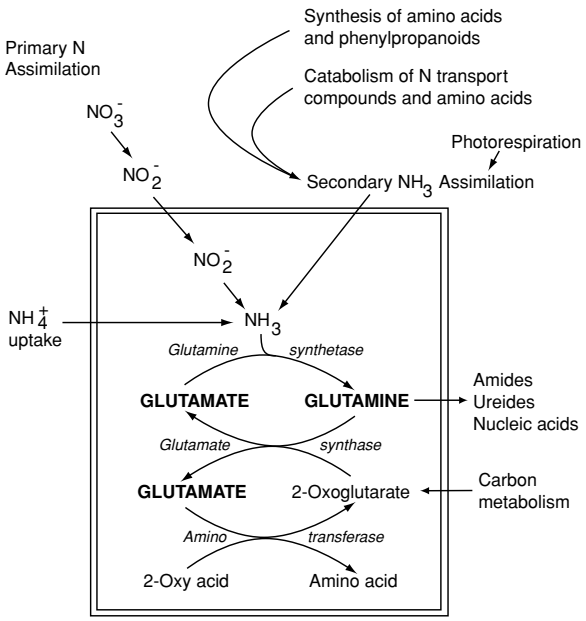


*Fig. 1.* The assimilation of ammonia through the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, showing the reactions taking place in the plastid inside the square box.

*et al.*, 1990; Woodall *et al.*, 1996). After isoelectric separation or SDS-gel electrophoresis, plastidic GS subunits may be represented by several polypeptides differing in their charges or size (Hirel *et al.*, 1984; Valpuesta *et al.*, 1989).

Phosphorylation and glycosylation of GS2 have been reported, although the significance of these modifications remains unclear. 5-Aminoimidazole-4-carboxiamide ribonucleoside (AICAR), a compound known to directly interfere with 14-3-3-binding to phosphoproteins having the 14-3-3-binding motif, was used as an in situ tool to identify 14-3-3-regulated metabolic steps in barley leaves (Athwal *et al.*, 2000, Huber *et al.*, 2002). Such experiments pointed to GS2 and mitochondrial glycine decarboxylase as 14-3-3-mediated control points of the N-assimilation and photorespiratory pathways, respectively (Man and Kaiser, 2001). The extractable GS2 activity was rapidly increased in response to AICAR, which also prevented protein degradation. GS2 in barley has a potential phosphorylation site and 14-3-3 binding site (Sehnke *et al.*, 2000). Riedel *et al.* (2001) showed an interaction of the tobacco octameric chloroplastic GS isoform with 14-3-3 proteins. Only GS2 that was strongly associated with 14-3-3 proteins was catalytically active. Modifications of the protein might be involved in its turnover during senescence, in response to light-to dark transition

or to the ATP/AMP ratio (Finnemann and Schjoerring, 2000; Comparot *et al.*, 2003). Kingston-Smith (2001) suggested that the lack of binding of 14-3-3 proteins in senescing leaves permits remobilization of nutrients within the plant and survival during transient nutrient stress such as carbohydrate limitation during senescence. The possibility that 14-3-3 proteins control the proteolytic degradation of their binding partners, as part of a sugar-sensing mechanism, has been suggested. The 14-3-3 protein isoforms were detected in the chloroplast stroma and the stromal side of the thylakoid membrane (Sehnke *et al.*, 2000). Whether 14-3-3 proteins regulate GS inside the chloroplast, or whether they support the import of GS into plastids in the manner described by Soll's group (May and Soll, 2000) is not known. It is interesting to note the similarity of regulation between a cytosolic enzyme such as nitrate reductase (NR), the chloroplastic ATP synthases, and GS. If the factors involved in the regulation are similar, a means to coordinate crosstalk between mitochondrial, cytosolic and chloroplastic C/N switches in response to factors such as oxygen or light deprivation may exist (Bunney *et al.*, 2001).

During the purification of GS to characterize its regulation by 14-3-3 proteins, a consistent 3- to 4-fold increase in total GS activity was observed in purified fractions as compared with the crude extract (Moorhead *et al.*, 2003). This apparent activation of GS was shown to be due to the separation of GS from a protein that was identified as a nucleotide pyrophosphatase. The latter protein catalyzed the hydrolysis of NADH, FAD and ATP. The inhibition of GS can be explained by the hydrolysis of Mg-ATP to 5′-AMP, which is a GS inhibitor. The nucleotide pyrophosphatase was reported to have identical properties to the NR-interfering protein that has nucleotide pyrophosphatase activity. GS from the stroma of spinach chloroplasts was also shown to be a target for chloroplastic thioredoxins *m* and *f* (Motohashi *et al.*, 2001; Balmer *et al.*, 2003). GS activity decreases when oxidized and is activated by thioredoxin in green algae (Florencio *et al.*, 1993). Less documented is the idea that modification of the holoenzyme structure from an octameric form to a tetrameric form may be a means to control GS2 activity during leaf ontogeny (Mack and Tischner, 1994).

GS2 is encoded by one nuclear gene per haploid genome. The deduced amino acid sequence of the protein includes a chloroplast-targeting transit peptide and the precursor is imported after translation in the cytoplasm (Tingey *et al.*, 1988). cDNAs and genes encoding the chloroplastic form of GS have been isolated from many species (Hirel and Lea, 2003). Fusion of a reporter gene to the GS2 promoter has shown that GS2 is predominantly expressed in photosynthetically active leaves (Edwards *et al.*, 1990). Mutants lacking GS have demonstrated the importance of the enzyme in photorespiratory ammonium assimilation as well as in the control of photosynthesis under drought stress (Häusler *et al.*, 1994; Wingler *et al.*, 2000).

In leaves, regulation of chloroplastic ammonia assimilation and reassimilation is directly related to plastid functionality, photorespiration and photosynthesis (Tobin *et al.*, 1985). A lowering of temperature, which caused a reduction in chloroplast photosynthetic activity, caused a reduction in GS2 activity (Woodall *et al.*, 1996). On the other hand, increases in temperature and irradiance favor oxygenation of RuBP and hence the flux through the photorespiratory pathway (Häusler *et al.*, 1994). During senescence, the degeneration of chloroplasts and concomitant loss of photosynthetic function coincide with a loss of GS2, and ammonia assimilation switches from the chloroplast to the cytosol (Masclaux *et al.*, 2000). Light is a key factor in the regulation of GS2 at the transcriptional and at the post-transcriptional level (Ireland and Lea, 1999). GS2 activity increases following dark-to-light transitions and during illumination of etiolated leaves (Yamaya *et al*., 1992). Light has been suggested to exert its effect indirectly through changes in photosynthetic carbon metabolites. Light-induction of chloroplastic GS2 could be mimicked by sucrose, fructose and glucose metabolites in *A. thaliana* (Oliveira and Coruzzi, 1999). The induction of mRNA for GS2 by light is mediated in part by phytochrome and in part by light-induced changes in levels of sucrose (Oliveira and Coruzzi, 1999). Analysis of reporter gene fusions with pea and *Phaseolus vulgaris* GS2 promoters in tobacco and *A. thaliana* allowed identification of conserved *cis*-acting elements involved in light regulation (Tjaden *et al.*, 1995).

The control of GS2 by nitrogen appears to be species-specific, with different responses to N availability being demonstrated. Ammonia enhances chloroplastic GS transcription in tobacco and rice (Hirel *et al.*, 1987; Kozaki *et al.*, 1992) but does not have an effect on the activity level. Nitrate enhances GS2 transcription in tobacco and maize after nitrogen starvation (Sakakibara *et al.*, 1992b; Migge and Becker, 1996; Lancien *et al.*, 1999). GS2 is preferentially accumulated in maize leaf mesophyll cells in response to

nitrate, whereas it is preferentially accumulated in the bundle sheath cells during the greening period of etiolated seedlings. This suggests that nitrate assimilation takes place in the mesophyll cells whereas the re-assimilation of the ammonia released during photorespiration takes place in the bundle sheath cells of maize (Sakakibara *et al.*, 1992a). When maize roots were treated with nitrate, the plastid GS levels rapidly increased after 30 minutes followed by a decline after 8 hours. The response to nitrate could be detected at concentrations as low as 10 μM. In tobacco roots, expression of the gene for plastid GS showed a transient increase in response to both nitrate and ammonia treatment, after a period of nitrogen starvation (Lancien *et al.*, 1999). The number of GS polypeptides in the plastids of roots of 7-d-old barley seedlings changed during root development and in response to $NO_3^-$ and $NH_4^+$ (Peat and Tobin, 1996). Glutamate was reported to have an inductive effect on GS2 gene expression in radish protoplasts (Watanabe *et al.*, 1997). However, the extreme conditions (50 mM glutamate) may have led to the induction of senescence-related genes. In *A. thaliana,* glutamate, glutamine and aspartate have an antagonistic effect on a sucrose induction of GS2 mRNA levels in the dark. These dark-mediated effects are exerted at the level of transcription, as amino acids have been shown to antagonize the sucrose induction of a GS2 promoter-ß-glucuronidase (GUS) gene construct (Oliveira and Coruzzi, 1999). The response of GS expression to carbon and amino acids is also reflected by changes in the levels of GS enzyme activity. The sucrose-induced GS gene expression antagonized by amino acids is similar to previous studies on NR and NiR genes in maize and tobacco (Vincentz *et al.*, 1993; Sivasankar *et al.*, 1997). Matt *et al.* (2002) showed that GS2 transcript levels and enzyme activities in tobacco plants are highest at the end of the light period and the first part of the dark period, suggesting that the GS2 mRNA transcription is under circadian control.

## III. Synthesis of Glutamate

### *A. Ferredoxin-Glutamate Synthase (GOGAT)*

Glutamate synthase, glutamine (amide): 2-oxoglutarate aminotransferase or GOGAT, catalyzes the reductant-dependent conversion of glutamine and 2-oxoglutarate to two molecules of glutamate (Fig. 1). Two different forms of GOGAT are present in plastids from higher plants, one that uses Fd as a source of reductant (Fd-GOGAT, EC 1.4.7.1) and the other that uses NADH (NADH-GOGAT, EC 1.4.1.14). Fd-GOGAT is the major enzyme for glutamate synthesis in photosynthetic tissues, whereas the NADH-GOGAT is predominant in non-photosynthetic tissues. Fd-GOGAT was first isolated from pea leaves (Lea and Miflin, 1974) and can represent up to 1% of total leaf protein (Marquez *et al.*, 1988). The enzyme has been shown to be dimeric in rice and monomeric in other plants (Ireland and Lea, 1999). Its molecular mass ranges between 115-kDa for the enzyme from rice to 145- to 180-kDa for the protein from other species. The enzyme in spinach contains one flavin mononucleotide and one [3Fe-4S] cluster per molecule (Hirasawa *et al.*, 1996). Using immunogold localization, Fd-GOGAT was found in chloroplast stroma of mesophyll, xylem parenchyma and epidermal cells in tomato (Botella *et al*., 1988). In maize, Fd-GOGAT was localized predominantly in the bundle sheath chloroplasts (Becker *et al.*, 1993b, 2000). In barley leaves, Fd-GOGAT is present in the chloroplasts of mesophyll and vascular tissue (Tobin and Yamaya, 2001). Fd-GOGAT was found in the chloroplasts of all the pine species investigated (Suárez *et al.*, 2002). Fd-GOGAT was also detected in root plastids (Tobin and Yamaya, 2001). Fd is present in roots, and mechanisms for the supply of reductant via the oxidative pentose-phosphate pathway have been proposed (Esposito *et al.*, 2003). In rice, Fd-GOGAT activity was highest in the youngest root cells in the tip and then decreased as the cells matured towards the root base. In the younger tissue, the enzyme was present in all cell types, but in older tissues it was only in the central cylinder. The application of ammonium ions did not affect the distribution of the Fd-GOGAT protein (Tobin and Yamaya, 2001). An involvement of thioredoxin in the activation of Fd-GOGAT has been proposed (Lichter and Haberlein, 1998). To investigate whether GOGAT itself is subject to post-translational regulation, novel methods are being developed for the assay of GOGAT (Stitt *et al.*, 2002).

The first cDNA sequence for Fd-GOGAT from maize was reported by Sakakibara *et al.* (1991). cDNAs clones were subsequently isolated from a number of species including maize, barley and *A. thaliana* (Suzuki *et al.*, 2001). The maize cDNA encodes a polypeptide of 1,616 amino acids, including a transit peptide sequence of 97 amino acids (Sakakibara *et al.*, 1991). Most early reports indicated that Fd-GOGAT is encoded by one nuclear gene, although Coshigano *et al.* (1998) showed

that *A. thaliana* has two genes, *GLU1* and *GLU2*. Evidence for two genes was also found with barley (Avila *et al.*, 1993). In *A. thaliana, GLU1* is preferentially expressed in leaves under photorespiratory conditions, whereas *GLU2* is predominant in roots. The low abundance of *GLU2* mRNA, particularly in leaves, may explain why the second gene has not been found in other species (Coshigano *et al.*, 1998). Histochemical analysis of an *A. thaliana GLU1* promoter in *Nicotiana tabacum* revealed that GUS expression is associated with mesophyll and vascular tissue of 14-day-old tobacco seedlings (Ziegler *et al.*, 2003). These expression analyses as well as T-DNA insertion mutations support a role for Fd-GOGAT in photorespiratory ammonium cycling, primary ammonium assimilation, and glutamate synthesis for intracellular transport of glutamine and glutamate (Ferrario-Mery *et al.*, 2001).

Illuminated pea chloroplasts carry out the GOGAT reaction (Lea and Miflin, 1974) and also support $O_2$ evolution in the presence of glutamine plus 2-oxoglutarate (Anderson and Walker, 1983) or ammonia plus 2-oxoglutarate (Woo and Osmond, 1982). The $O_2$ evolution was attributed to light-coupled GOGAT activity and the combined reaction of both GS and GOGAT. A substantial proportion of Fd-GOGAT activity is detectable in etiolated tissues, and light-inducibility was shown for several species (Suzuki and Rothstein, 1997). In pine seedlings, although Fd-GOGAT activity was increased during germination, dark or light had no effect on this activity (Garcia-Gutierrez *et al.*, 1995, 1998). The most pronounced stimulation of Fd-GOGAT by light occurred at the mRNA level in both maize and tobacco (Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992). In *A. thaliana*, the level of *GLU1* mRNA increased in response to light or sucrose. The effect of light on *GLU1* could be partially replaced by sucrose (Coschigano *et al.*, 1998). On the other hand, GOGAT activity decreased when the concentration of sugar was lowered in tobacco (Stitt *et al.*, 2002). The induction of enzyme activity was suggested to be a phytochrome-mediated response (Becker *et al.*, 1993a), which also involves a specific blue/UV-A light receptor (Migge *et al.*, 1998).

Fd-GOGAT activity increased in maize in response to addition of nitrate and ammonium ions (Sakakibara *et al.*, 1992b). Expression of *GLU1* was stimulated by nitrate in tobacco (Scheible *et al.*, 1997). An interaction between light and N sources was demonstrated with maize leaves, where Fd-GOGAT activity and amount of the polypeptide increased 3- to 5-fold following transfer of etiolated seedlings to medium containing nitrate or ammonia in the light but not in the dark (Suzuki *et al.*, 1996). A corresponding 5-fold increase in mRNA encoding the enzyme was also detected under the same conditions. In tobacco leaves, a small decrease in the expression of the genes encoding chloroplastic Fd-GOGAT and GS following N-starvation was observed, but the effect was much less marked than for both NR and NiR. Both GS and GOGAT mRNA levels were restored by the application of nitrate and glutamine, while ammonia or glutamate only increased Fd-GOGAT mRNA (Migge and Becker, 1996). In soybean cotyledons or leaves, in tomato seedlings and in tobacco leaves, nitrate and ammonium did not have such an effect on nitrogen-starved plants (Migge *et al.*, 1998; Lancien *et al.*, 1999; Turano and Muhitch, 1999). Growing tobacco plants under non-photorespiratory conditions (elevated $CO_2$) had no effect on Fd-GOGAT mRNA or protein synthesis (Migge *et al.*, 1997).

### *B. NADH-Glutamate Synthase (GOGAT)*

NADH-GOGAT is the predominant form of this activity in non-photosynthetic tissues of higher plants. In green leaves, this activity is low in comparison to Fd-GOGAT (Wallsgrove *et al.*, 1982; Hecht *et al.*, 1988). NADH-GOGAT was purified from rice suspension-culture cells and root nodules and shown to be a monomer with a molecular mass of 196- to 200-kDa. Tissue print immunoblots utilizing specific antisera indicated that NADH-GOGAT is located in the large and small vascular parenchyma cells (metaxylem and metaphloem parenchyma cells) and mestome sheath cells of the young leaf blade before emergence (Hayakawa *et al.*, 1994). In rice roots, the NADH-GOGAT immunogold-labeling density was high in the plastids of cells of the epidermis and exodermis, cortical parenchyma and vascular parenchyma (Hayakawa *et al.*, 1999). In rice, NADH-GOGAT is active in developing organs, such as unexpanded non-green leaves and developing grains (Yamaya *et al.*, 1992). Hayakawa *et al.* (1994) hypothesized that NADH-GOGAT is probably involved in the utilization of remobilized nitrogen, because the enzyme is located in the specific cell types that are important for solute transport from the phloem and xylem elements.

NADH-GOGAT cDNA and genes have been isolated from alfalfa, *A. thaliana* and rice (S. Goto *et al.*, 1998; Trepp *et al.*, 1999; Lancien *et al.*, 2002). A 99- to 101-amino acid presequence was identified in rice and alfalfa proteins (S. Goto *et al.*, 1998). A T-DNA knockout mutation in *A. thaliana* indicated a role for the enzyme

in ammonium assimilation under non-photorespiratory conditions (Lancien *et al.*, 2002).

NADH-GOGAT activity increased in response to nitrate treatment after nitrogen starvation in tobacco (Lancien *et al.*, 1999). Addition of nitrate, ammonia, glutamine or asparagine to rice cells or wheat suspension-culture cells induced an accumulation of NADH-GOGAT mRNA and protein (Watanabe *et al.*, 1996). In rice cell cultures, induction by ammonium occurred at the transcriptional level and was mimicked by okadaic acid, an inhibitor of phosphatases (Hirose and Yamaya, 1999). In maize, the activity increased when nitrogen-starved rice seedlings were transferred to medium containing 1 mM ammonium chloride (Yamaya *et al.*, 1995). In soybean, the enzyme activity in the roots increased 14-fold following the addition of ammonium salts to nitrogen-starved seedlings and 7-fold with potassium nitrate. Smaller increases were detected at the protein and mRNA level (Turano and Muhitch, 1999). A putative role for NADH-GOGAT in the export of glutamine to the phloem in senescing tissues and roots reflects its expression in vascular tissues. NADH-GOGAT was rapidly induced in roots in nitrate-supplied or nitrate-deficient medium, which indicated that the endogenous level of nitrate is normally low as the result of rapid reduction of absorbed nitrate (X. Wang *et al.*, 2002).

### *C. Sources for Primary Nitrogen Assimilation*

As revealed by the presence of the two main routes for glutamate and glutamine synthesis in the plastids, a provision of nitrogen, energy, reducing power as well as carbon skeletons is necessary for the functioning of the pathway. The pattern of nitrogen flow in plant cells depends on the availability of carbon and nitrogen, the tissue and plant concerned, and a variety of environmental conditions. There is a heterogeneity between plastids from different sources, which reflects the differences in anabolic and catabolic processes that can occur simultaneously (Emes and Neuhaus, 1997). Fluxes through biochemical pathways need to be coordinated. To unravel the interconnection between pathways, methods for broad metabolite profiling combined with genomics and proteomics analyses are currently being developed (Stitt and Fernie, 2003).

Nitrogen assimilation is integrated with respiratory activity and causes mobilization of carbon. Carbon flow must be regulated to ensure sufficient supply of organic acids for amino acid biosynthesis in the plastid. Amino acid synthesis places a heavy demand for organic carbon as glycolate from photorespiration in photosynthetic tissues and from glycolysis and the tricarboxylic acid cycle in non-photosynthetic and photosynthetic tissues (Bowsher *et al.*, 1992). Nitrogen assimilation also depends on $CO_2$ concentration and carbohydrate status. The proportion of fixed carbon required for nitrogen assimilation will change according to developmental stage, nitrogen availability and the nature of the product from ammonia assimilation. Numerous studies have indicated that the rate of net photosynthesis and the amount of photosynthetic components are correlated with the nitrogen content of the leaf (Kumar *et al.*, 2002). High nitrogen increases the number of chloroplasts per mesophyll cell as well as the protein density (predominantly Rubisco) in the stroma (Sivasankar *et al.*, 1998). The relationship between the components of the photosynthetic system may change over the range of nitrogen content, reflecting adaptation of the photosynthetic system. Interestingly, decreased expression of Rubisco in tobacco caused a decrease in photosynthesis rates and an inhibition of nitrate and ammonium assimilation as well as an inhibition of amino acid biosynthesis (Stitt *et al.*, 2002).

2-Oxoglutarate is required for glutamate synthesis and is a key organic acid for plant ammonium assimilation. The demand for this carbon skeleton varies according to the tissue, age of the tissue, time of day and other factors. This organic acid can originate as a net product from the reaction catalyzed by isocitrate dehydrogenases (Lancien *et al.,* 2000), but the enzymatic origin of 2-oxoglutarate is still a matter of discussion (Lancien *et al.*, 2000; Hodges *et al.*, 2003). Through these enzymes, 2-oxoglutarate can be either synthesized inside the plastid or outside within the mitochondria or the cytosol. It can also be produced via transamination reactions in the photorespiratory pathway or by aminotransferases in the plastid (Lancien *et al.*, 2000). It has been argued that at high reduction levels of $NADP^+$ and $NAD^+$ observed at limiting $CO_2$ in the light, i.e., when photorespiratory glycine is the main mitochondrial substrate, isocitrate oxidation in mitochondria via $NAD^+$-dependent isocitrate dehydrogenase is suppressed (Igamberdiev and Gardestrom, 2003). Under photorespiratory conditions, citrate would have to be transported to the cytosol, where the cytosolic $NADP^+$-isocitrate dehydrogense supplies 2-oxoglutarate for the photorespiratory ammonia refixation. Other studies using gene expression analysis in response to nitrate and ammonia, and the analysis of potato and tobacco mutants for cytosolic isocitrate dehydrogenase, have underlined the flexibility of the system for 2-oxoglutarate supply in plants. Results of these

studies suggested a role of the mitochondrial $NAD^+$-dependent isocitrate dehydrogenase in the supply of 2-oxoglutarate for ammonia assimilation (Lancien *et al.*, 1999, 2000; Igamberdiev and Gardestrom, 2003). Because the precursor for ammonia assimilation is synthesized in the cytosol and/or in the mitochondria, plastids rely on a transport system for the import of 2-oxoglutarate into the plastids and for the export of the product of ammonia assimilation, glutamate, from the plastids into the cytosol. The chloroplast envelope membranes contain at least two distinct dicarboxylate transporters with partially overlapping substrate specificities, the 2-oxoglutarate/malate-translocator (DiT1) and the glutamate/malate-translocator (DiT2) (Weber and Flugge, 2002). DiT1 is specific for dicarboxylates (malate, succinate, fumarate, glutarate and 2-oxoglutarate), whereas DiT2, in addition to the substrates transported by DiT1, also accepts the amino acids glutamate and aspartate. A two-translocator model for the transport of 2-oxoglutarate and glutamate was proposed that explained the stimulation of 2-oxoglutarate transport into isolated chloroplasts and the stimulation of the (ammonia, 2-oxoglutarate)-dependent oxygen evolution in isolated chloroplasts by malate (Woo and Osmond, 1982). According to this model, 2-oxoglutarate is imported into the plastid in counter-exchange with malate by DiT1 and is subsequently converted to glutamate by GS/GOGAT. Glutamate is then exported to the cytosol by DiT2, again in counter-exchange with malate. The result is a counter-exchange of 2-oxoglutarate with glutamate without net malate transport (Weber and Flugge, 2002).

Recent analysis of an *A. thaliana* T-DNA insertional mutant in the chloroplastic 2-oxoglutarate/glutamate transporter AtpDCT1 revealed that disruption of the transporter caused a photorespiration-deficient phenotype. The AtpDCT1 mutant was non-viable in normal air but grew normally in high $CO_2$ (Taniguchi *et al.*, 2002). This finding suggested that AtpDCT1 is a necessary component for photorespiratory nitrogen recycling. A small gene family encoding malate/2-oxoglutarate transporters was described in *A. thaliana* (Renne *et al.*, 2003). Mutant AtpOMT1, with an insertion in a malate/2-oxoglutarate transporter gene, did not show a photorespiratory-deficient phenotype. This observation suggested that the knockout of only one transporter is not sufficient for a phenotype (Taniguchi *et al.*, 2002). Renne *et al.* (2003) presented a model for maize in which both glutamate/malate DiT1 and DiT2 transporters, expressed in bundle sheath of maize cells, are involved in ammonia reassimilation resulting from photorespiration, which occurs in the bundle sheath cells in $C_4$ plants. These transporters were proposed to function also in primary ammonia assimilation that works as a two-step process, spatially separated between bundle sheath cells and mesophyll cells. Nitrate is reduced to ammonia and converted into glutamine in mesophyll cells. Glutamine is transported to the bundle sheath cells and taken up into the plastids by the glutamine/glutamate translocator (Yu and Woo, 1988). One molecule of glutamate is shuttled back to the mesophyl cells and DiT2 exports the other one (Renne *et al.*, 2003).

The main function of the oxidative pentose-phosphate pathway (OPPP) in plastids is to supply reductant equivalents (NADPH) for nitrite reduction, ammonia assimilation, amino acid and fatty acid synthesis (Bowsher *et al.*, 1992). The requirement for photosynthetic energy is reflected by the marked stimulation of nitrogen assimilation by light in many species. In photosynthetic cells, 80% of the reductant required for nitrogen assimilation comes directly from Fd, where photochemically-reduced Fd reduces $NADP^+$ to NADPH. The mitochondria play an important role to prevent over-excitation of chloroplasts in limiting $CO_2$, which intensifies under high light. As discussed in the 2-oxoglutarate production section, accumulation of reductant can affect 2-oxoglutarate and lead to the accumulation of glutamine and ammonia in the chloroplast, hence the need exists for an efficient system to oxidize excess reductant. The mitochondrially localized steps in photorespiration provide strong support for chloroplast photosynthesis at both limiting and optimal $CO_2$ concentrations. Under limiting $CO_2$ conditions, the mitochondrial oxidation of glycine ensures not only the dissipation of excess redox equivalents from chloroplasts but also the regeneration of significant amount of glycerate for re-entry to the Calvin-Benson cycle (Raghavendra and Padmasree, 2003). In roots, during the induction of nitrate assimilation, there is an increase in the plastidial content of Fd and Fd-$NADP^+$ reductase (FNR) (Bowsher *et al.*, 1993) and in the activity of the two oxidative reactions of the plastidial OPPP, glucose 6-phosphate dehydrogenase (Glc6PDH) and 6-phosphogluconate dehydrogenase (Bowsher *et al.*, 1992). The OPPP generates NADPH, which is used to reduce Fd by FNR in the reverse direction of the reaction in photosynthesis. Glucose 6-phosphate can support nitrite reduction in pea root plastids and a close link between Glc6PDH and glutamate synthesis was shown in root plastids (Esposito *et al.*, 2003). A competition between metabolic processes for reductant from the OPPP could limit the flux through pathways such as nitrite reduction, ammonia

assimilation and glutamate synthesis in root plastids (Emes and Neuhaus, 1997; Bowsher and Tobin, 2001). This is the case during the pathway of carbohydrate oxidation within the amyloplast, which represents a drain on the flux of carbon to starch when there is a demand for reducing power by GOGAT during amino acid production (Tetlow *et al.*, 1996).

Nitrite, ammonia and glutamate are the major nitrogen sources in plastids for glutamate and glutamine biosynthesis. During photorespiration, ammonia is released following glycine decarboxylation in the mitochondria and is transported to the chloroplast where it is used by the GS/ GOGAT pathway for primary amino acid synthesis. Ammonia can be produced from nitrate reduction and inorganic nitrogen absorption from the soil in roots. Plants maintain a highly sensitive nitrate detection system to control enzymes for nitrate metabolism (Scheible *et al.*, 1997; R. Wang *et al.*, 2003). A retrieval system exists in the chloroplast envelope to recover ammonia that has been produced by photorespiration, but chloroplastic ammonia transporters have yet to be identified in the plastid membrane (Williams and Miller, 2001). It was also proposed that ammonia can cross the membrane through a potassium channel and aquaporin (Williams and Miller, 2001). Nitrite produced via nitrate reduction is also transported into the chloroplast. It is unclear if a plastid membrane nitrite transporter family exists in higher plants, although such nitrite transporters are thought to exist (Galvan *et al.*, 2002). Orthologs to algal nitrite transporters are difficult to identify in plants because of limited sequence conservation. Furthermore, the function of nitrite transport to the plastid could be carried out by proteins unrelated to the formate-nitrite transporter family described in algae (Galvan *et al.*, 2002).

Photorespiration involves a cycling of nitrogen and carbon metabolites between the mitochondria, peroxisomes and chloroplasts. Glutamate produced by GS/GOGAT pathway can be used by the photorespiratory cycle to regenerate organic acids for the pathway. Recently, a peroxisomal alanine:2-oxoglutarate aminotransferase (AOAT) with glutamate:glyoxylate aminotransferase (GGAT) activity was knocked out in an *A. thaliana* mutant *aoat1-1*. The mutant had a phenotype resembling that of a photorespiratory-deficient mutant (Igarashi *et al.*, 2003). In air, glutamate and serine levels increased, whereas only small differences were observed in high $CO_2$. This suggested a major role of the enzyme in photorespiration and amino acid metabolism.

In experiments monitoring amino acids levels in response to nitrogen or metabolite feeding, glutamine levels often rise or fall but 2-oxoglutarate and glutamate remain unaltered (Stitt and Fernie, 2003). However, glutamate levels can fluctuate strongly in photorespiratory or metabolic mutants that lack Fd-GOGAT, GS2 and GGAT and NADH-GOGAT. These observations suggest that the flow of reduced nitrogen into metabolism is regulated downstream of glutamine and pinpoint GOGAT and/or glutamate dehydrogenase (which catalyses the reversible reaction of glutamate reduction into 2-oxoglutarate) as targets, as well as the transporters of glutamate and 2-oxoglutarate. Fluctuation in the glutamine content may reflect the balance between ammonia and 2-oxoglutarate availability (Ferrario-Mery *et al.*, 2001). Yu and Woo (1988) characterized a specific glutamine translocator from spinach and oat chloroplasts, which was different from the dicarboxylate translocator. The putative glutamine/glutamate translocator was suggested to act in combination with both DiT1 and DiT2 to export glutamine in exchange for 2-oxoglutarate, with no net glutamate and malate transport. This model would provide the cytosol with glutamine as a precursor for biosynthesis of asparagine, for example. The coupling of glutamine export from plastids to the availability of glutamate in the cytosol has been proposed to act as a control mechanism circumventing glutamine-depletion of the GS/GOGAT-cycle (Weber and Flugge, 2002).

In *Escherichia coli*, nitrogen metabolism is controlled via GS activity at both the transcriptional and post-transcriptional levels and involves glutamine and 2-oxoglutarate sensing by a PII protein (Jiang *et al.*, 1998). In plants, PII is as a chloroplast protein (Moorhead and Smith, 2003; Smith *et al.*, 2003). The contribution of PII to nitrogen signaling and its interacting partners remain to be determined in plants. PII in *A. thaliana* is transcriptionally up-regulated by light and sucrose and down regulated by glutamate and glutamine (Hsieh *et al.*, 1998). In cyanobacteria, PII clearly plays a role in the uptake of nitrate and nitrite (H.M. Lee *et al.*, 2000), and it has been speculated that PII may play a similar role in plants. In the presence of bound ATP, the plant PII binds 2-oxoglutarate with a $K_d$ close to the organic acid concentration in the chloroplast (Kamberov *et al.*, 1995; Smith *et al.*, 2003), whereas 2-oxoglutarate prevented binding of ADP to the protein (Smith *et al.*, 2003). Moorhead and Smith (2003) suggested that this could allow the plant PII to be a precise sensor of fluctuations in the concentration of 2-oxoglutarate, a signal molecule for carbon status. They suggested that the PII signaling system may include further subtleties to monitor and coordinate

cellular energy status with nitrogen assimilation. However, further work is needed to provide physiological evidence that PII senses and interprets N, C and energy metabolism in plastids.

## IV. The Aspartate Pathway

The essential amino acids, lysine, threonine, methionine and isoleucine are synthesized via a common pathway (Fig. 2). Aspartate is the starting compound, which is formed by transamination of oxaloacetate derived from the tricarboxylic acid cycle in the mitochondria or through operation of phospho*enol*pyruvate carboxylase or phospho*enol*pyruvate carboxykinase in the cytoplasm (Leegood and Walker, 2003). Early detailed localisation studies and the feeding of radioactive precursors clearly indicated that illuminated chloroplasts had the capacity to carry out all the reactions shown in Fig. 2, except the final step of methylation of homocysteine to form methionine (Mills *et al.*, 1980; Wallsgrove *et al.*, 1983). As will be seen in the later discussion, it is encouraging to those of us involved in the early experiments, to learn that the encoding of N-terminal, chloroplast-targeting, transit peptides has been confirmed in the cDNA sequences of all the enzymes so far studied.

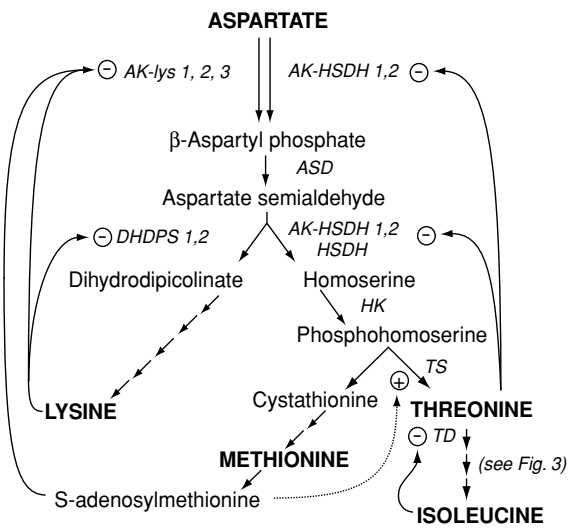


*Fig. 2.* The biosynthetic pathway of the aspartate-derived amino acids. All the reactions except the final step in the synthesis of S adenosylmethionine take place in the plastids. AK, HSDH, DHDPS and TD represent the major points of feedback inhibition (−) by the end products lysine, threonine, S adenosylmethionine and isoleucine. TS is stimulated (+) by S adenosylmethionine. AK, aspartate kinase; ASD, aspartate semialdehyde dehydrogenase; HK, homoserine kinase; HSDH homoserine dehydrogenase; DHDPS, dihydrodipicolinate synthase; TS, threonine synthase; TD, threonine deaminase. The numbers represent the different isoenzymes and probable genes for each protein. Adapted from Rognes *et al.* (2003) and generously supplied by Dr. Sven Rognes.

### *A. Aspartate Kinase (AK) and Homoserine Dehydrogenase (HSDH)*

Aspartate kinase (AK, EC 2.7.2.4) catalyses the ATP-dependent formation of β-aspartyl phosphate, which is required for the synthesis of all four essential amino acids. Aspartyl phosphate is converted to aspartate semialdehyde by aspartate semialdehyde dehydrogenase (EC 1.2.1.11) in an NADPH-dependent reaction (Paris *et al.*, 2002a). Aspartate semialdehyde is at the first branch point in the pathway and is a substrate for either homoserine dehydrogenase or dihydrodipicolinate synthase (Fig. 2). Homoserine dehydrogenase (HSDH, EC 1.1.1.3) catalyses the reduction of aspartate semialdehyde to form homoserine.

AK was purified and characterised from a range of plant species, although initially there were difficulties in obtaining absolutely pure preparations (Azevedo *et al.*, 1997; Matthews, 1999; Azevedo and Lea, 2001; Azevedo, 2002). Two distinct forms of AK were separated by standard chromatographic methods, one of which is sensitive to feedback inhibition by lysine and one by threonine. Although the ratio of lysine-sensitive AK to threonine-sensitive AK activities varies with plant tissue and developmental age, the lysine-sensitive form is normally predominant in higher plants. However, the threonine-sensitive AK is more active in soybean cotyledons and callus culture and in *Coix lacryma-jobi* seeds (Azevedo and Lea, 2001). Following an initial suggestion from Aarnes and Rognes (1974), it became apparent that threonine-sensitive AK and threonine-sensitive HSDH co-purified and are activities present on the same protein (Matthews, 1999). The activities of both lysine- and threonine-sensitive AK and HSDH are located in the chloroplast (Wallsgrove *et al.*, 1983).

Lysine-sensitive AK is present as a monofunctional protein and has a $K_i$ for lysine of 200 to 600 μM. In addition, S adensosyl-methionine inhibits AK activity synergistically in the presence of lower concentrations of lysine (Rognes *et al.*, 1980). The importance of lysine-sensitive AK in the regulation of lysine synthesis is still not clear, as it is well established that DHDPS is more sensitive to feedback inhibition by lysine (see later section). However, a series of mutants was isolated that contain lysine-insensitive forms of AK and high concentrations of soluble threonine. A detailed

analysis of mutants of barley and maize also indicated that two forms of lysine-sensitive AK were present that were under the regulation of distinct genes (Azevedo and Lea, 2001; Azevedo, 2002).

The mechanism of lysine inhibition of plant AK has not been clearly established, although some information is available from microbial systems. The ACT domain, an amino acid-binding domain named after bacterial AK, chorismate mutase, and TyrA (prephenate dehydrogenase), is a regulatory domain mostly found in enzymes directly or indirectly involved in amino acid metabolism (Chipman and Shaanan, 2001). The ACT domain is in the C-terminal sequence of all three *A. thaliana* lysine-sensitive AK enzyme proteins, but in the intermediate regulatory domain of both threonine-sensitive AK-HSDH proteins (Hsieh and Goodman, 2002). In bacteria, the regulation of the activity of AK by lysine and threonine is predominantly at the level of transcription, while there is little evidence for such a mechanism in plants.

Following over-expression in *E. coli* (Paris *et al.*, 2002b, 2003), the protein structure of *A. thaliana* AK-HSDH was shown to comprise a 66-amino acid N-terminal region, a 276-amino acid AK region, a 246-amino acid intermediary threonine regulatory domain and a 328-amino acid C-terminal HSDH region. The intermediary regulatory domain of threonine-sensitive AK-HSDH contains two similar sub-domains, each with a common loop-α-helix-loop-β-strand-loop-β-strand motif in an analogous manner to threonine deaminase (see later section). Paris *et al.* (2003) carried out a series of mutations of key glutamine residues, which had been proposed to be in the threonine binding region. Steady-state kinetic experiments on wild-type and mutant enzymes demonstrated that each regulatory domain of the monomers of AK-HSDH contained two non-equivalent threonine binding sites, constituted in part by Gln443 and Gln524. The results also demonstrated that interaction of threonine with Gln443 led to inhibition of AK activity and facilitated the binding of a second threonine on Gln524. Interaction of this second threonine with Gln524 led to inhibition of HSDH activity.

Two different cDNAs were initially isolated from *A. thaliana* that encode a mono-functional lysine-sensitive AK (Frankard *et al.*, 1997; Tang *et al.*, 1997). Yoshioka *et al.* (2001) screened a gene trap library of *A. thaliana* and isolated a third gene encoding a lysine-sensitive AK, designated as *AK-lys3*, which was highly expressed in the xylem of leaves and hypocotyls and stele of roots. Significant expression of this gene was also observed in trichomes after bolting. Slight expression of *AK-lys3* was detected in vascular bundles and mesophyll cells of cauline leaves, inflorescence stems, sepals, petals, and stigmas. These results indicated that the *AK-lys3* gene is not expressed uniformly but in a spatially specific manner.

At least two genes encoding bifunctional threonine-sensitive AK-HSDH were identified in several plants and in maize the number may be higher (Matthews, 1999). In *A. thaliana*, a gene encoding AK-HSDH was initially isolated by Ghislain *et al.* (1994). Zhu-Shimoni *et al.* (1997) studied the expression of the *A. thaliana* threonine-sensitive AK-HSDH gene using a series of promoter-GUS constructs introduced into tobacco. Expression of the AK-HSDH gene was confined to young tissues that contained actively growing cells, including apical meristems, lateral buds, young (but not mature) leaves, trichomes, vascular and cortical tissues of stems, developing anthers and ovaries, and maturing seeds. In a series of related investigations, Zhu-Shimoni and Galili (1997, 1998) demonstrated that light and sucrose stimulated the expression of the AK-HSDH-GUS constructs in transgenic tobacco cotyledons and of AK-HSDH mRNA in *A. thaliana*, while nitrogen in the form of glutamine, asparagine or ammonium nitrate had no effect on expression.

More recently a second gene encoding AK-HSDH was isolated and characterised from *A. thaliana* (Rognes *et al.*, 2003) and located on chromosome 4. The new gene has been named *akthr2* and the first gene isolated by Ghislain *et al.* (1994), which is located on chromosome 1, renamed as *akthr1*. Using a GUS expression system, Rognes *et al.* (2003) showed expression of *akthr2* in rapidly dividing and growing cells, e.g., vascular tissue, young leaves and reproductive organs. Some small differences in the expression of *akthr1* and *akthr2* were detected. In particular, the latter was not expressed during seed development and maturation. However, the two genes were simultaneously expressed in young leaves, pollen grains, style, stigma, carpels and ovarian tissues. Possible reasons for the apparent redundancy of the two genes are discussed by Rognes *et al.* (2003).

## B. Lysine Synthesis

The first enzyme unique to lysine synthesis, dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52), catalyses the condensation of pyruvate and aspartate semialdehyde to form 4-hydroxy-2,3,4,5-tetrahydrodipicolinate. DHDPS was purified and characterised from a range of plant species and shown to be very

sensitive to inhibition by low concentrations of lysine (Azevedo *et al.*, 1997; Azevedo and Lea, 2001). The crystal structure of DHDPS from *Nicotiana sylvestris,* with and without inhibitory lysine bound to the enzyme, was solved to a resolution of 2.8 Angstroms. The molecule is a homotetramer composed of two tightly bound dimers. Comparison with the structure of DHDPS from *E. coli,* which is 100-fold less sensitive to lysine, showed that a profound rearrangement of the dimers forming the tetramer occurs following the binding of lysine (Blickling *et al.*, 1997).

Genes encoding DHDPS were isolated from maize, *A. thaliana,* tobacco, soybean, poplar and wheat (Matthews, 1999). A range of mutagenized forms of maize DHDPS were isolated that contained single amino acid changes that reduced inhibition by lysine (Shaver *et al.*, 1996). Substitution of arginine for tryptophan at position 53, or asparagine for isoleucine at position 80 in mutant forms of *A. thaliana* and tobacco DHDPS, prevented inhibition of the enzyme by lysine. The possible structure of the lysine-binding allosteric site of plant DHDPS has been discussed in more detail by Vauterin *et al.* (2000).

Initially only one gene encoding DHDPS was isolated from *A. thaliana,* and the expression of promoter-GUS constructs in transformed *A. thaliana* and tobacco was found to be cell-type specific and mainly in fast growing tissues where protein synthesis rates were high (Vauterin *et al.*, 1999). However, more recently a second gene (*dhdps-2*) that exhibits 84% identity at the nucleotide level with the previously cloned *dhdp-1* cDNA was characterised by two independent laboratories (Craciun *et al.*, 2000; Sarrobert *et al.*, 2000). Both groups confirmed that the *dhdps-1* gene is located on chromosome 3, while the *dhdps-2* gene is on chromosome 2. The *dhdps-2* gene was expressed in *E. coli* to form a functional enzyme that was strongly inhibited by lysine, with a 50% loss of activity at 30 μM lysine. DHDPS-1, which is slightly more sensitive, is inhibited 50% by 10 μM lysine.

Craciun *et al.* (2000) observed that expression of the *dhdps-2* gene in *A. thaliana* was strikingly similar to that observed for the *dhdps-1* gene. In vegetative parts of the plants, GUS expression driven by the *dhdps-2* promoter was detected in meristem and vasculature tissues. Roots showed expression in the root tips, mainly in the elongation zone and in the meristems of emerging lateral roots. Expression of the *dhdps-2* gene was strongly detected in the vasculature of stems and leaves but only slightly in the mesophyll cells of the leaves. In reproductive organs, strong *dhdps-2* expression was observed in anthers, developing pollen, carpels, and developing seeds. Sarrobert *et al.* (2000) observed some differences in the expression of *dhdps-2,* which they showed to be predominant in the elongation zone of the root tips and pollen grains. No *dhdps-2*-promoter driven GUS activity was detected in the leaves or stems. The key question is whether the two enzymes encoded by the two genes play a different role in the growth of the plant. Knockout mutants of *A. thaliana* with insertions in the *dhdps-2* gene exhibit close to normal growth but have a reduced lysine and greatly increased threonine content (Craciun *et al.*, 2000; Sarrobert *et al.*, 2000).

Following the formation of dihydrodipicolinate, a further six enzymes are required for the synthesis of lysine. Some basic information is available concerning 2,3-dihydrodipicolinate reductase (EC 1.3.1.26) and *meso*-2,6-diaminopimelate decarboxylase (4.1.1.20), which are located in the chloroplast (Azevedo *et al.*, 1997; Matthews, 1999). Otherwise, the enzymes have been largely ignored by plant researchers. It was recently shown that expression of a gene with homology to diaminopimelate decarboxylase was increased following infection of roots of *A. thaliana* with the endoparasitic nematode, *Meloidogyne incognita* (Vercauteren *et al.*, 2001).

It is clear that synthesis of lysine in plants is very tightly regulated at the reaction controlled by DHDPS. Plants that accumulate high concentrations of lysine have only been obtained by mutation of the lysine-binding site of DHDPS or by the insertion of a new *dhdps* gene encoding a form of DHDPS less sensitive to lysine (Galili, 2002). AK is inhibited by concentrations of lysine 25-fold higher than concentrations that totally inhibit DHDPS. As both AK and DHDPS are plastid-located, it is difficult to see how lysine can reach concentrations that could affect the activity of the lysine-sensitive AK. However, there is evidence that lysine-sensitive AK does play a key regulatory role. When the flux through the aspartate pathways was disturbed, e.g., by mutation of lysine-sensitive AK (Bright *et al.*, 1982), insertion of an unregulated form of AK (Galili, 2002) or a reduction in DHDPS activity (Craciun *et al.*, 2000; Sarrobert *et al.*, 2000), threonine accumulated to a high concentration. Galili (2002) proposed that the enzymes and end-product amino acids of the aspartate pathway may not be uniformly distributed throughout the plastids. Such a hypothesis requires some form of compartmentalization within the stroma of the plastids, which could be achieved by physical separation or substrate channelling through multienzyme complexes.

### C. Threonine Synthesis

Homoserine kinase (HSK, EC 2.7.1.39) catalyzes the phosphorylation of homoserine and is required for the biosynthesis of threonine, isoleucine and methionine. The reaction is similar to that catalyzed by AK, with a difference in that the phosphoryl acceptor on homoserine is an alcohol rather than a carboxylic acid. This distinction results in a significant difference in the products. *O*-Phosphohomoserine is thermodynamically stable whereas β-aspartyl phosphate is not. HSK belongs to the GHMP superfamily, which includes **G**alactokinase, **H**omoserine kinase, **M**evalonate kinase and **P**hosphomevalonate kinase. Recent studies on the crystal structure of the bacterial enzyme revealed the presence of a highly specific homoserine binding site and a novel nucleotide binding site (Krishna *et al.*, 2000).

In plants, the enzyme is a dimer of approximately 75-kDa in mass and requires potassium ions for activity. No isoenzymes have been detected. Although evidence was presented that the enzyme activity may be inhibited by end-product amino acids, the concentrations used may not have been at physiological levels (Matthews, 1999). A gene encoding a 38-kDa HSK protein with a N-terminal transit peptide was isolated from *A. thaliana* and appears to be present in only one copy. An active enzyme protein was expressed in *E. coli*, which was not subject to regulation by end-product amino acids (M. Lee and Leustek, 1999).

Threonine synthase (TS, EC 4.2.99.2) is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the ultimate step in threonine biosynthesis, the βγ-replacement reaction of *O*-phosphohomoserine that yields threonine and inorganic phosphate. Amino acid sequence alignments revealed that TS can be grouped into a plant and fungal subfamily (Garrido-Franco *et al.*, 2002). The crystal structures of TS from *A. thaliana* (Laber *et al.*, 1999; Thomazeau *et al.*, 2001) and yeast (Garrido-Franco *et al.*, 2002) were established.

TS is not regulated by end-product feedback inhibition, but rather by a strong stimulation of TS activity by S adenosyl-methionine (SAM), a product of the methionine biosynthetic pathway. The allosteric binding of SAM leads to an 8-fold increase in the rate of catalysis and to a 25-fold decrease in the $K_m$ value for *O*-phosphohomoserine. Curien *et al.* (1998) concluded that the dramatic modification in kinetic properties of plant TS involved binding of at least two SAM molecules per dimeric enzyme and that the transition occurred at a much faster rate in the presence of the substrate. A potential binding site for SAM molecules was identified in the N-terminal domain of the TS protein structure (Thomazeau *et al.*, 2001).

The potato *StTS* gene encodes a TS protein with a molecular mass of 57-kDa. The amino terminal sequence contains a number of serine and threonine residues, which are common features of transit peptides. Gene expression was differentially regulated in a tissue specific manner, with the *StTS* transcripts being most abundant in source leaves and flowers. Less expression was detected in sink leaves, stems, stolons and roots, and the lowest level of expression was found in tubers. Photosynthesis-related metabolites such as sucrose, oxaloacetate and phosphate, nitrogenous compounds, or intermediates/products such as *O*-phosphohomoserine, threonine, homoserine, asparagine, and glutamine did not influence gene expression (Casazza *et al.*, 2000).

In plants, *O*-phosphohomoserine is a common substrate for threonine and methionine synthesis. The pathway of methionine synthesis involves the condensation of cysteine and *O*-phosphohomoserine to form cystathionine, which is subsequently converted to homocysteine and then methionine by the enzymes cystathionine γ-synthase, cystathionine β-lyase, and methionine synthase, respectively. The subject of the synthesis of the sulphur containing amino acids will be covered in Chapter 19 (Pilon-Smits and Pilon) and has also been the subject of a number of recent reviews (Amir *et al.*, 2002; Curien *et al.*, 2003; Hesse and Hoefgen, 2003).

It is interesting to note, however, that *in vitro* activity measurements indicate that in plants TS has a 250- to 500-fold higher affinity for *O*-phosphohomoserine than the competing enzyme of the methionine pathway, cystathionine γ-synthase. This would suggest that most of the carbon from aspartate is channelled to threonine rather than methionine. The complex regulation of the branch point at *O*-phosphohomoserine has been discussed in detail by Amir *et al.* (2002), and Curien *et al.* (2003) devised a computer model to account for the fluxes towards threonine and methionine synthesis.

## V. Synthesis of Branched Chain Amino Acids

The pathway for the synthesis of isoleucine, leucine and valine is shown in Fig. 3. Although isoleucine derives some carbon from aspartate, and pyruvate is the sole

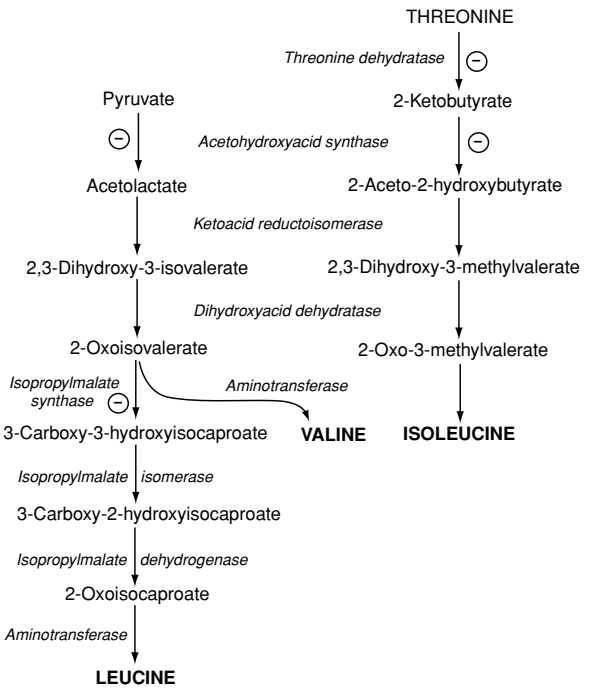


*Fig. 3.* The biosynthetic pathway of the branched chain amino acids inside plastids. (−) indicates a point of feedback inhibition of enzyme activity. Adapted from Singh (1999) and generously supplied by Dr. Bijay Singh.

source of carbon for both leucine and valine, the three amino acids share common enzymes in their synthesis. Chloroplasts carry out light-dependent synthesis of the branched chain amino acids derived from pyruvate (Schulze-Siebert *et al.*, 1984; Schulze-Siebert and Schultz, 1989), confirming early evidence for localisation of the enzymes in leaf chloroplasts and root plastids (Miflin, 1974).

## A. Threonine Deaminase (TD)

TD (EC 4.2.1.16) is the first and only unique enzyme in the biosynthetic pathway for isoleucine. The enzyme deaminates and dehydrates threonine to produce 2-ketobutyrate and ammonia. One isoenzyme that is predominantly present in younger developing tissues is inhibited by isoleucine and is termed "biosynthetic". A second, "biodegradative" isoenzyme is insensitive to inhibition by isoleucine and occurs in older, senescing tissues (Singh, 1999). The biosynthetic TD in *A. thaliana* is a tetramer composed of identical 60-kDa subunits. The inhibitory binding of isoleucine to TD induces dimerization of the enzyme, whereas tetramerization is restored by addition of high concentrations of valine (Halgand *et al.*, 2002). Kinetic and binding experiments demonstrated that each regulatory domain of monomers of *A. thaliana* TD possesses two different effector-binding sites, constituted in part by Tyr-449 and Tyr-543. Tyr-449 belongs to a high-affinity binding site whose interaction with a first isoleucine molecule induces conformational modifications, yielding a protein with an enhanced ability to bind a second isoleucine at a lower-affinity binding site containing Tyr-543. Isoleucine interaction with this latter binding site is responsible for conformational modifications leading to final inhibition of the enzyme. Tyr-449 interacts with both regulators, isoleucine and valine. However, the interaction of valine with the high-affinity binding site induces different conformational modifications, leading to the reversal of isoleucine binding and reversal of inhibition (Wessel *et al.*, 2000).

The biosynthetic form of TD was identified as a major soluble protein in tomato flowers, and the gene encoding a 55-kDa protein was isolated and characterised (Samach *et al.*, 1991). A 51-amino acid transit peptide, rich in serine and threonine, was identified from the cDNA sequence. The expression of the TD gene was found to be 250- to 500-fold higher in floral organs as compared with leaves, roots or seeds. Immunolocalisation studies indicated that the enzyme protein was quite specifically located in the parenchyma cells of petals, stamens and sepals. Later studies showed that dramatic increases in the expression of TD could be induced in the parenchyma of leaves and flowers by wounding or application of methyl jasmonate (Samach *et al.*, 1995). In experiments to determine the effects of leaf damage by the insect *Manduca sexta* on gene expression in the leaves of its natural host, *Nicotiana attenuata*, expression of biosynthetic TD gene showed the strongest and most specific response to insect attack. mRNA transcripts encoding TD accumulated to high concentrations after herbivory and the application of methyl jasmonate to leaves (Hermsmeier *et al.*, 2001). The strong herbivore-induced expression of TD may reflect the high demand for enzyme activity for the synthesis of defense compound precursors derived from 2-ketobutyrate.

## B. Acetohydroxyacid Synthase (AHAS)

AHAS (EC 4.1.3.18), also known as acetolactate synthase, carries out a series of parallel reactions required for the biosynthesis of leucine, valine and isoleucine. In the pathway that produces isoleucine, the enzyme catalyses the condensation of pyruvate with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate. In the

reaction that produces valine and leucine, AHAS catalyses the condensation of two molecules of pyruvate to form 2-acetolactate. The enzyme requires thiamine pyrophosphate, flavine adenine dinucleotide and a divalent cation for both condensation reactions (Singh, 1999). AHAS is the target of a range of potent, low-dose herbicides and for this reason the enzyme has been the subject of intense study, particularly with the respect of changes to the amino acid sequence of the protein that confer herbicide resistance (Wittenbach and Abell, 1999; Tan and Medd, 2002; Jander *et al.*, 2003; Kochevenko and Willmitzer, 2003; Pang *et al.*, 2003). The majority of this work is beyond the scope of this chapter.

An unusual feature of plant AHAS is inhibition by all three of the branched-chain amino acids, unlike most bacterial and fungal enzymes that are sensitive to valine only. Moreover, the plant enzyme is subject to synergistic inhibition by the combination of leucine plus valine. Initially there was some confusion as to the localisation of the binding site(s) of the feedback-inhibitor, end-product amino acids, until it became clear that the native plant AHAS enzyme contained not only a catalytic subunit but also a smaller regulatory subunit (Y.-T. Lee and Duggleby, 2001). A molecular model of the AHAS regulatory subunit from *A. thaliana* was proposed in which the amino acid sequence contains two domains created by an internal duplication, with one binding valine or isoleucine and the other leucine. Reconstitution of the catalytic subunit with one domain of the regulatory subunit yielded an enzyme that was inhibited by leucine but not by valine or isoleucine. Saturation of the second domain with leucine in fact enhances the affinity for valine (Y.-T. Lee and Duggleby, 2002).

Only one gene encoding the catalytic subunit of AHAS was isolated from *A. thaliana*, but the presence of multigene families was detected in maize and tobacco (Mazur *et al.*, 1987; Singh, 1999). Putative transit peptides were identified in all cDNA clones so far sequenced, confirming the localisation of AHAS activity in chloroplasts (Miflin, 1974). Unlike the gene for TD, as discussed earlier, there is no evidence that AHAS is over-expressed in flowers. Most studies showed an overall pattern of constitutive expression with higher levels being detected in young meristematic tissue (Singh, 1999).

### C. Ketolacid Reductoisomerase (KARI)

KARI (EC 1.1.1.86), also known as acetohydroxy acid isomeroreductase, isomerises and then reduces the two acetohydroxyacids to produce dihydroxyacids. The enzyme was purified from spinach and barley, and studies on the spinach enzyme over-expressed in *E. coli* indicated that the enzyme probably exists as a dimer of 59-kDa monomers (Wessel *et al.*, 1998). The enzyme has similar high affinity for the two substrates, which appear to compete for the same binding site. However, the $V_{max}$ for the two plant enzymes is 6- to 11-times higher for 2-aceto-2-hydroxybutyrate than for 2-acetolactate (Dumas *et al.*, 1992; Durner *et al.*, 1993). The binding of $Mg^{2+}$ ions and NADPH causes an initial conformational change at the interface of the two domains of each monomer, which alters the structure of the active site to promote substrate binding (Halgand *et al.*, 1999).

A gene encoding KARI was isolated from spinach and *A. thaliana* and only one copy could be detected per haploid genome. The gene in *A. thaliana* contains nine introns and encodes a protein precursor of 591 residues that includes a putative transit peptide of 67 amino acids (Dumas *et al.*, 1993).

### D. Dihydroxyacid Dehydratase

Dihydroxyacid dehydratase (EC 4.2.1.9) also carries out two reactions in which 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate is dehydrated to form 2-oxoisovalerate or 2-oxo-3-methylvalerate, respectively. A range of auxotrophic mutants that require isoleucine, leucine and valine for growth were isolated (Singh, 1999), which indicated that it is likely that only one copy of the gene is present (Singh, 1999). The enzyme was purified to homogeneity from spinach and shown to be a dimer with a subunit of 60-kDa. The enzyme contains a [2Fe-2S] cluster, which is a novel finding for an enzyme of the hydrolyase class (Flint and Emptage, 1988).

### E. Leucine Synthesis

Leucine is synthesised from 2-oxoisovalerate, the keto-acid that is transaminated to valine, in four steps. Isopropylmalate synthase (EC 4.1.3. 12) catalyses the first reaction which is the conversion of 2-oxoisovalerate to 3-carboxy-3-hydroxyisocaproate (2-isopropylmalate). Isopropylmalate synthase is strongly feedback inhibited by leucine, indicating that it is a major control point in leucine synthesis (Hagelstein and Schultz, 1993). Junk and Mourad (2002) screened a number of *A. thaliana* libraries for genes encoding isopropylmalate synthase and somewhat surprisingly found evidence of four distinct loci. Three clones, *IMS1*, *IMS2* and *IMS3,* were characterised and

shown to complement a leucine-requiring *E. coli* mutant. Gene expression studies indicated that *IMS1* and *IMS2* were expressed in roots, leaves, stems and flowers, with *IMS2* being particularly strong in roots and leaves. *IMS3* was expressed at a high level in roots but not in flowers (Junk and Mourad, 2002).

Isopropylmalate isomerase (EC 4.2.1.33) catalyses the conversion of 3-carboxy-3-hydroxyisocaproate (2-isopropylmalate) to 3-carboxy-2-hydroxyisocaproate (3-isopropylmalate), the enzyme has been little studied in plants. Isopropylmalate dehydrogenase (EC 1.1.1.85) catalyses the NAD-dependent dehydrogenation of 3-isopropylmalate followed by decarboxylation of the intermediate, to produce 2-oxoisocaproate. A cDNA encoding the enzyme was isolated from rape (Ellerstrom *et al.*, 1992) and potato (Jackson *et al.*, 1993). The rape gene encodes a 52-kDa protein, which has a putative transit peptide and was imported into chloroplasts and cleaved to the mature size. The potato gene encodes a protein of 357 amino acids, which included a 29-amino acid, putative transit peptide. The potato gene was expressed in all tissues of the plant and its expression was increased by leucine, leucine plus threonine or sucrose, in contrast to the situation in yeast and prokaryotes (Jackson *et al.*, 1993).

### *F. The Final Transamination Step*

The final metabolic step in the production of the amino acids leucine, isoleucine and valine is a transamination reaction catalyzed by the branched-chain amino acid aminotransferases (BCAT, EC 2.6.1.42). The crystal structure of the *E. coli* enzyme and the mechanism of binding of the substrates were determined by M. Goto *et al.* (2003). Hagelstein *et al.* (1997) isolated two different forms of the aminotransferase from spinach chloroplasts, one which was able to use 2-oxoisovalerate as a substrate and thus form valine and another which could use either 2-oxo-3-methylvalerate or 2-oxoisocaproate as substrates to form isoleucine and leucine, respectively.

Two different cDNAs were isolated from potato that encoded BCAT (*BCAT1* and *BCAT2*). Southern analysis indicated that *BCAT1* and *BCAT2* were not in identical regions of the genome and that *BCAT1* may exist in multiple copies. The deduced amino acid sequences for both cDNAs contained putative transit peptides for plastid or mitochondrial localization. Analysis of gene expression indicated an induction of *BCAT1*, relative to *BCAT2*, in leaf callus cultures exposed to treatment with α-naphthalene acetic acid and 6-benzylaminopurine (Campbell *et al.*, 2001). More recently, seven putative *BCAT* genes were identified in *A. thaliana* and six of the cDNA sequences were cloned (Diebold *et al.*, 2002). The deduced amino acid sequences exhibited between 47.5% and 84.1% identity to each other and 30% identity to the homologous enzyme from yeast. Analysis of the subcellular localization of BCATs in *A. thaliana* using GFP fusion proteins indicated that three of the genes encoded plastid forms and one gene encoded a mitochondrial form of the enzyme. Localization of the enzymes encoded by the two remaining genes was not clear. Diebold *et al.* (2002) proposed that the chloroplast aminotransferases are involved in the biosynthetic reactions and that a separate pathway of branched chain amino acid degradation takes place in the mitochondria, in a manner similar to that previously discussed for lysine (Galili, 2002).

## VI. Synthesis of the Aromatic Amino Acids

The aromatic amino acids, phenylalanine, tyrosine and tryptophan, are synthesised via the shikimate pathway (Schmid and Amrhein, 1995, 1999; Herrmann and Weaver, 1999). The initial common pathway requires seven enzyme-catalysed reactions, which synthesize chorismate from the carbohydrate metabolites phospho*enol*pyruvate and erythrose 4-phosphate (Fig. 4). Details of the basic properties of the enzymes isolated from various plant sources were described by Schmid and Amrhein (1999). Chorismate is the precursor of the three aromatic amino acids and, eventually, a vast range of secondary aromatic metabolites such as alkaloids, lignins and phenols (Haslam, 1993; Boerjan *et al.*, 2003; Knaggs, 2003). Details of these enzymes were also covered by Siehl (1999).

Haslam (1993) proposed that between 20 to 30% of the carbon photosynthetically assimilated by a plant passes down the shikimate pathway to chorismate. There is considerable evidence from enzyme localisation, and molecular studies that indicate the presence of plastid targeting sequences, that all the enzymes of the shikimate pathway are present in the chloroplast (Schmid and Amrhein, 1999; Bischoff *et al.*, 2001). Intact, illuminated chloroplasts are able to synthesise phenylalanine, tyrosine and tryptophan from $^{14}CO_2$ plus pathway intermediates such as shikimate (Bickel *et al.*, 1978; Bickel and Schultz, 1979; Schulze-Siebert and Schultz, 1989; Leuschner and Schultz, 1991). Although erythrose 4-phosphate is an intermediate in the Calvin-Benson cycle, the origin of

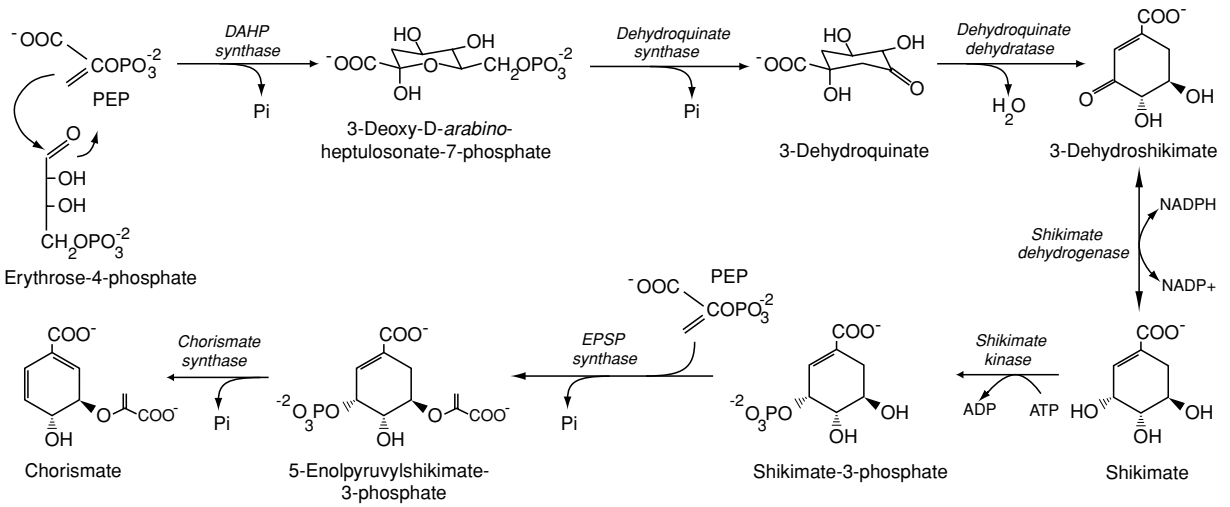


*Fig. 4.* The biosynthetic pathway of chorismate, via shikimate. All the reactions take place in the plastids. Adapted from Siehl (1999) and generously supplied by Dr. Daniel Siehl.

phospho*enol*pyruvate is less clear. Bagge and Larsson (1986) proposed that phospho*enol*pyruvate is synthesised in the cytoplasm and transported across the chloroplast envelope. The presence of at least two specific phospho*enol*pyruvate translocators in *A. thaliana* plastid envelope was recently confirmed (Knappe *et al.*, 2003). The photosynthetic synthesis of phenylalanine, tyrosine and tryptophan in chloroplasts from $^{14}CO_2$ is subject to feedback inhibition, with each amino acid inhibiting its own synthesis at physiological concentrations. Tryptophan at very high concentrations inhibits the synthesis of all three amino acids (Bickel and Schultz, 1979; Bagge and Larsson, 1986).

## *A. Chorismate Synthesis*

3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) is the first enzyme in the pathway and catalyses the condensation of phospho*enol*pyruvate and erythrose 4-phosphate (Konig *et al.*, 2004). Two different isoenzymes exist in plants, one in the cytoplasm that requires $Co^{2+}$ and a chloroplast form that is able to use $Mn^{2+}$ or, in some plants, other metal ions. The mature enzyme has a molecular mass of 53- to 54-kDa and exists as oligomers. Although DAHP synthase in bacteria is subject to feedback regulation, somewhat surprisingly there is no evidence that the plant enzymes are inhibited by any of the three aromatic amino acids. However, arogenate, the penultimate metabolite in phenylalanine and tyrosine synthesis, is an inhibitor of the enzyme (Herrmann and Weaver, 1999; Siehl, 1999).

Following the initial isolation of a cDNA encoding DAHP synthase from potato, cDNAs were isolated from a range of plants and all were shown to have N-terminal transit sequences (Schmid and Amrhein, 1999). Plants have at least two genes encoding the enzyme and in *A. thaliana* there may be three genes (Herrmann and Weaver, 1999). Transport of a DAHP synthase precursor into the chloroplast and cleavage of the terminal sequence of 6-kDa was demonstrated (J.M. Zhao *et al.*, 2002). The chloroplast enzyme from *A. thaliana* requires reduced ferredoxin/thioredoxin for activation and is therefore linked to electron flow from photosystem I (Entus *et al.*, 2002).

3-Dehydroquinate (DHQ) synthase (EC 4.6.1.3) catalyses the cyclisation step in the pathway. Conversion of DAHP to DHQ requires $Co^{2+}$ for activity (Nichols *et al.*, 2004). A cDNA encoding an enzyme containing 442 amino acids was isolated from tomato and found to have an N-terminal transit sequence containing serine and threonine. Interestingly, sequence identity was higher between the proteins from tomato and *E. coli* than between *E. coli* and other bacteria (Bischoff *et al.*, 1996).

In higher plants, the third and fourth steps are cataysed by a bifunctional enzyme, 3-dehydroquinate dehydratase (DHQase)-shikimate:$NADP^+$ oxidoreductase (SORase) (DHQase-SORase, EC 4.2.1.10–EC 1.1.1. 25), which converts 3-dehydroquinate to shikimate via 3-dehydroshikimate. In bacteria, activities as DHQase and SORase (also known as shikimate dehydrogenase) exist as separate enzyme molecules (Maes *et al.*, 2004). The enzyme activities were

detected in a number of plant species and the respective proteins in pea and tobacco (Schmid and Amrhein, 1999) and *Pinus taeda* (Ossipov *et al.*, 2000) were purified to near homogeneity. Two classes of cDNAs encoding the enzymes were isolated from tomato that were derived from alternatively spliced transcripts and differed by 57 bp within the coding regions but were otherwise identical. Expression of the two polypeptides in *E. coli* indicated that only the shorter polypeptide was enzymically active and thus removal of the potential exon is essential. A very short, encoded N-terminal sequence (13 amino acids), absent from the mature enzyme, did not show any of the characteristics commonly found in plastid transit peptides (Bischoff *et al.*, 2001). However, previous studies had clearly shown that the enzyme is localised within plastids (Bonner and Jensen, 1994).

Shikimate kinase (EC 2.7.1.71) catalyses the ATP-dependent phosphorylation of shikimate to shikimate 3-phosphate (Gu *et al.*, 2002). The spinach chloroplast enzyme was purified to near homogeneity and has a molecular mass of 31-kDa. Although initial studies suggested that the enzyme is regulated by thioredoxin, it was later suggested that energy charge was the main controller of activity (Schmidt *et al.*, 1990).

One cDNA clone encoding shikimate kinase was isolated from tomato and contained an N-terminal transit sequence. Plastid localisation was confirmed by the uptake and processing of the enzymically active precursor protein by isolated intact chloroplasts (Schmid *et al.*, 1992). Analysis of the genome suggested that three genes encoding a plastid-located shikimate kinase may be present in *A. thaliana* (*Arabidopsis* Genome Initiative, 2000; Rippert and Matringe, 2002b).

5-Enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) catalyses the condensation of a second molecule of phosphoenolpyruvate with shikimate 3-phosphate to form EPSP (McDowell *et al*., 2004). The enzyme has been extensively studied because it is the target of the major commercial herbicide, glyphosate. Enzyme kinetic and X-ray crystallographic studies indicated that glyphosate binds at the phosphoenolpyruvate site (Schonbrunn *et al*., 2001). A range of amino acid substitutions to this site demonstrated that the inhibitory action of glyphosate can be greatly reduced without significantly affecting the ability of phosphoenolpyruvate to act as a substrate (Baerson *et al*., 2002; Eschenburg *et al*., 2002).

Up to three different isoenzymic forms of EPSP synthase have been isolated from a range of plant species (Schmid and Amrhein, 1999). The two EPSP synthase isoenzymes of maize were purified to homogeneity and their location in plastids confirmed (Forlani, 1997). Several genes and cDNA clones encoding EPSP synthase were isolated and sequenced. The identity of the protein sequences of the mature enzyme is 85% between *A. thaliana* and petunia and 93% between petunia and tomato. All plant EPSP synthase sequences include a putative N-terminal plastid-targeting peptide, but the amino acid content exhibits considerable variation. The protein synthesised in petunia is reduced in mass from 55- to 48-kDa following transport into the chloroplast (Schmid and Amrhein, 1999).

The construction of glyphosate-tolerant crops for commercial use (Shaner, 1999; Lutman and Berry, 2003) is beyond the remit of this chapter. However, it is worth noting that early successes involved nuclear transformation with genes encoding an EPSP synthase with an appropriate plastid import sequence. More recently, EPSP synthase has been targeted directly to the plastid genome and the enzyme protein can accumulate to 10% of total leaf protein (Ye *et al.*, 2001).

The last common step in the synthesis of all three aromatic acids is the formation of chorismate by a 1,4-*trans* elimination of phosphate from EPSP by chorismate synthase (CS, EC 4.6.1.4). Although this reaction does not involve a net redox change, the enzyme has an absolute requirement for reduced flavin mononucleotide, which is not consumed during the reaction. Two invariant histidine residues found in the active site of the enzyme play a key role in the CS catalysed reaction (Ahn *et al.*, 2004; Kitzing *et al.*, 2004).

One cDNA clone encoding CS was initially isolated from *Corydalis sempervirens* and shown to contain an N-terminal transit sequence, the removal of which was essential for enzyme activity (Henstrand *et al.*, 1995). Three distinct classes of cDNAs were isolated from tomato, but two were identical except for a 12-bp deletion within the coding region. The two were derived from the primary transcript of the same gene *LeCS2* by alternative spicing of the third intron (Gorlach *et al.*, 1995). All three transcripts contained a transit sequence, but again the enzyme protein was not active until this was removed (Braun *et al.*, 1996).

As stated above, plant DAHP synthase, unlike the enzyme in bacteria, is not inhibited by any of the three major end-product amino acids. The enzyme is inhibited by arogenate with an $I_{50}$ of 150 μM, although it is not clear whether arogenate ever reaches this concentration under normal physiological conditions. Evidence that DAHP synthase may be under some regulatory control is provided by the demonstration of massive accumulations of shikimate following the application

of glyphosate, which could be reduced to some extent by the presence of arogenate (Siehl, 1999).

Expression of the genes encoding the seven enzymes required for the synthesis of chorismate in various organs of the tomato plants was studied in detail by Amrhein and his colleagues (Schmid and Amrhein, 1999; Bischoff *et al.*, 2001). Three distinct expression patterns were established. (i) For the *LeDHS1* gene encoding DAHP synthase, equal levels of mRNA transcripts were identified in all organs tested. (ii) For the second gene encoding DAHP synthase *LeDHS2* and those encoding DHQ synthase and DHQase-SORase, the highest relative abundance of transcripts was detected in the roots, with lower levels in stems, flowers, and cotyledons, and the lowest in the leaves. (iii) For shikimate kinase, EPSP synthase and the two CS genes *LeCS1* and *Le CS2,* transcript expression was highest in the roots and flowers, lower in the stems and lowest in the leaves and cotyledons.

The application of a fungal elicitor to cultured cells, or a pathogenic fungus to intact plants induced expression of mRNA encoding the enzymes required for the synthesis of chorismate. However, where two genes were clearly identified for DAHP synthase and chorismate synthase, only one was induced by the fungal elicitors (Schmid and Amrhein, 1999; Bischoff *et al.*, 2001). In contrast, using an enzymological approach, Forlani (2002) found that two distinct isoenzymes of EPSP synthase in maize were induced by an elicitor from *Fusarium acuminatum*, but there was a clear difference in the time courses of induction of the two.

## *B. Tryptophan Synthesis*

The synthesis of tryptophan is required not only for incorporation into proteins but also as an intermediate in the synthesis of acridones, auxin, glucosinolates and alkaloids. The enzymes involved in the pathway were characterised on the basis of their microbial counterparts (Xie *et al.*, 2003) and found to be located in plastids. In addition, precursor enzyme proteins were imported and processed by chloroplasts and the cleavage sites of the additional transit peptides were determined (J. Zhao and Last, 1995). Confirmation of the precise sequence of enzymes required for tryptophan synthesis (Fig. 5) has come from the work of Last and colleagues, who isolated and characterised a series of auxotrophic mutant lines of *A. thaliana* that lacked individual enzymes in the pathway (Radwanski and Last, 1995).

Anthranilate synthase (AS, EC 4.1.3.27) catayses the amination of chorismate using glutamine as a substrate and the removal of the *enol*pyruvyl sidechain. The plant enzyme contains an $\alpha$ subunit of 64- to 67-kDa in mass and a $\beta$ subunit of 25.5-kDa in mass and probably exists in an $\alpha_2\beta_2$ configuration. The $\alpha$ subunit carries out the conversion of chorismate to anthranilate, while the $\beta$ subunit catalyses a glutamine-dependent amidotransferase reaction. The activity of the enzyme is very sensitive to feedback inhibition by tryptophan in the range 1 to 5 $\mu$M, which binds to a Leu-Leu-Glu-Ser sequence in the $\alpha$ subunit (Romero *et al.*, 1995).

Two distinct genes encode the $\alpha$ subunit of AS in *A. thaliana, Ruta graveolens* and rice, all of which have a putative N-terminal transit peptide. The predicted amino acid sequences exhibit a 70 to 80% identity with each other (Niyogi and Fink, 1992; Bohlmann *et al.*, 1995; Tozawa *et al.*, 2001). In *A. thaliana* the expression of *ASA1* mRNA was 10-fold higher than *ASA2* in roots, leaves and stems, and was induced by wounding and bacterial infection. Low levels of *ASA2* mRNA were detected in the siliques, which were not increased by external stimuli (Niyogi and Fink, 1992). In rice the abundance of the mRNA transcripts of *OASA2* was higher than *OASA1* except in the panicles and was induced by external stimuli (Tozawa *et al.*, 2001). It was proposed that the rice *OASA*1, *A. thaliana ASA2* and the *R. graveolens ASα1* belong to the same family of constitutively expressed genes and that rice *OASA2, A. thaliana ASA1* and the *R. graveolens ASα2* belong to the same family of inducible genes.

At least two distinct genes were identified that encode the $\beta$ subunit of AS in *A. thaliana* (Niyogi *et al.*, 1993) and rice (Kanno *et al.*, 2004), which contain putative N-terminal transit peptides. The *OASB1* mRNA transcripts were present in higher abundance than *OASB2* in both the roots and leaves of rice. Using a wheat germ translation system, Kanno *et al.* (2004) reconstituted different combinations of $\alpha$ and $\beta$ subunits encoded by the different genes. Considerable differences were noted in the kinetic properties of the reconstituted enzymes, particularly with respect to the $K_i$ for tryptophan, which varied from 5.3 to 135 $\mu$M.

As the availability of tryptophan frequently limits the nutrient quality of plant foodstuffs, relatively successful attempts have been made to increase the soluble concentration of tryptophan by the introduction of genes encoding a feedback-insensitive form of AS, targeted to the chloroplast using a transit peptide (Cho *et al.*, 2000; Tozawa *et al.*, 2001). More recently Zhang *et al.* (2001) introduced a feedback-insensitive tobacco *ASA2* gene directly into the plastid genome by site-specific insertion. Increased concentrations of soluble tryptophan in leaves and total tryptophan in seeds were achieved.

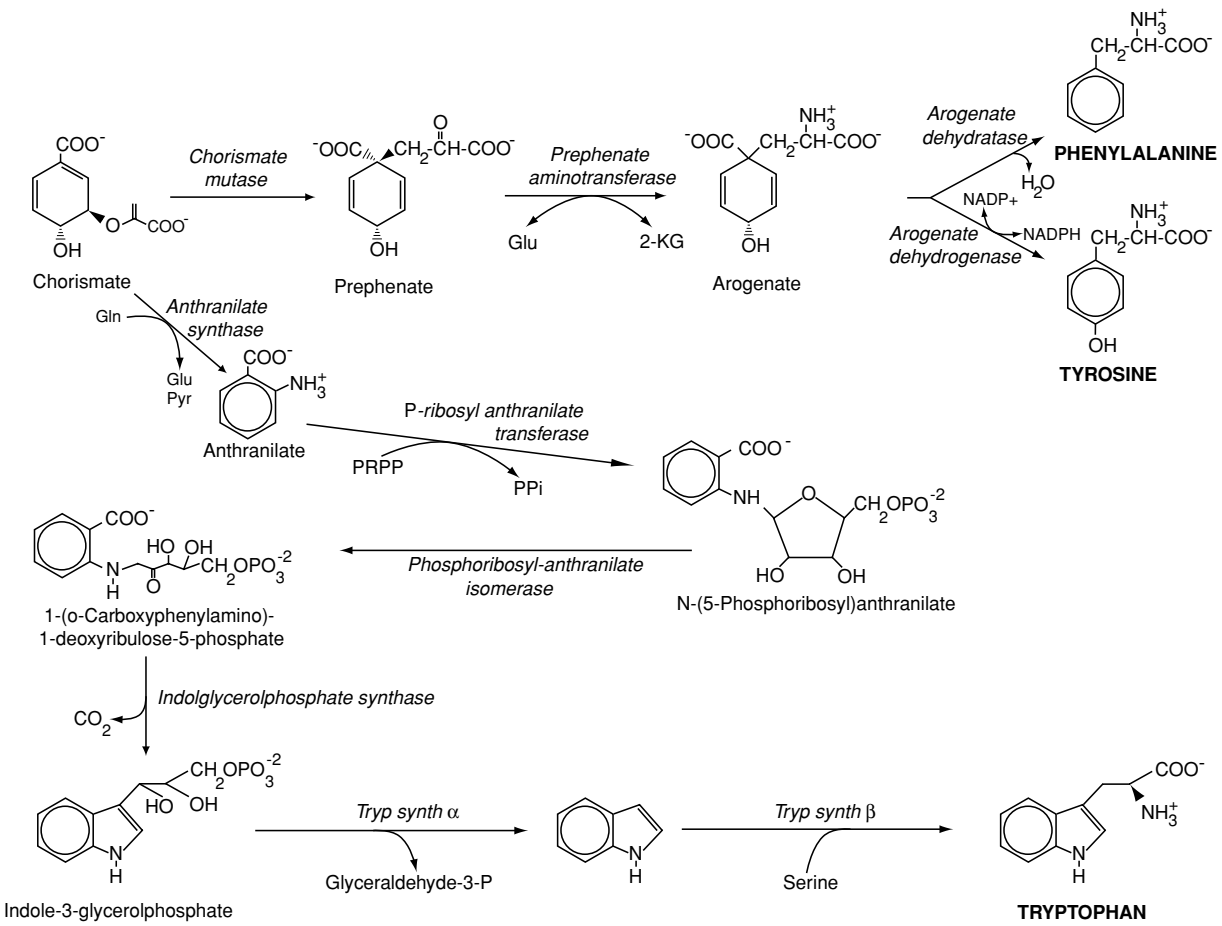


*Fig. 5.* The pathway of synthesis of tryptophan, phenylalanine and tyrosine from chorismate. All the reactions take place in the plastids. Adapted from Siehl (1999) and generously supplied by Dr. Daniel Siehl.

The authors argued that this was the first example of the return of a nuclear gene, which presumably had arisen before endosymbiosis, back to the plastids in order to modify an endogenous biosynthetic pathway (Zhang *et al.*, 2001).

Phosphoribosyl-anthranilate transferase (PAT, EC 2.4.2.18) catalyses addition of a phophoribosyl group to the amino nitrogen of anthranilate to form phosphoribosyl-anthranilate. Although the enzyme from bacteria was extensively characterised (Mayans *et al.*, 2002), the enzyme activity has not been studied in plants. However, blue-fluorescent mutants of *A. thaliana* were isolated that have very low PAT activity and accumulate anthranilate compounds throughout the plant (Radwanski and Last, 1995). The gene encoding the enzyme was isolated from *A. thaliana* and encodes a transit peptide. PAT1 protein was found in approximately equal amounts in all tissues, including roots, leaves and flowers. Two introns greatly enhance the expression of the *PAT-1* gene, and they acted post-transcriptionally to increase the steady-state level of mRNA (Rose and Last, 1997).

Phosphoribosyl-anthranilate isomerase (PAI, EC 5.3.1.24) catalyses the conversion of N-(phosphoribosyl) anthranilate to 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate. The enzyme is related to N′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamideribonucleotide isomerase, which is involved in histidine biosynthesis. Both enzymes have a $(\beta\alpha)_8$ barrel fold and catalyze Amadori rearrangements of a thermolabile aminoaldose into the corresponding aminoketose (Henn-Sax *et al.*, 2002). Three genes encoding the enzyme were isolated from *A. thaliana* Columbia, which showed over 90% sequence identity at the nucleotide level. All three genes are expressed in the plant and each has a putative N-terminal transit peptide (Li *et al.*, 1995b). Later work examined the expression of the three genes using

a promoter-GUS reporter gene system. Each gene was differentially expressed in a tissue- and cell-type specific manner. *PAI1* and *PAI3* were 10-fold more strongly expressed than *PAI2*, but the latter displayed a stronger response to environmental stresses such as UV light and elicitors (He and Li, 2001).

Indole-3-glycerolphosphate synthase (EC 4.1.1.48) catalyzes ring closure of the N-alkylated anthranilate to the 3-alkyl indole derivative, a reaction requiring Lewis acid catalysis (Forsyth and Matthews, 2002; Hennig *et al.*, 2002). In bacteria, the protein may be fused with PAI to form a bifunctional enzyme and, in fungi, also with the β-subunit of AS to form a trifunctional protein (Challen *et al.*, 2002). A cDNA clone was isolated from *A. thaliana* that encodes a protein of 41-kDa in mass. The predicted amino acid sequence has 22 to 38% identity with microbial counterparts, but also contains a putative N-terminal transit peptide (Li *et al.*, 1995a).

Tryptophan synthase (TrpS, EC 4.2.1.20) catalyses the conversion of indole-3-glycerolphosphate to tryptophan in the presence of serine. The fully functional enzyme contains α and β subunits in a $\alpha_2\beta_2$structure (Raboni *et al.*, 2003). The α-protein alone is able to convert indole-3-glycerolphosphate to indole and the β-protein is able to convert indole to tryptophan. A single gene encoding the α-subunit of TrpS was initially isolated from maize. The mRNA transcript was abundant in pith and young leaves but low in roots and absent in seeds (Kramer and Koziel, 1995). In *A. thaliana,* gene *TSA1* encodes a putative N-terminal transit peptide in addition to a processed α-protein of 28.8-kDa in mass. The gene has 53% identity with the maize protein. It was suggested that one or possibly two further genes encoding TrpS α-subunits occur in *A. thaliana* (Radwanski *et al.*, 1995).

The final step of tryptophan synthesis is complicated in maize, as the intermediate in the TrpS reaction, indole, is used for synthesis of the cyclic hydroxamic acid, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a defense compound against pathogens. The *Bx1* gene, which is closely related to *TSA,* was characterised in maize and encodes a protein capable of converting indole-3-glycerol phosphate to indole at a high rate. Plants carrying mutations in the *Bx1* gene were not able to synthesise 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one and were susceptible to pathogen attack (Frey *et al.*, 1997).

In addition, maize seedlings damaged by beet armyworm larvae release a specific blend of terpenoids and indole that is attractive to *Cortesia marginiventris*, a parasitic wasp that attacks the larvae. Volicitin is a specific elicitor present in the secretions of the larvae that induces expression of a third *TSA*-type gene termed *Igl* that encodes another enzyme capable of converting indole-3-glycerol phosphate to indole. The other two *TSA* genes in maize are not induced by volicitin (Frey *et al.*, 2000).

Two genes encoding the TrpS β-subunit were isolated from *A. thaliana* and termed *TSB1* and *TSB2.* Both contain putative N-terminal transit peptides, are nearly identical in sequence and are closely related to the enzyme of bacterial origin. Levels of mRNA transcripts and promoter-GUS fusion studies indicated that *TSB1* was transcribed 10-fold higher than *TSB2. TSB1* mRNA transcripts were most abundant in leaves and less so in flowers and roots (Pruitt and Last, 1993). Similarly, two genes were isolated from maize that were 98% identical and the expression levels of these two genes were very similar (Wright *et al.*, 1992). It was proposed that one of the enzymes in maize is involved in synthesis of indole, which is a precursor of pathogen defence compounds (Frey *et al.*, 1997). Only one β-subunit gene was cloned from *Campotheca acuminata,* a plant that produces indole alkaloids derived from tryptophan. The *CaTSB1* gene, although containing a transit peptide, was expressed mainly in the vascular tissue of the shoot and the cortex of roots and only at low levels in green leaves (Lu and McKnight, 1999).

There is strong evidence that AS is the main target for feedback regulation, because it is inhibited by micromolar concentrations of tryptophan. In addition, plants that contain a deregulated AS, accumulate tryptophan. In mutants that are deficient in enzymes further down the pathway, and are therefore unable to synthesize tryptophan, there is evidence for increased expression and activity of AS (Radwanski and Last, 1995). J. Zhao *et al.* (1998) examined the effects of various forms of stress on the expression of the genes described above that are involved in the synthesis of tryptophan. Starvation for the aromatic and branched chain amino acids and methionine induced accumulation of mRNA encoding all of the tryptophan-synthesizing enzymes tested. However, treatment with the herbicide aciflurofen or an abiotic elicitor also induced synthesis of mRNA encoding the same tryptophan synthesizing enzymes. The authors therefore concluded that up-regulation of gene expression is a general stress response to allow for the synthesis of secondary defence metabolites (J. Zhao *et al.*, 1998).

### *C. Phenylalanine and Tyrosine Synthesis*

Confirmation of the pathway of phenylalanine and tyrosine synthesis in higher plants has been fraught with

difficulty. The route is not the same as found in most bacteria, where prephenate is converted to the respective keto-acids of phenylalanine and tyrosine and the final step involves an aminotransferase reaction (Chen *et al.*, 2003). It is now clear from the work of Jensen and colleagues that in most plants, the aminotransferase reaction takes place first and that prephenate is transformed to arogenate, which can then be converted by separate reactions into either phenylalanine or tyrosine (Fig. 5) (Bonner *et al.*, 1995).

Chorismate mutase (CM, EC 5.4.99.5) catalyses the Claisen rearrangement of chorismate to form prephenate, which involves the pericyclic rearrangement of the oxygen involved in the ether linkage (Marti *et al.*, 2004). High quality enzyme studies clearly demonstrated that two separable isoenzymic forms of CM occur in higher plants. CM1, which is localised in the chloroplast, is inhibited by phenylalanine and tyrosine and activated by tryptophan, while CM2 is located in the cytoplasm and is unaffected by any end-product amino acid. Full details of the properties of the two isoenzymes isolated from various plant sources were presented by Siehl (1999).

cDNA clones encoding the two CM isoenzymes were isolated from *A. thaliana* (Eberhard *et al.*, 1996b) and the corresponding genes were expressed at similar levels throughout the plant. The deduced amino acid sequences were 50% identical, but the *CM1* gene sequence included a putative N-terminal chloroplast-targeting sequence while the *CM2* sequence did not. The abundance of mRNA transcripts of the two *A. thaliana* genes were very similar in five different organs tested, with roots exhibiting the highest levels and leaves the lowest, although the differences were not large. When the CM1 and CM2 proteins were expressed in *E. coli,* they exhibited the feedback inhibition properties described above. A cDNA clone encoding an unregulated CM was also isolated from tomato, which lacked a plastid targeting sequence (Eberhard *et al.*, 1996a). Mobley *et al.* (1999) carried out a further investigation into the genes encoding CM in *A. thaliana* and isolated a third gene designated *CM3,* which included a putative transit peptide. When the CM3 protein was expressed in yeast, the enzyme was found to be subject to feedback inhibition by phenylalanine and tyrosine but to have a lower $K_m$ for chorismate than CM1.

Prephenate aminotransferase (EC 2.6.1.57) activity was found in a variety of plants (Siehl, 1999) and the enzyme from *Anchusa offinalis* was purified to homogeneity and shown to be a heterotetramer of 220-kDa in mass (De-Eknamkul and Ellis, 1988). Aspartate and glutamate were the preferred amino donors, although differences between plant species were found. No evidence was found that the enzymes were sensitive to feedback inhibition (Siehl, 1999). The enzyme from all species tested was particularly stable at high temperatures (Bonner *et al.*, 1995).

Arogenate dehydrogenase (EC 1.3.1.43) catalyses the $NADP^+$- or $NAD^+$-dependent oxidation of arogenate to form tyrosine and is located in the chloroplast. ADH from all sources was inhibited by micromolar concentrations of tyrosine. Two isoenzymic forms could be separated chromatographically (Siehl, 1999). Rippert and Matringe (2002a) isolated two genes designated *tyrAAT1* and *tyrAAT2* from *A. thaliana* that encode an ADH with a putative N-terminal transit peptide. Both genes were expressed in *E. coli* and kinetics of the recombinant enzyme were studied (Rippert and Matringe, 2002b). The product of the *tyrAAT1* gene was unusual in that the single polypeptide chain contained two very similar domains. When genes encoding single domains were expressed separately, each product was fully functional. In contrast, *tyrAAT2* encoded a peptide with only one domain. Both forms of arogenate dehydrogenase were very sensitive to inhibition by tyrosine, which was competitive with respect to arogenate. A comparison of the kinetic properties indicated that the repeated domains of tyrAAT1 conferred upon the enzyme a 4-fold greater catalytic efficiency ($kcat/K_m$) than that of the single-domain tyrAAT2 (Rippert and Matringe, 2002b).

Arogenate dehydratase (EC 4.2.1. 91) catalyses the removal of the hydroxyl group of arogenate to form phenylalanine in the chloroplast (Jung *et al.*, 1986). The enzyme in sorghum seeds is inhibited by phenylalanine with a $K_i$ of 24 μM and activated by tyrosine (Siehl and Conn, 1988). Evidence for six genes encoding arogenate dehydratase in *A. thaliana* was obtained (*Arabidopsis* Genome Initiative, 2000; Rippert and Matringe, 2002b).

The chloroplastic form of chorismate mutase, CM1, is inhibited by phenylalanine and tyrosine together and activated by phenylalanine alone, as would be expected for an enzyme controlling the branch point in a pathway (Siehl, 1999). A fungal elicitor increased the activity of other key enzymes in parsley cells but had no effect on CM activity, while wounding increased CM1 activity in potatoes (Kuroki and Conn, 1988). In oat leaves, the activity of one of the two isozymes located in the chloroplast was increased by a fungal elicitor (Matsukawa *et al.*, 2002). Molecular studies with *A. thaliana* indicated that accumulation of mRNA encoding the chloroplastic *CM1,* but not *CM2,* was induced by fungal

elicitors and fungal infection (Eberhard *et al.*, 1996b). Mobley *et al.* (1999) also showed that wounding and pathogen increased the expression of *CM1* but had little effect on *CM2* and *CM3*. Taking into account the differences in $K_m$ values and induction profiles, it is possible (assuming that the two genes are expressed in the same cells) that CM3 encodes a housekeeping enzyme that synthesizes phenylalanine and tyrosine under normal growth conditions and that CM1 encodes an enzyme that is required under stress condition for the synthesis of defence compounds (Mobley *et al.*, 1999).

A second stage of regulation affects the metabolism of the common metabolite, arogenate. Tyrosine and phenylalanine are potent inhibitors of the specific enzyme responsible for their own synthesis, arogenate dehydrogenase and arogenate dehydratase, respectively. Rippert and Matringe (2002b) argued that competitive inhibition of arogenate dehydrogenase by tyrosine, for which the enzyme has a greater affinity than for arogenate, provided complete inhibition of the enzyme and thus total diversion of metabolites towards synthesis of phenylalanine.

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# Chapter 19

# Sulfur Metabolism in Plastids

Elizabeth A.H. Pilon-Smits* and Marinus Pilon
*Biology Department, Colorado State University, Fort Collins, CO 80523, U.S.A.*

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## Summary

Sulfur is an essential element for plant primary metabolism as a structural component of proteins and lipids, antioxidants, regulatory molecules, metal-binding molecules and cofactors/coenzymes. The various steps involved in the reduction of sulfate and its assimilation into cysteine happen predominantly or exclusively in plastids. Cysteine holds a central position in S metabolism and is used for the biosynthesis of a variety of other reduced S compounds including methionine, S-adenosylmethionine, glutathione and phytochelatins, the coenzymes thiamine, biotin, lipoic acid and Coenzyme-A, the Molybdenum cofactor and Fe-S clusters. In this chapter we will give an overview of S metabolism in higher plants, focusing on the role of plastids. The regulation of S metabolism is discussed, as well as the involvement of S metabolic pathways in metabolism of other oxyanions. We conclude with an overview of results from genetic engineering of S pathway enzymes.

## I. Introduction

Sulfur (S) is an essential macronutrient for plants and is present at 0.1 to 1% of plant dry weight depending on the plant family and soil type (Marschner, 1995). Sulfur is generally less limiting for plant growth than other macronutrients such as N or P, but nevertheless, positive responses to S fertilization have been reported from many areas in the world including most agricultural areas (Hoeft and Walsch, 1975). Sulfur deficiency manifests itself as chlorosis of younger leaves and stunted growth (Marschner, 1995).

The main form of S taken up by plants is sulfate, which is the most oxidized form of S (valence state +6), and the predominant bio-available form in oxic soils. The form of S present in biomolecules is mostly reduced S, although S also occurs in its oxidized form in sulfolipids and various sulfated compounds (for a review, see Leustek *et al.*, 2000). The reduction of

*Author for correspondence, email: epsmits@lamar.colostate.edu

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sulfate needed for the synthesis of many S compounds happens exclusively in plastids and predominantly in the photosynthetic tissues. Cysteine (Cys) is the first organic form of S after sulfate reduction. Cys holds a central position in S metabolism and is used for biosynthesis of a variety of other reduced S compounds including methionine (Met), S-adenosylmethionine (SAM), glutathione (GSH) and phytochelatins (PCs), the coenzymes thiamine, biotin, and Coenzyme-A, the Molybdenum cofactor (MoCo) and Fe-S clusters.

Sulfur compounds hold essential functions in plant primary metabolism as structural components of proteins and lipids, as antioxidants, as regulatory molecules, metal-binding molecules, and as cofactors/coenzymes for biochemical reactions. Next to this structural role, the S atom is often directly required for the function of S-containing molecules. For instance, a thiol (-SH) group in proteins or peptides may have a key function due to its redox capacity and its metal-binding properties, or be directly involved in enzyme catalysis.

In addition to the roles of S compounds in primary plant metabolism of all plants, certain S compounds are not essential but play a role in stress resistance or in ecological interactions between plants and other organisms. For instance, some S compounds are signaling molecules in plant-microbe interactions (Lerouge *et al.*, 1990; Denarie and Cullimore, 1993). Other S compounds (e.g. glucosinolates) may also protect plants from herbivory or microbial infection (Ernst, 1990; Sendl, 1995; Lacomme and Roby, 1996). The sulfated compound gallate glucoside controls seismonasty, which may also function in deterring herbivory (Varin *et al.*, 1997). Accumulation of S compounds (e.g. sulfate or dimethylsulfoniumpropionate, DMSP) may also protect plants from osmotic stresses (Ernst, 1990).

The study of plant S metabolism has relevance for human society because S is a macronutrient and can be limiting for agricultural productivity. Also S compounds may contribute to plant biotic and abiotic stress resistance, positively affecting crop yields. The S content and allocation by crop plants contribute to animal and human food quality. Sulfur compounds can influence the quality of plant products by means of their flavors and odors (Thompson *et al.*, 1986), by influencing the quality of flour (Byers *et al.*, 1987), or by their medicinal activity (Hell, 1997). In addition, there are volatile S compounds emitted by plants that can influence climate (Kelly *et al.*, 1994).

In this chapter we will give an overview of S metabolism in higher plants focusing on the role of plastids, the main site of S reduction. The regulation of S metabolism is discussed, as well as the involvement of S metabolic pathways in metabolism of other oxyanions. We conclude with an overview of results from genetic engineering of S pathway enzymes.

## II. Sulfur Compounds and Their Properties

The role of S in molecules is very diverse, because S can exist in multiple oxidation states (+6, +4, 0, −2), each with different chemical properties (Beinert, 2000). In this section we will give an overview of the major S-containing groups and their properties and functions in biomolecules. We include a list of (classes of) S-containing biomolecules and their functions in Table 1. In the next section we will describe how these various S compounds are synthesized, and describe their individual roles in more detail. For an overview of S compounds in plants see also Buchanan *et al.* (2000, pp 826–830).

Thiols contain a –SH group. Examples are Cys and Cys-containing peptides (e.g. GSH) and proteins, as well as lipoic acid and Coenzyme-A. Thiol groups are redox active. Two –SH groups can form one S-S disulfide bond, giving up two electrons and two protons. This reversible reaction is used in cells to stabilize protein structure (especially secreted proteins) and to regulate enzyme activity (especially intracellular enzymes). The redox activity of thiol groups is also used by S compounds such as GSH to keep cell components in a reduced state and scavenge free radicals (Kunert and Foyer, 1993). The thiol group can also be

*Abbreviations:* ATP – adenosine triphosphate; APS – adenosine phosphosulfate; APSe – adenosine phosphoselenate; CbS – cystathionine-β-lyase; CgS – cystathionine-γ-synthase; Cys – cysteine; CysD – cysteine desulfurase; DMS – dimethylsulfide; DMSe – dimethylselenide; DMSP – dimethyl sulfoniumpropionate; DMSeP – dimethyl selenoniumpropionate; ECS – γ-glutamylcysteine synthetase; ER – endoplasmic reticulum; GR – glutathione reductase; GS – glutathione synthetase; GSH – glutathione (reduced); GSSG – glutathione (oxidized); Met – methionine; MMT – methionine methyltransferase; MoCo – molybdenum cofactor; NADPH – nicotinamide adenine dinucleotide phosphate; OAS – O-acetylserine; OPH – O-phosphohomoserine; PAPS – phosphoadenosine phosphosulfate; PC – phytochelatin; PS – phytochelatin synthase; RT-PCR – reverse transcription polymerase chain reaction; SAM – S-adenosylmethionine (also called AdoMet); SAT – serine acetyltransferase; SeCys – selenocysteine; SMT – selenocysteine methyltransferase; TS – threonine synthase; SQDG – sulfolipid: 6-sulfo-α-D-quinovosyl diacylglycerol.

*Table 1.* Overview of the main S-containing biomolecules in plants and their functions

| Molecule | S group | Function |
|---|---|---|
| Cysteine | thiol | amino acid, metabolic intermediate |
| Cysteine derivatives | | |
| methyl-cysteine | thiol | |
| cystathionine | thioether | |
| cystine | disulfide | |
| glutathione | thiol | reduction processes, stress resistance |
| phytochelatins | thiol | metal tolerance |
| Cys in proteins | thiol, disulfides | |
| Mo cofactor | thioether | cofactor of enzymes |
| Biotin | thioether | carbon dioxide transfer |
| Thiamine | thioether | coenzyme A formation? |
| FeS clusters | sulfide | cofactor of enzymes |
| Lipoic acid | thiol | coenzyme |
| Coenzyme A | thiol | coenzyme |
| Methionine | thioether | amino acid, metabolic intermediate |
| Methionine derivatives | | |
| methyl-methionine | methylsulfonium | transport, metabolic intermediate |
| S-adenosylmethionine | methylsulfonium | methyl donor, ethylene precursor |
| dimethylsulfide | thioether | release of excess S?, ecological function? |
| Met in proteins | thioether | |
| Sulfolipids | sulfonic acid | thylakoid membrane component |
| Sulfated compounds | sulfate ester | |
| flavonoids | sulfate ester | drought resistance? |
| brassinosteroids | sulfate ester | plant growth regulator |
| gallate glucoside | sulfate ester | plant growth regulator? |
| glucosinolates | sulfate ester | ecological role in defense? |
| peptides | sulfate ester | |
| choline sulfate | sulfate ester | S transport? salt resistance? |

modified during group transfer reactions such as those that involve Coenzyme-A. Furthermore, thiol groups can have a direct catalytical role in the active site of enzymes. The thiol group of Cys also has metal-binding properties and is responsible for the metal-binding capacity of many metal-binding proteins such as metallothioneins (Zhou and Goldsbrough, 1994) and metal transporters such as P-type ATPases of which there are eight in *Arabidopsis* (Axelsen and Palmgren, 2001). Specialized thiol compounds such as phytochelatins (PCs) are used by cells to detoxify excesses of various metals (Cobbett, 2000).

Iron-sulfur clusters contain S as sulfide ($S^{2-}$) (Beinert *et al.*, 1997). The most abundant forms in plants are 2Fe-2S or 4Fe-4S complexes. These cofactors are bound to thiol groups of proteins. Iron-sulfur clusters are redox active due to the capacity of Fe to reversibly take up an electron. This is used, for instance, in components of the chloroplast electron transport chain (Raven *et al.*, 1999). FeS clusters can also have a catalytic role in enzymes other than redox activity, as occurs in aconitase.

Other S groups are found in methylsulfonium compounds, which contain S as $(CH_3)_2S^+-$. Examples are S-adenosylmethionine (SAM, also called AdoMet), DMSP and S-methylmethionine. Furthermore, S occurs in its most oxidized state as sulfate esters ($-SO_4^-$) in a variety of sulfated compounds including sulfated hormones, glucosinolates, peptides and flavonoids. Sulfur can also be present as a thioether ($-S-$), as in methionine, thiamine and biotin. Sulfonic acids, which contain S as $-SO_3^-$, are found in glucose-6-sulfonate, cysteic acid, and sulfolipids. Sulfoxides, as $-SO-$, occur in compounds such as the secondary plant compound allicin.

## III. Biosynthesis and Functions of S Compounds

The flow of S in plants can be summarized as follows. Most S is taken up as sulfate, which is first activated and then reduced to sulfite and finally sulfide. Sulfide is subsequently incorporated into cysteine (Cys). Two pathways branch from this sulfate reduction pathway. Activated sulfate can be used to produce a range of sulfated compounds, and sulfite is used for the production of sulfolipids. Cys holds a central position, from which

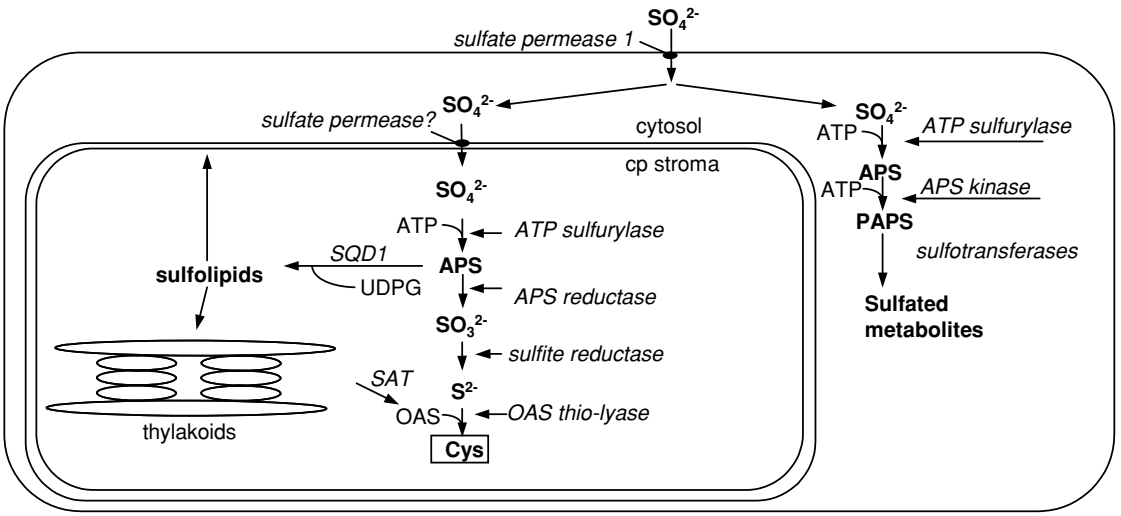


*Fig. 1.* Sulfur assimilation in the plastid and cytosol of higher plants from sulfate to Cys. SAT: serine acetyl transferase; OAS: O-acetylserine.

S can go in several directions: (i) S can be transferred to methionine and its derivatives; (ii) S from Cys can be released and incorporated into various cofactors or coenzymes, and (iii) Cys can be incorporated into proteins or into the peptide glutathione and its derivatives. Cys can also be stored in methylated form. The various pathways of S metabolism, and the important role of plastids in these processes are discussed in more detail below. They are also depicted in Fig. 1 and 2.

## *A. From Sulfate to Cysteine*

The main form of S taken up into plants by roots is sulfate. Sulfate is transported over membranes via secondary active transport by sulfate-$H^+$ cotransporters, driven by the proton motive force created by ATPase (Smith *et al.*, 1995). Fourteen genes encoding sulfate transporters (sulfate permeases) have been reported for *Arabidopsis thaliana*, which can be divided over five groups. These genes differ in tissue-specific expression

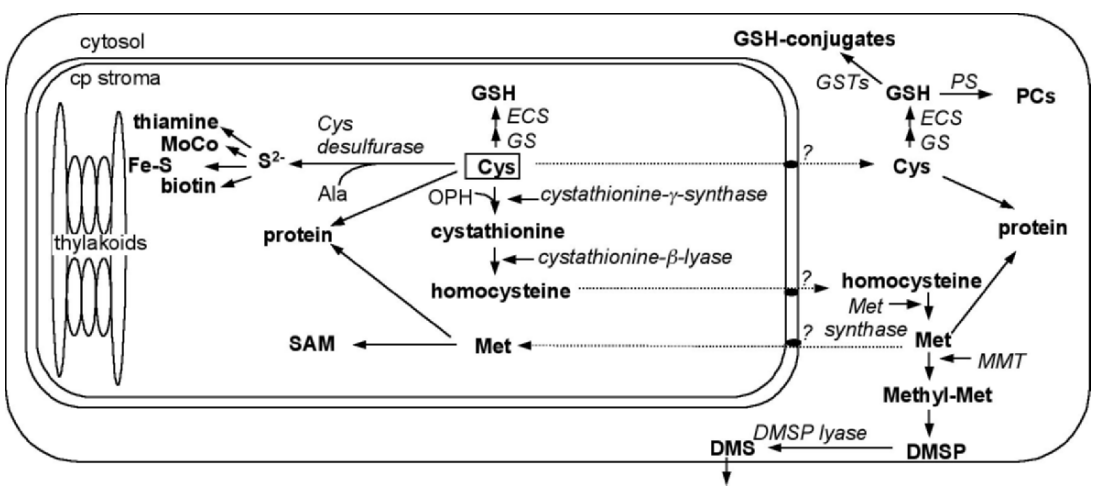


*Fig. 2.* Overview of Cys metabolism to other S compounds in plastid and cytosol of higher plants. ECS: γ-glutamylcysteine synthetase; GS: glutathione synthetase; PS: phytochelatin synthase; GSTs: glutathione-S-transferases; MMT: Met methyltransferase; DMSP: dimethylsulfoniumpropionate; DMS: dimethylsulfide.

and subcellular localization of the gene product (for a review see Hawkesford, 2003).

The assimilation of sulfate into Cys takes place mainly in the chloroplast. In C4 plants this happens in the chloroplasts of the bundle sheath cells (Schmutz and Brunold, 1984). On its way from the soil to the chloroplast, sulfate enters the plant via group-1 high-affinity sulfate transporters in the plasma membrane (Smith *et al.*, 1995; Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002). Translocation of sulfate to the shoot via the xylem appears to be facilitated by sulfate transporters from groups-4, -3 and -2 in *Arabidopsis* roots, involved in vacuolar efflux (sultr4;1 and 4;2) and xylem loading (sultr3;5 and 2;1) respectively (Takahashi *et al.*, 1997, 2000; H. Takahashi, personal communication). Sulfate is taken up from the xylem into leaf mesophyll cells, perhaps by the combined action of group 2 and 3 sulfate transporters (Takahashi *et al.*, 1999; Grossman and Takahashi, 2001). In the cytosol the sulfate concentration is in the mM range (Schroppel-Meier and Kaiser, 1988). From the cytosol, sulfate is transported into the chloroplasts. There may be a $H^+$-sulfate co-transporter in the chloroplast envelope, but so far none has been identified unequivocally (Hawkesford, 2003). Alternatively, or additionally, sulfate may be taken up into chloroplasts in exchange for phosphate (Hampp and Ziegler, 1977; Mouroux and Douce, 1979). Another source of S for leaves in areas with high industrial activity is $SO_2$ from the air. Most of $SO_2$ entering leaves is oxidized to sulfate; a smaller fraction may also be reduced to $H_2S$ gas and emitted as a detoxification mechanism (Wilson *et al.*, 1978).

Sulfate is activated by reaction with ATP to form adenosine-5-phosphosulfate (APS). This reaction is catalyzed by ATP sulfurylase. The predominant isoform of this enzyme is located in the plastids, but there is also a minor cytosolic form. The two isoforms are regulated differently (Rotte and Leustek, 2000). In the cytosol the resulting APS is further phosphorylated by APS kinase to PAPS, which is used as a sulfate donor by cytosolic sulfotransferases. These sulfation reactions produce a variety of sulfated compounds including hormones (e.g. brassinosteroids, gallate glucosides), glucosinolates (Poulton and Moller, 1993), peptides and flavonoids (Ananvoranich *et al.*, 1994; Lacomme and Roby, 1996; Hell, 1997).

In the chloroplasts APS is reduced by APS reductase (also called APS sulfotransferase) to sulfite (Setya *et al.*, 1996). The two electrons probably come from glutathione (GSH) (Prior *et al.*, 1999). Sulfite may either be used for the production of sulfolipids or further reduced to sulfide, both in the chloroplast. When used for sulfolipid production, sulfite is first coupled to UDP-glucose to form UDP-sulfoquinovose, catalyzed by the SQD1 enzyme (Sanda *et al.*, 2001). UDP-sulfoquinovose is then coupled to diacylglycerol to form the sulfolipid SQDG (sulfolipid 6-sulfo-α-D-quinovosyl diacylglycerol), which is an important component of chloroplast membranes and may contain as much as one third of leaf S (Harwood and Nicholls, 1979; Schmidt, 1986). These sulfolipids are unique to plastids and required for plastid functions including photosynthesis (Yu and Benning, 2003).

The further reduction of sulfite to sulfide is mediated by sulfite reductase, a plastidic enzyme (Bork *et al.*, 1998). The six electrons needed for this step are thought to come from ferredoxin (Yonekura-Sakakibara *et al.*, 2000). Sulfide is incorporated into Cys by coupling to O-acetylserine (OAS). This reaction is mediated by the enzyme OAS thiol lyase, also called cysteine synthase. The OAS needed for this reaction is produced by serine acetyltransferase (SAT). As only plastidic forms of APS reductase and sulfite reductase have been found, reduction of sulfate to sulfide is thought to occur exclusively in plastids. Because of the higher reducing power in the photosynthetic chloroplasts, most of sulfate reduction probably happens in chloroplasts, although non-green plastids also perform sulfate reduction. Cys synthesis from sulfide and OAS occurs predominantly in the chloroplast, although there are also cytosolic and mitochondrial isoforms of serine acetyltransferase and OAS thiol lyase (Noji *et al.*, 1998). After formation, Cys is rapidly converted to other compounds in the chloroplast or other compartments. Therefore, the Cys concentration in the cell is quite low (μM range).

### *B. Cysteine in Proteins*

Much of Cys is incorporated into proteins, either in the plastids or in the cytosol. Cys is coupled to a specific tRNA by an amino acyl-tRNA synthetase, delivered to a growing polypeptide chain and incorporated. Cys residues in proteins often serve an important role in protein structure and function. The structural importance is due to the capacity of two Cys thiol groups to form a disulfide bond, which can contribute to protein tertiary and quaternary structure. Disulfide bonds are formed mainly in the ER and are especially important for the structural integrity of secreted proteins, because these proteins occur in more oxidized environments (Braakman *et al.*, 1992). In intracellular proteins, thiols are mostly in a reduced state, where conditions are more reducing. The reducing power of these Cys thiol groups can be used to reduce other cell components.

For instance, in chloroplasts the redox capability of Cys in thioredoxin is crucial for the regulation of photosynthetic enzymes (Buchanan *et al.*, 2002). In the light, ferredoxin is reduced by electrons from photosystem I and in turn reduces thioredoxin, which activates stromal Calvin-Benson cycle enzymes via reduction of thiol groups. In the dark the regulatory thiol groups on these enzymes become oxidized for lack of reduced ferredoxin and thioredoxin, rendering the enzymes inactive. In this way the light reactions and the Calvin-Benson cycle of photosynthesis are synchronized to be active only in the light.

### C. From Cysteine to Methionine and its Derivatives

The S in Cys can be transferred to methionine (Met) in three steps. First cystathionine-γ-synthase (CgS) couples Cys to O-phosphohomoserine (OPH) to form cystathionine. The enzyme cystathionine-β-lyase (CbL) then splits cystathionine into homocysteine, ammonia and pyruvate. These first steps occur in plastids. In the third step homocysteine is methylated by Met synthase to form Met. Met synthase has so far only been found in the cytosol. If there indeed is no plastidic isoform, then homocysteine must be transported out of the plastid and Met has to be transported back into the plastid via as yet unknown transporters (Ravanel *et al.*, 1998a).

Met can be incorporated into proteins either in plastids or in the cytosol. Most of the synthesized Met is converted to SAM via reaction with ATP by SAM synthetase. This may occur in the chloroplast or cytosol, but probably happens in the cytosol. SAM is then transported into plastids via an unknown transporter. SAM is involved in many reactions in different cell compartments including the plastids. It serves as methyl donor for the synthesis of a variety of plant compounds and is the precursor for the plant growth regulators ethylene and polyamines (Ravanel *et al.*, 1998a). SAM can be reconverted to homocysteine in the cytosol. Antisense plants for one of the enzymes involved, S-adenosyl-L-homocysteine hydrolase (SAHH), showed aberrant growth and flower morphology and reduced DNA methylation (Tanaka *et al.*, 1997) suggesting that this enzyme regulates the expression of genes involved in plant growth and flower development.

Met can be methylated in the cytosol to S-methyl-Met by the enzyme Met methyl transferase (MMT), using SAM as a methyl donor. Methyl-Met can be transported long-distance via the phloem where it can reach concentrations that are higher than GSH (Bourgis *et al.*, 1999). Other possible fates of methyl-Met are reconversion to Met or further conversion to dimethylsulfoniumpropionate (DMSP) (James *et al.*, 1995). This compound can serve as an osmoprotectant in *Spartina alterniflora* (Kocsis *et al.*, 1998). It also serves as the substrate for production of volatile dimethylsulfide (DMS) by DMSP lyase (Dacey *et al.*, 1987). Sulfur volatilization is a common property of plants, and may serve as a valve to get rid of excess S. Other such valves may be excretion in glands or in the rhizosphere. An alternative function for the emission or excretion of S compounds may be that they serve an allelopathic or antimicrobial function, or perhaps as signaling compound to symbiotic partners.

### D. From Cysteine to Glutathione and its Derivatives

About 2% of the organic reduced S in the plant is present in the form of non-protein thiols, and around 90% of this fraction is glutathione (γ-Glu-Cys-Gly, GSH) (Rennenberg, 1982; de Kok and Stulen, 1993). Glutathione is synthesized enzymatically in both the plastids and the cytosol (Noctor *et al.*, 1998a). Glu and Cys are combined by γ-glutamylcysteine synthetase (γ-ECS), followed by addition of Gly by glutathione synthetase (GS). Both enzymes require ATP.

Cellular GSH levels in plants can be in the millimolar range, with more than 50% localized in the chloroplasts (Foyer and Halliwell 1976; Rennenberg and Lamoureux, 1990). Two GSH molecules can be oxidized reversibly to one molecule of GSSG, yielding two protons and two electrons, which may be used to reduce other cell components. GSSG can be reduced back to GSH by glutathione reductase (GR), using NADPH as a source of protons and electrons. An isoform of glutathione reductase occurs in the chloroplast and another in the cytosol, but the predominant form is plastidic (Foyer and Halliwell, 1976). Most of glutathione is present in the reduced form in unstressed cells (Kunert and Foyer, 1993).

As described above, the thiol group of Cys enables it to reduce other molecules and to bind metals. These properties enable GSH to play many important roles in the cell (for reviews see Kunert and Foyer, 1993; Noctor *et al.*, 1998b). Next to maintaining a reducing environment, GSH functions include storage and transport of reduced S, protection of cells against oxidative stress, detoxification of xenobiotics and heavy metals (Cobbett *et al.*, 1998), and redox regulation of gene expression.

Glutathione may be used in the cytosol as a substrate for synthesis of phytochelatins (Grill *et al.*, 1989).

Phytochelatins (PCs) are metal-binding peptides with the general structure $(Glu\text{-}Cys)_nGly$, where typically n = 2 to 5 (Cobbett, 2000). They are synthesized by phytochelatin synthase, which catalyzes the elongation of the $(\gamma\text{-}Glu\text{-}Cys)_n$ by transferring a $\gamma$-GluCys group from GSH to PC (or to another GSH). Phytochelatin synthase is constitutively expressed but needs to be activated by metals. Hence, PCs are only produced in response to metal stress and probably mainly function in metal tolerance, especially to Cd and As (Cobbett, 2000; Pickering *et al.*, 2000). Following binding of metals by PCs, the complex is transported to the vacuole by an ABC-type transporter and further complexed by sulfide. The sulfide is produced in the cytosol from cysteine sulfinate and imported into the vacuole (Cobbett, 2000). Phytochelatins were shown recently to be transported from root to shoot in plants (Gong *et al.*, 2003). The mode of PC transport over the cell membrane is yet to be elucidated. Recently a rice GSH transporter with a wide substrate specificity was reported, which was hypothesized to transport GSH derivatives over the cell membrane (Zhang *et al.*, 2004) and may also transport PCs.

Another fate of GSH in the cytosol is to serve as a substrate for GSH-conjugation by GSH-S-transferases (GSTs, Marrs, 1996). The GSH-S-transferases occur as a family with hundreds of members, each with different substrate specificity. Their substrates vary from secondary plant compounds like anthocyanins to xenobiotics such as the herbicide atrazine (for a review about GSH transferases, see Marrs, 1996). GS-conjugates can be transported to the vacuole by the GS conjugate vacuolar pump, an ABC transporter (Rea, 1999), where they may be metabolized to Cys-conjugates (Lamoureux and Rusness, 1993).

GSH can be transported in the phloem (Blake-Kalff *et al.*, 1998). As mentioned above, a GSH transporter has been reported that may be involved in the transport of GSH and its derivatives over the cell membrane (Zhang *et al.*, 2004). Glutathione can be broken down on the outer surface of the plasma membrane (and perhaps other locations) by $\gamma$-glutamyl transpeptidase, releasing Cys-Gly, which is subsequently broken down into Cys and Gly by a dipeptidase (Storozhenko *et al.*, 2002).

### *E. Cysteine as a S Donor for Cofactors and Coenzymes*

Cysteine can be converted to Ala and sulfide by Cys desulfurases (CysD). These are NifS-like proteins, which are related in structure to the NifS protein from *Azotobacter vinelandii* (Zheng *et al.*, 1993). In *Arabidopsis*, one NifS-like enzyme has been reported in plastids (Leon *et al.*, 2002; Pilon-Smits *et al.*, 2002), while a second form may be present in mitochondria (Kushnir *et al.*, 2001). Cys desulfurase enzymes function to provide reduced S for the production of Fe-S clusters as well as several coenzymes (Mihara and Esaki, 2002). In chloroplasts, Fe-S clusters play a key role in photosynthesis as well as reduction reactions. The capacity of the Fe atom in Fe-S clusters to reversibly take up an electron provides the electron carrier capacity of many components of the electron transport chain in the thylakoid membrane. Fe-S clusters are required for the function of the cytochrome $b_6f$ complex (one 2Fe-2S cluster in the Rieske FeS protein), photosystem I (three 4Fe-4S clusters) and ferredoxin (one 2Fe-2S cluster) (Raven *et al.*, 1999). The capacity of the Fe atom in Fe-S clusters to be reversibly reduced and oxidized is also used in chloroplast reduction pathways such as nitrite reductase and sulfite reductase (Lancaster *et al.*, 1979; Krueger and Siegel, 1982).

Assembly of the FeS cluster into freshly imported ferredoxin precursor, obtained by in vitro translation, was demonstrated using isolated intact chloroplasts (Li *et al.*, 1990). The reaction proceeds in the absence of cytosol (Pilon *et al.*, 1995). These experiments strongly suggest the presence of an Fe-S cluster formation machinery in chloroplasts. Since Cys was identified as a source for Fe-S formation in chloroplasts (Takahashi *et al.*, 1986; Takahashi *et al.*, 1990), a protein with Cys desulfurase activity is likely involved in this process. Indeed, the plastidic Cys desulfurase from *A. thaliana* shows capacity for in vitro reconstitution of apo-Fd to Fd, and thus may serve a role in providing sulfide for Fe-S cluster formation (H. Ye, M. Pilon and E.A.H. Pilon-Smits, unpublished results).

Cys desulfurase may also provide reduced S for synthesis of the coenzymes biotin, thiamine, Molybdenum cofactor (MoCo), lipoic acid and Coenzyme-A, by analogy with bacterial pathways (Amrani *et al.*, 2000; Leimkuhler and Rajagopalan, 2001; Mihara *et al.*, 2002). In plants, MoCo is an essential component of nitrate reductase and several other enzymes. MoCo deficiency leads to reduced nitrate reductase activity and N depletion, as well as reduced phytohormone synthesis (Stallmeyer *et al.*, 1999). Cys provides S for production of the pantetheine moiety of Coenzyme-A, which has many important functions in primary plant metabolism (Kupke *et al.*, 2003). Thiamine and lipoic acid are involved in the decarboxylation of pyruvate to acetyl-Coenzyme-A. Biotin is the carrier for activated $CO_2$ in the conversion of acetyl-CoA to malonyl-CoA in fatty

acid synthesis (Wood and Barden, 1977). The thiamine biosynthesis pathway is thought to be located in the chloroplast (Belanger *et al.*, 1995).

### *F. Other Fates of S in Cysteine*

Two Cys molecules can be reduced reversibly via a disulfide bridge to form cystine, which may function in keeping other cell components reduced or play a role in storage of S. Another fate for Cys is methylation (or alkylation) to S-methyl-Cys (alkyl-Cys). This reaction probably occurs in the cytosol by Cys methyltransferase (Thompson and Gering, 1966). The function could be S storage, because this is a non-protein amino acid.

There are a variety of additional secondary S compounds that may be derived from Cys and that occur only in certain species. These compounds may play an ecological role by warding off microbial infection or herbivory (Virtanen, 1965; Ernst, 1990; Sendl, 1995). Relatively little is known about the biosynthesis of many of these compounds, some of which have important applications as pharmaceuticals (Hell, 1997).

## IV. Regulation of S Metabolism

### *A. Rate-Limiting Steps in S Pathways*

Sulfate assimilation is regulated by S status. When the amount of S in the plant is low, many enzymes involved in S acquisition and reduction are up-regulated, including sulfate permease, ATP sulfurylase and APS reductase (Gutierrez-Marcos *et al.*, 1996; Takahashi *et al.*, 2000). Expression of the gene encoding APS reductase is most closely correlated with S status and this enzyme is suspected to be a rate-controlling enzyme for the pathway (Leustek *et al.*, 2000). There is also indication that ATP sulfurylase may be limiting for sulfate uptake and assimilation, because over-expression of the gene resulted in higher plant levels of both reduced and total S (Pilon-Smits *et al.*, 1999). Another potentially limiting enzyme for Cys formation may be serine acetyltransferase, because over-expresion in cytosol and plastids resulted in 3-fold and 6-fold higher Cys levels, respectively (Wirtz and Hell, 2003). The OAS thiol lyase protein is present at levels 2 orders of magnitude higher than serine acetyltransferase in chloroplasts (Droux *et al.*, 1998) and hence not expected to be limiting. This is in agreement with the finding that transgenic plants that over-express OAS thiol lyase do not contain more Cys (Saito *et al.*, 1994).

The regulatory mechanism for sulfate uptake and reduction to cysteine includes regulation at the transcriptional level in response to plant S status. When S limitation is sensed, up-regulation of several sulfate transporters as well as ATP sulfurylase and APS reductase occurs. The main shoot-to-root molecule that signals plant S status to the root appears to be GSH (Lappartient and Touraine, 1996; Lappartient *et al.*, 1999), although Cys and intracellular sulfate have also been reported to control uptake of sulfate (Smith, 1975). When these compounds accumulate, S uptake and reduction are down-regulated. Another regulatory molecule is OAS, which up-regulates sulfate uptake and assimilation and can overrule internal S status (Neuenschwander *et al.*, 1991; Smith *et al.*, 1997).

Some regulation of Cys formation also occurs at the level of the two enzymes serine acetyltransferase and OAS thiol lyase (Bogdanova and Hell, 1997; Droux *et al.*, 1998), which can exist as a complex as well as separately. When complexed with serine acetyltransferase, OAS thiol lyase does not synthesize Cys. On the other hand, serine acetyltransferase requires complexation with OAS thiol lyase to produce OAS. The formation of the complex is favored by sulfide, while OAS destabilizes the complex. Thus, when sulfide accumulates, serine acetyltransferase is activated and produces OAS, which then activates OAS thiol lyase. When the resulting reduced organic S compounds accumulate, the rates of further uptake and reduction of sulfate decrease.

The rate-controlling enzyme for Met synthesis from Cys is thought to be cystathionine-γ-synthase. Both cystathionine-γ-synthase and threonine synthase (TS) use OPH as a substrate, and the in vitro affinity for OPH is two orders of magnitude higher for threonine synthase than for cystathionine-γ-synthase (Curien *et al.*, 1998; Ravanel *et al.*, 1998b; Amir *et al.*, 2002). If a similar affinity occurs in vivo, then most of the carbon flux would go towards threonine (Amir *et al.*, 2002), making cystathionine-γ-synthase a likely rate-limiting step for Met synthesis. This hypothesis is supported by results from mutant and transgenic *Arabidopsis* and potato plants with reduced threonine synthase activity, which showed a substantial increase in Met levels and a small decrease in threonine levels, suggesting that reduced threonine synthase activity increases the flow of carbon towards Met (Bartlem *et al.*, 2000; Zeh *et al.*, 2001). Furthermore, mutant and transgenic *Arabidopsis* plants with increased cystathionine-γ-synthase activity accumulated up to 40 times more Met (Inba *et al.*, 1994; Chiba *et al.*, 1999; Suzuki *et al.*, 2001; Hacham *et al.*, 2002; J. Kim *et al.*, 2002). On the

other hand, antisense *Arabidopsis* plants with a 5- to 20-fold reduction in cystathionine-γ-synthase concentration showed no more than a 35% reduction in Met levels (Gakiere *et al.*, 2000; J. Kim and Leustek, 2000).

Neither cystathionine-γ-synthase nor cystathionine-β-lyase appear to be feed-back inhibited by end products at the protein level (Ravanel *et al.*, 1998a,b). However, cystathionine-γ-synthase appears to be feed-back regulated by Met at the transcript level (Giovanelli *et al.*, 1985). In *A.thaliana,* cystathionine-γ-synthase transcript levels were reduced by Met by autoregulation. A cystathionine-γ-synthase amino acid sequence encoded by exon-1 can act in *cis* to destabilize its own mRNA when activated by Met or one of its metabolites (Chiba *et al.*, 1999). A regulatory role for cystathionine-γ-synthase rather than cystathionine-β-lyase is supported by the observation that over-expression of cystathionine-β-lyase does not enhance metabolic flux toward Met (Maimann *et al.*, 2001).

The regulatory enzyme for GSH synthesis under unstressed conditions is thought to be γ-glutamylsynthetase (Noctor *et al.*, 1996, 1998a,b). Under metal stress, γ-glutamylsynthetase activity is up-regulated both at the transcription level and the enzyme activity level, and GSH synthetase may become co-limiting (Zhu *et al.*, 1999a). Since Cys levels are very low in cells, the supply of Cys is another potential limiting factor for GSH formation (Noctor *et al.*, 1996). This limitation may be alleviated to some extent by metal-induced up-regulation of sulfate uptake and assimilation (Leustek *et al.*, 2000; Nocito *et al.*, 2002).

A substantial part of the synthesized Met is used for the formation of SAM. This molecule can act as an enzyme activator and thus influence metabolic fluxes through pathways. For instance, SAM activates threonine synthase, leading to enhanced threonine synthesis and reduced Met synthesis (Ravanel *et al.*, 1998a). Also, as a precursor of the plant growth regulators ethylene and polyamines, SAM influences overall plant growth and development.

### *B. Regulation of S Metabolism in Response to the Environment*

As mentioned above, S limitation induces sulfate uptake and assimilation at the transcriptional level, with GSH as an important signal molecule. While uptake and reduction of S are enhanced under S limitation, the synthesis of secondary S compounds (e.g. sulfation) is down-regulated, and secondary S compounds such as glucosinolates are even broken down to provide S for essential compounds (Maruyama-Nakashita *et al.*, 2003). Sulfur limitation also affects the expression of seed storage proteins (H. Kim *et al.*, 1999), the rate of photosynthesis (Wykoff *et al.*, 1998) and protein turnover (Gilbert *et al.*, 1997). Conversely, when photosynthesis is reduced, sulfate assimilation is reduced as well. Accumulation of AMP and ADP were reported to inhibit ATP sulfurylase (Schwenn and Depka, 1977), offering a partial explanation of the mechanism involved.

The S assimilation pathway appears to also be developmentally regulated, and to be most active in young tissues (Rotte and Leustek, 2000), perhaps because in this time of rapid growth most reduced S is needed for protein synthesis. Light also stimulates S assimilation, as more reduced Fd is available for S reduction.

The S assimilation pathway is also regulated in coordination with nitrogen (N) assimilation and the ratio of reduced S to reduced N is typically maintained at 1:20 (Buchanan *et al.*, 2000). Reduced S compounds activate the key enzyme of N reduction, nitrate reductase. Similarly, reduced N compounds stimulate the key enzymes of S reduction, ATP sulfurylase (Reuveny *et al.*, 1980) and APS reductase (Koprivova *et al.*, 2000) at the transcription level. The same reduced S and N compounds feed-back inhibit the key enzymes of their own biosynthetic pathways. Stimulation of S uptake and assimilation under low-S conditions does not happen when N is limiting, suggesting there is an N compound necessary for de-repression of S pathway enzymes (Yamaguchi *et al.*, 1999).

Sulfur deprivation leads to oxidative stress due to a lack of GSH. This was demonstrated by the observation that pathways involved in stress resistance were up-regulated under S limitation in *Arabidopsis* in a transcriptome profiling study (Maruyama-Nakashita *et al.*, 2003). Under other conditions of oxidative stress such as the presence of heavy metals, there is an increased demand for reduced S compounds like GSH, Cys and PCs. Hence, genes involved in uptake and reduction of sulfate are up-regulated at the transcription level under these conditions (Leustek *et al.*, 2000; Nocito *et al.*, 2002), as are genes involved in formation of GSH and PCs (Xiang and Oliver, 1998).

The regulatory proteins involved in S sensing and signal transduction in higher plants are not known. In *Chlamydomonas reinhardtii* several genes (*SAC1* to *SAC3*) were identified that are involved in sensing S deficiency and mediating many physiological responses (Davies *et al.*, 1994). *SAC1* encodes a membrane protein with similarity to sodium dicarboxylate transporters (Davies and Grossman, 1998; Ravina *et al.*, 2002). *SAC1*-deficient mutants are unable to

sense S deficiency and lack the associated increases in S-associated enzyme activities (Ravina *et al.*, 2002). *SAC2* appears to regulate APS reductase activity post-transcriptionally (Ravina *et al.*, 2002), while *SAC3* encodes a putative serine-threonine kinase (Davies *et al.*, 1999). In higher plants no homolog of *SAC1* has been found, but there are 12 homologs of *SAC3* (Ravina *et al.*, 2002). Some of these proteins may be involved in regulating plant S metabolism. Transcriptome analysis in *Arabidopsis* under S deficiency also yielded a number of putative transcription factors that were up-regulated under S deficiency and may function in signaling cascades of stress response (Maruyama-Nakashita *et al.*, 2003).

## V. Involvement of S Pathways in Metabolism of Other Oxyanions

There is abundant evidence that enzymes of the S pathway also metabolize analogs of the related element selenium (Se). All sulfate transporters tested can also transport selenate (Smith *et al.*, 1995; Hawkesford, 2003). Sulfate transport is inhibited by sulfite, selenate, arsenate, chromate, molybdate, and tungstate, again indicating that sulfate transporters may be involved in transport of these related oxyanions (Wilson and Bandurski, 1958; Leustek, 1996). Indeed, over-expression of a type-1 sulfate transporter resulted in enhanced accumulation of Se, Cr, V and W (E.A.H. Pilon-Smits *et al.*, unpublished results), indicating that sulfate permease mediates uptake of these oxyanions in vivo. ATP sulfurylase, the enzyme that activates sulfate by binding it to ATP, was also shown to react with selenate as well as molybdate in vitro (Wilson and Bandurski, 1958). Over-expression of ATP sulfurylase in plastids resulted in higher Se accumulation, more reduced organic Se, and higher Se tolerance (Pilon-Smits *et al.*, 1999), demonstrating the involvement of this enzyme in selenate reduction in vivo. More recently, over-expression of ATP sulfurylase was shown to also result in enhanced accumulation of the oxyanions of As, Cr, Cu, Mo, V and W (Wangeline *et al.*, 2004). It is not clear at this point whether the end-products of ATP and these other oxyanions are stable. Results from in vitro studies indicate that besides sulfate only selenate can be further metabolized by the sulfate reduction pathway (Wilson and Bandurski, 1958).

Over-expression of OAS thiol lyase did not affect Se accumulation or tolerance (de Souza *et al.*, 2000b), probably because this enzyme is not rate-limiting for these processes, although it is likely involved in SeCys formation. Over-expression of cystathionine-γ-synthase in plastids led to enhanced formation of DMSe, the volatile form of Se analogous to DMS, suggesting this enzyme is involved in, and rate-limiting for, Se volatilization from SeCys (van Huysen *et al.*, 2003). Knockout of Met methyltransferase abolished DMSe production in *Arabidopsis* (Tagmount *et al.*, 2002), showing the involvement of this enzyme in Se volatilization. The analog of DMSP, dimethylselenoniopropionate (DMSeP) was shown to be the precursor of volatile DMSe production (de Souza *et al.*, 2000a).

Based on these results, the entire S assimilation and volatilization pathway appears to be able to use the Se analogs as substrates. The same may be true for most other enzymes in the S pathway. The activity of most S-related enzymes on the Se analogs of their substrates may in most cases be a non-functional but unavoidable side-effect. However, it is also feasible that in some cases these activities have a function in plants. Selenium volatilization may be a way to get rid of excess toxic Se. Another potential way to detoxify Se is the conversion of SeCys to alanine and selenide, thereby preventing non-specific incorporation of SeCys in proteins. Plants may indeed use this strategy, because a plastidic NifS-like protein from *Arabidopsis* was shown to have 300-fold higher in vitro activity with selenocysteine (SeCys lyase activity) than with Cys (Pilon-Smits *et al.*, 2002). Furthermore, its over-expression enhanced Se tolerance and accumulation (Van Hoewyk *et al.*, 2005). Expression of a related mouse NifS-like protein (a SeCys lyase) in *Arabidopsis* also resulted in enhanced Se accumulation, and either enhanced or reduced Se tolerance depending on the intracellular location of the SeCys lyase protein. In these cases, Se incorporation in proteins was reduced (Pilon *et al.*, 2003).

Many organisms (e.g. mammals, bacteria and *Chlamydomonas*) have essential Se metabolism. These organisms need Se because enzymes such as glutathione peroxidase, iodothyronine deiodinase, and formate dehydrogenase require a SeCys in their active site for activity (Stadtman, 1990, 1996). In bacteria, Se is also an essential component of special tRNAs that have a Se-containing uracyl analog in the wobble position (Mihara *et al.*, 2002). For essential Se metabolism, selenate is taken up and assimilated to SeCys by the sulfate reduction pathway described above. SeCys is then converted into selenide and alanine by SeCys lyase activity (Mihara *et al.*, 1999, 2000, 2002). The selenide formed is the substrate for selenophosphate synthase

(Lacourciere and Stadtman, 1998; Lacourciere *et al.*, 2000). The resulting selenophosphate is used for the formation of SeCys-tRNA, which is used in the translation of UGA *opal* codons in specific mRNAs encoding SeCys-containing enzymes (Böck *et al.*, 1991) and for the incorporation of Se into the specific tRNAs (Mihara *et al.*, 2002).

The question whether Se is essential for higher plants is still unanswered. A seleno-form of glutathione peroxidase was found in *Chlamydomonas reinhardtii* (Fu *et al.*, 2002), but there is no unequivocal evidence for selenoenzymes in higher plants. It has been suggested that essential Se metabolism has been lost in higher plants (Novoselev *et al.*, 2002). There is one report of a glutathione peroxidase in *Aloe vera* in which the authors concluded that this enzyme is a selenoprotein (Sabeh *et al.*, 1993). However, this has not been confirmed. There is also some evidence that the machinery for incorporation of Se into selenoproteins is present in plants. A SeCys-tRNA has been found in *Beta vulgaris* that recognizes the UGA anticodon (Hatfield *et al.*, 1992). More studies are needed to investigate whether these proteins and activities are evolutionary relics, unavoidable by-products of S metabolism, or whether essential Se metabolism exists in plants as it does in many animals, algae and bacteria.

SeCys can be methylated to form Se-methyl-SeCys (Neuhierl and Bock, 1996; Neuhierl *et al.*, 1999), which is not incorporated into proteins but accumulates in the free form. The enzyme involved, SeCys methyltransferase (SMT) appears to play an important role in Se tolerance and perhaps also accumulation in certain plant species that hyper-accumulate Se (LeDuc *et al.*, 2004). A related protein is present in non-accumulating plants, as judged from immunoblotting using antibodies raised against a hyper-accumulator SeCys methyltransferase, and from RT-PCR (E.A.H. Pilon-Smits, unpublished results). Whether this protein plays any role in Se metabolism or rather in S metabolism (as a Cys methyltransferase) remains to be elucidated.

## VI. Transgenic Approaches to Study and Manipulate S Metabolism

As already mentioned above, in several instances transgenic approaches have been used successfully to study the involvement and rate-limitation of enzymes in the biochemical pathways of S and related oxyanions. This has given insight into fundamental biological processes but has also potential for breeding plants with favorable properties for human use. For instance, enhanced capacity of plants to extract S from soil may give higher yields on marginal soils, and may enhance plant biotic and abiotic stress resistance. Higher levels of S compounds may also give plants a better flavor, or higher medicinal value. Since S volatilization can influence weather, manipulation of S volatilization may be used to try to promote rain in dry areas. Because of the involvement of S metabolism in accumulation and tolerance of other trace elements (other oxyanions and thiol-bound metals), it may be possible to engineer plants with enhanced capacity to tolerate and accumulate these pollutants from the environment (phytoremediation) by means of manipulating S metabolism.

We will end with a short overview of results obtained so far using plant biotechnology of S metabolism. Via over-expression of sulfate transporters, plants were created that show higher accumulation of S, Se, Cr, V and W (E.A.H. Pilon-Smits and N. Terry, unpublished results). Over-expression of ATP sulfurylase in Indian mustard (*Brassica juncea*) resulted in higher accumulation of S, Se, As, Cr, Cu, Mo, V and W (Pilon-Smits *et al.*, 1999; Wangeline *et al.*, 2004). Over-expression of (bacterial) serine acetyltransferase in tobacco resulted in higher Cys levels and enhanced resistance to oxidative stress (Blaszczyk *et al.*, 1999), while over-expression of OAS thiol lyase in tobacco increased stress resistance to $H_2S$ and sulfite (Youssefian *et al.*, 1993; Saito *et al.*, 1994). Expression of a S-rich seed albumin led to higher Met levels in seeds and increased nutritional value (Molvig *et al.*, 1997). Selenium volatilization and Se tolerance were enhanced by over-expressing cystathionine-$\gamma$-synthase or SeCys methyltransferase (van Huysen *et al.*, 2003; LeDuc *et al.*, 2004). Enhanced Se tolerance and accumulation was achieved by over-expression of enzymes with SeCys lyase activity (Pilon *et al.*, 2003). Over-expression of the GSH-synthesizing enzymes $\gamma$-glutamylcysteine synthetase and GSH synthetase resulted in enhanced metal tolerance and accumulation (Zhu *et al.*, 1999a,b), while over-expression of GSH reductase resulted in increased resistance to oxidative stress in tobacco and poplar trees (Aono *et al.*, 1993; Foyer *et al.*, 1995).

Together these results obtained from manipulation of S metabolism in plants have rendered new information about the enzymes involved in S metabolism and their importance as rate-limiting steps in the various pathways. Also, some of the transgenics obtained may find uses in agriculture, horticulture or environmental cleanup of polluted sites.

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# Chapter 20

# Regulation and Role of Calcium Fluxes in the Chloroplast

Carl Hirschie Johnson*
*Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, U.S.A.*

Richard Shingles
*Department of Biology, Johns Hopkins University, Baltimore, MD 21218, U.S.A.*

William F. Ettinger
*Biology Department, Gonzaga University, Spokane, WA 99258, U.S.A.*

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## Summary

Ionized free calcium ($Ca^{++}$) is a crucial—and practically ubiquitous—regulator of biological processes. There is much information about the regulation of cytosolic $Ca^{++}$ levels and fluxes in plants, but very little is known about its regulation in the chloroplast, despite clear evidence for the importance of calcium in this organelle. The chloroplast contains calcium-binding proteins, such as calmodulin, calcium-regulated kinases, and calcium transport proteins. Although calmodulin-binding proteins are found within the chloroplast, none of the calmodulin sequences in the *Arabidopsis* genome would predict targeting of calmodulin to the chloroplast. This chapter summarizes evidence for $Ca^{++}$ fluxes across chloroplastidal membranes, especially those across the inner membrane and the thylakoid membranes. Light stimulates the uptake of $Ca^{++}$ into the chloroplast, but this $Ca^{++}$ is apparently rapidly bound and/or transported into the thylakoids because the $Ca^{++}$ level within the stroma does not change significantly in the light. The onset of darkness, however, elicits a profound transient increase of stromal $Ca^{++}$ levels. Therefore, $Ca^{++}$ levels are not homeostatically regulated to be constant—rather, changes in the light/dark conditions in the environment influence the level of $Ca^{++}$ in the chloroplast.

*Author for correspondence, email: carl.h.johnson@vanderbilt.edu

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## I. Introduction

### A. Regulation of Ionized Calcium in Plants

Fluxes of ionized calcium ($Ca^{++}$) are one of the primary signaling pathways in plant, animal, and microbial cells. In plants, the effects of $Ca^{++}$ fluxes are usually thought to be modulated by $Ca^{++}$-binding proteins, including calmodulin (CaM) and a class of $Ca^{++}$-dependent but calmodulin-independent protein kinases called calcium-dependent protein kinases (CDPK). CDPKs are found in plants and some protozoa but are absent from animals and fungi (Roberts and Harmon, 1992). In contrast, the $Ca^{++}$/CaM-dependent protein kinases that are abundant in animals seem to be rare in plants (Sanders *et al.*, 2002).

A remarkable number of physiological stimuli elevate cytosolic $Ca^{++}$ levels in plant cells, including light, abscisic acid, gibberellins, touch, osmotic and oxidative stress, fungal elicitors, temperature shocks, nodulation factors, and so on (Knight *et al.*, 1991; Bush, 1995; Trewavas and Malho, 1997; Sanders *et al.*, 1999, 2002). One of the most important of these stimuli that relates to a plant's response to its environment is the increase of cytosolic $Ca^{++}$ elicited by light/dark signals (Millar *et al.*, 1994; Baum *et al.*, 1999; Frohnmeyer *et al.*, 1999).

Although much is known about the regulation of cytosolic and organellar $Ca^{++}$ in plants, prior research has focused on the mechanisms by which cytosolic $Ca^{++}$ is controlled (Bush, 1995; Sanders *et al.*, 1999, 2002), whereas the study of $Ca^{++}$ in organelles has tended to be relegated to determining the potential role of the organelles in regulating cytosolic $Ca^{++}$ levels. In the past few years, a plethora of new information has emerged about the regulation of cytosolic $Ca^{++}$ in plants, particularly the roles of the plasma membrane, the vacuole, and the endoplasmic reticulum (ER) in this process (Sanders *et al.*, 2002). At the plasma membrane, there are a variety of $Ca^{++}$-permeable channels and at least one $Ca^{++}$-pump (an ATPase) that regulate cytosolic $Ca^{++}$ levels by regulating the flux between intracellular $Ca^{++}$ (sub-μM $Ca^{++}$ concentrations) and extracellular $Ca^{++}$ (millimolar $Ca^{++}$ concentrations). The vacuole and ER serve as intracellular $Ca^{++}$ stores to tune cytosolic $Ca^{++}$ levels. Channels, exchangers, and pumps are implicated in the regulation of $Ca^{++}$ fluxes across these endomembranes. $Ca^{++}$ fluxes across the vacuolar and ER membranes appear to be regulated by the messengers inositol triphosphate, cyclic ADP-ribose, and the NADP metabolite nicotinic acid adenine dinucleotide phosphate (Wu *et al.*, 1997; Sanders *et al.*, 1999; Navazio *et al.*, 2000; Sanders *et al.*, 2002).

A profound question that emerges in any discussion of second messenger signaling is the "specificity" issue (Trewavas and Malho, 1997; McAinsh and Hetherington, 1998). How can a simple messenger like $Ca^{++}$ be involved in so many different signal transduction pathways and still convey stimulus specificity to each separate pathway? There are a number of potential answers to this question, including differential tissue specificity, differential dynamics of the $Ca^{++}$ signal, and/or the involvement of different secondary signals. Undoubtedly, however, a major means by which specificity is conferred is by the spatial properties of $Ca^{++}$ signals. One of these spatial properties is the differential compartmentalization of $Ca^{++}$ regulation and fluxes among different organelles. This chapter will address the specificity issue in the context of the chloroplast.

### B. Calcium and Chloroplasts

Unlike the cases of the plasma membrane, vacuole, and ER, very little is known about $Ca^{++}$ regulation within the chloroplast or fluxes across chloroplast membranes (Melkonian *et al.*, 1990; Sanders *et al.*, 2002). Also, unlike the plasma membrane, tonoplast, and ER membrane, the chloroplast is not delimited and defined by a single membrane. Thus, the chloroplast has a set of membranes and subcellular compartments within which $Ca^{++}$ fluxes can be modulated independently. The outer envelope is freely permeable to $Ca^{++}$, in part due to the chloroplast outer envelope solute channel OEP24, which is highly permeable to $Ca^{++}$ ions (Flugge and Benz, 1984; Pohlmeyer *et al.*, 1998; Bolter and Soll, 2001), but the inner envelope is an important site for the regulation of $Ca^{++}$ fluxes as well (Roh *et al.*, 1998, and see below). The thylakoid membrane is the other membrane system at which $Ca^{++}$ fluxes are regulated (Ettinger *et al.*, 1999, and see below). At least two bulk phases are relevant for the regulation of $Ca^{++}$

*Abbreviations*: ARAMEMNON – integrates a list of *Arabidopsis* proteins that are predicted to be membrane localized with multiple signal peptide prediction programs to enhance the analysis of their subcellular localization (Schwacke *et al.*, 2003); BCaM – biotinylated CaM; CaM – calmodulin; CDPK – calcium-dependent protein kinase; DCMU – diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea); ER–endoplasmic reticulum; LD – light/dark cycle, e.g., LD 8:16 = 8 h light, 16 h dark; LD 16:8 = 16 h light, 8 h dark; OEC – oxygen-evolving complex of photosystem II; PSORT – is a computer tool that predicts the targeting of proteins to subcellular compartments based upon the N-terminal amino acid sequence (Nakai and Horton, 1999).

levels, the stroma and the thylakoid lumen. Based on the voluminous literature on $Ca^{++}$ as a signaling molecule, it is our working hypothesis that $Ca^{++}$ fluxes within the chloroplast can play signaling roles. In spite of the fact there is no direct evidence to support this hypothesis, the chloroplast contains CDPKs and CaM-binding proteins (see I.B.2 below) that presumably are involved in signaling, so this working hypothesis is very likely to be correct.

### *1. Calcium is Required within the Chloroplast*

Independent of the likelihood of $Ca^{++}$ signaling within the chloroplast, calcium in both its free and bound forms is absolutely required for several essential processes inside the chloroplast. In the thylakoid, calcium ions are necessary for the function and structural assembly of the oxygen-evolving complex (OEC) of photosystem II (PSII). The PSII OEC is a multimeric complex in the thylakoid lumen responsible for light—dependent oxygen evolution in plants. Functional assembly of PSII and the OEC requires that all essential polypeptides and cofactors are present in the stroma, thylakoid membrane or thylakoid lumen. Significantly, the construction of the OEC inside the thylakoid lumen requires the assembly of the OE33, OE23, and OE17 polypeptides and essential inorganic ions $Mn^{++}$, $Ca^{++}$, and $Cl^{-}$ to the OEC in a light-dependent process (Becker *et al.*, 1985; Ghanotakis *et al.*, 1984; Miller and Brudvig, 1989). Additionally, in saturating light the reaction center D1 protein of PSII is rapidly damaged in a process known as photoinhibition (Mattoo *et al.*, 1989). Damage resulting from photoinhibition is repaired by the proteolytic degradation of the D1 protein, followed by the disassembly of other remaining PSII proteins and OEC polypeptides, the resynthesis of D1, and the reassembly of a new PSII core and OEC from existing polypeptides and ions (Broussac *et al.*, 1990; Hundal *et al.*, 1990a, b; Virgin *et al.*, 1990). Therefore, both the initial assembly of PSII and its subsequent reassembly after photoinhibition require calcium availability in the thylakoid lumen. Furthermore, $Ca^{++}$ in the thylakoid lumen has been implicated in the stabilization of the high redox potential form of cytochrome *b–559* (McNamera and Gounaris, 1995). All of these processes require the availability of $Ca^{++}$ in the thylakoid lumen.

Within the stroma, many proteins bind $Ca^{++}$ (Melkonian *et al.*, 1990). For example, fructose-1,6-*bis*phosphatase (a key Calvin-Benson cycle enzyme) is activated by low concentrations and inhibited by high concentrations of $Ca^{++}$ (Hertig and Wolosiuk, 1983; Kreimer *et al.*, 1988). Moreover, NAD kinase, which catalyzes the conversion of NAD to NADP and is therefore crucial to providing NADP for photosynthetic reduction to NADPH, is $Ca^{++}$-activated, light-regulated, and is present in the chloroplast stroma (Muto *et al.*, 1981; Jarrett *et al.*, 1982). Finally, high $Ca^{++}$ concentrations within the stroma tend to inhibit photosynthetic $CO_2$ fixation (Portis and Heldt, 1976; Demmig and Gimmler, 1979) at sites other than FBPase.

### *2. Chloroplastidic $Ca^{++}$ and/or CaM-binding Proteins: $Ca^{++}$ Signaling within the Chloroplast?*

The initial description of NAD kinase activation in pea (Muto and Miyachi, 1977) eventually led to the discovery of calmodulin in plants (Anderson *et al.*, 1980). Calmodulin mediates the activation of many calcium-dependent enzymes and is present in both the cytosol and chloroplasts of peas (Jarrett *et al.*, 1982). Putative calmodulin binding domains have been identified on a number of calcium-dependent enzymes such as phosphodiesterase, adenylate cyclase, calcium-dependent protein kinases and protein phosphatase (Zielinski, 1998).

A number of CaM-binding proteins can be detected in the pea chloroplast stroma, thylakoid membranes

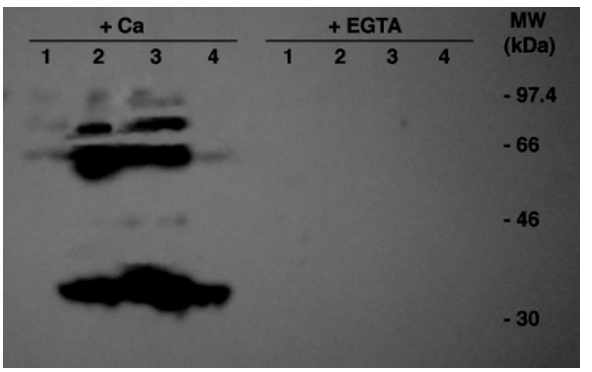


*Fig. 1.* CaM-binding proteins associated with chloroplasts. Proteins extracted from the chloroplast stroma, thylakoid membrane and inner and outer envelope membranes of peas were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was incubated with biotinylated-CaM in the presence of 5 mM $Ca^{++}$ or 5 mM EGTA. The protein-BCaM conjugates were reacted with streptavidin horseradish peroxidase and detected by chemiluminescence. Lane 1 = stromal proteins, Lane 2 = thylakoid proteins, Lane 3 = inner envelope proteins, Lane 4 = outer envelope proteins. C.H. Johnson, R. Shingles and W.F. Ettinger, unpublished data

and inner and outer envelope membranes (Fig. 1). Biotinylated CaM (BCaM) reacts strongly with proteins found associated with the membranes of the chloroplast. Binding was dependent upon the presence of $Ca^{++}$ as addition of 5 mM EGTA completely abolished BCaM binding. The 95 kDa protein that binds to BCaM (Fig. 1) also appears to cross react with a peptide antibody against a putative $Ca^{++}$-ATPase (see next paragraph). Further identification of other CaM-binding proteins extracted from isolated chloroplasts has yet to be performed.

Current evidence also supports a widespread distribution of CaM-regulated $Ca^{++}$-ATPase activities in plant cells (Evans *et al.*, 1991). In *Arabidopsis* these transporters are members of the ACA family and are characterized by having a CaM-binding domain on the N-terminal end of the enzyme (Malmstrom *et al.*, 1997). PEA1 (ACA1) was localized to the chloroplast inner envelope using antibodies that only recognized a single 90- to 95-kDa polypeptide (Huang *et al.*, 1993), and this is probably the 95 kDa protein seen in Figure 1. PEA1/ACA1 has a predicted CaM binding motif at its N-terminus. Because no $Ca^{++}$-stimulated ATPase activity or ATP-stimulated $Ca^{++}$ transport has been detected with isolated inner envelope membranes (Huang *et al.*, 1993; Roh *et al.*, 1998), the "sideness" of CaM-binding and direction of $Ca^{++}$ pumping by the PEA1/ACA1 $Ca^{++}$-ATPase are not known. Therefore we do not know if PEA1/ACA1 interacts with cytosolic or stromal $Ca^{++}$ and/or CaM.

Zielinski reviewed the identity of known and putative plant calmodulin-regulated proteins in 1998. Subcellular localization of many proteins was not given in that review due to lack of information at that time. Since 1998, the completion of the *Arabidopsis* genome sequence has allowed us to identify genes coding for CaM-binding proteins and calcium-dependent protein kinases and to predict their subcellular targeting. PSORT is a tool that predicts the targeting of proteins to subcellular compartments based upon the N-terminal amino acid sequence (Nakai and Horton, 1999). In addition, the database ARAMEMNON integrates a list of *Arabidopsis* proteins that are predicted to be membrane localized with multiple signal peptide prediction programs to enhance the analysis of their subcellular localization (Schwacke *et al.*, 2003). There are a number of CaM-binding proteins and calcium-dependent protein kinases that are predicted to be targeted to the stromal compartment of chloroplasts and the thylakoid membranes (Table 1). Localization of these proteins needs to be confirmed by molecular biological and biochemical techniques.

PSORT predicts a CaM-binding ATPase to be targeted to the chloroplast stroma. However, more rigorous analysis by ARAMEMNON suggests that this protein may actually be membrane-associated. The role of the CaM-binding ATPase is unclear but the protein is unrelated to proteins in the ACA family of $Ca^{++}$-ATPase proteins discussed above. Other CaM-binding proteins that may be localized to the chloroplast stroma

*Table 1.* *Arabidopsis* $Ca^{++}$-dependent proteins predicted to be chloroplast localized. Protein sequences translated from the *Arabidopsis* genome were analyzed for chloroplast targeting domains using PSORT* (Nakai and Horton, 1999) and ARAMEMNON (Schwacke *et al.*, 2003) to predict membrane proteins.

| Type | Gene locus | PSORT prediction | ARAMEMNON prediction |
|---|---|---|---|
| Calmodulin-binding proteins | | | |
| ATPase | At3g56690 | stroma | chloroplast |
| Chaperonin 10 | At5g20720 | stroma | no consensus |
| NAD kinase | At1g21640 | stroma | no consensus |
| Uncharacterized protein | At1g27460 | thylakoid | no consensus |
| Cyclic nucleotide and calmodulin-regulated ion channels | | | |
| Ion channel-like protein | At5g14870 | thylakoid | ** |
| Voltage-gated ion channel protein | At3g17690 | thylakoid | chloroplast** |
| Putative ion channel protein | At3g17700 | thylakoid | chloroplast |
| Calcium-dependent protein kinases | | | |
| Serine/threonine phosphatase | At3g50530 | stroma | chloroplast |
| $Ca^{++}$-dependent kinase | At5g66210 | stroma | no consensus |
| $Ca^{++}$-dependent kinase | At4g23650 | stroma | chloroplast** |
| $Ca^{++}$-dependent kinase | At5g12180 | thylakoid | chloroplast |
| $Ca^{++}$-dependent kinase | At2g17890 | stroma | no consensus |
| $Ca^{++}$-dependent kinase | At5g12480 | thylakoid | chloroplast |

*Only sequences with a score higher than 0.800 have been included.
**Proteins that also show strong mitochondrial targeting sequences.

include chaperonin 10 and NAD kinase. Chaperonin 10 has been shown to be highly expressed in green tissues and has a C-terminal CaM binding domain (Yang and Poovaiah, 2000) suggesting that the $Ca^{++}$/CaM messenger system may be involved in Rubisco assembly in the chloroplast.The NAD kinase may be the same as the protein studied by Muto and Miyachi (1977) and is important for pyridine nucleotide biosynthesis in the chloroplast.

The family of cyclic nucleotide calmodulin-regulated ion channels (CNGC) is involved in voltage-gated cation transport, particularly $K^+$ and $Ca^{++}$ (Leng *et al.*, 1999). Three members of the family were predicted to be localized to the thylakoid membrane by PSORT, but only two proteins were predicted to be localized to the chloroplast membranes by ARAMEMNON (Table 1). These proteins may control the movement of cations across the thylakoid membrane and hence regulate photosynthesis by compartmentalization of $Ca^{++}$ within the chloroplast.

The existence of multiple CaM isoforms in plants suggests that they may interact with different CaM-binding proteins (Zhang and Lu, 2003). Differences in subcellular localization may also partition certain CaM members to interact only with specific CaM-binding proteins found within organelles, such as the chloroplast. The evidence for $Ca^{++}$/CaM interaction within the chloroplast is strong based on potential CaM-binding proteins found in the chloroplast (Fig. 1 and Table 1). However, it is interesting to note that of the 24 identified calmodulins in the *Arabidopsis* database, none are predicted to be chloroplastic. Whether CaM is produced in the cytosol and imported into chloroplasts or is synthesized within the chloroplast remains to be investigated.

In 1982, Hetherington and Trewavas reported the first calcium-dependent, CaM-independent protein kinase activity in plants. Since Ca++ regulates the activity of CDPKs, this leads us to infer that these proteins are involved in $Ca^{++}$-mediated signal transduction. Four distinct domains typify CDPK family members, an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain and a CaM-like domain (Cheng *et al.*, 2002). Although possessing a CaM-like domain, CDPK activity in plants is generally CaM-independent (Cheng *et al.*, 2002; Sanders *et al.*, 2002). However, there is a report that light-induced phosphorylation of an endogenous thylakoid membrane protein is inhibited by EGTA, by CaM antagonists (trifluoperazine and W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide), and by a naturally occurring plant CaM-binding protein, BP-10 (Li *et al.*, 1998). The inhibitory effects of BP-10 can be eliminated by addition of CaM. The results imply that $Ca^{++}$ and CaM may regulate the kinase(s) that catalyze thylakoid membrane protein phosphorylation and hence are most likely to participate in and regulate photosynthesis. There are a number of CDPKs that are predicted to be localized to the chloroplast stroma and thylakoid membrane (Table 1). The presence of several $Ca^{++}$-binding proteins and CDPKs predicted to be targeted to the chloroplast suggests an important role for these proteins in $Ca^{++}$ signaling in this organelle. Further biochemical characterization of the proteins encoded by these genes will provide insight as to how $Ca^{++}$, CaM and $Ca^{++}$-binding proteins regulate photosynthetic processes within the chloroplast.

## II. $Ca^{++}$ Fluxes Across Chloroplast Membranes

The roles that chloroplast calcium fluxes may play in regulating processes within the chloroplast and in the regulation of cytosolic calcium concentration are underappreciated. Several studies have measured $Ca^{++}$ transport into intact chloroplasts. $Ca^{++}$ movement into intact wheat chloroplasts indicated that uptake occurs via an $H^+/Ca^{++}$ antiport mechanism (Muto *et al.*, 1982). $Ca^{++}$ influx across the envelope of intact illuminated spinach chloroplasts was also reported to occur via a uniport-type carrier (Kreimer *et al.*, 1985a,b). These studies were based on measurements of free $Ca^{++}$ with the metallochromic indicator, Arsenazo III that is responsive in the high μM range. Chloroplasts have been reported to contain between 4 to 23 mM total $Ca^{++}$ (Portis and Heldt, 1976). However the resting concentration of free $Ca^{++}$ in the stroma of chloroplasts is sub-μM (Kreimer *et al.*, 1988; Johnson *et al.*, 1995). Since the majority of $Ca^{++}$ in chloroplasts is bound to membranes and proteins, and the internal environment of isolated chloroplasts cannot be controlled, estimates of rates of transport into chloroplasts are difficult due to the buffering of $Ca^{++}$ in intact chloroplasts. Therefore, studies using isolated membranes have been undertaken to provide further insight into the transport of $Ca^{++}$ into chloroplasts.

### *A. $Ca^{++}$ Fluxes Across Outer and Inner Envelope Membranes*

The chloroplast outer envelope membrane has been assumed to be permeable to low molecular weight solutes

(<600 Da) due to the presence of porins (Flugge and Benz, 1984). Characterization of three pore forming proteins in the outer envelope found differences in substrate specificity and gating (Bolter and Soll, 2001). In general, however, the chloroplast inner envelope membrane is considered to be the primary permeability barrier to solute transport.

The initial rate of $Ca^{++}$uptake has been measured using chloroplast inner envelope membrane vesicles loaded with the calcium-sensitive fluorophore, fura-2 (Roh *et al.*, 1998). Inner envelope membranes prepared by extrusion were shown to be predominantly right-side-out in orientation (Shingles and McCarty, 1995). $Ca^{++}$ uptake in these membrane vesicles was rapid, reaching completion in about 20 s (Fig. 2). Correcting for buffering by the membranes, the initial rate of $Ca^{++}$ influx was determined to be 9.0 μmoles $min^{-1}mg^{-1}$ protein, indicating a high capacity for $Ca^{++}$ movement across the inner envelope membrane. Under certain conditions $Ca^{++}$ efflux from intact chloroplasts could be observed (Kreimer *et al.*, 1985a). $Ca^{++}$ efflux measured in membrane vesicles that are largely inside-out was less than 4.5 μmoles $min^{-1}mg^{-1}$ protein, indicating a sidedness for $Ca^{++}$ transport.

An inwardly directed, potential-stimulated gradient across the inner envelope membrane drives $Ca^{++}$ uptake in right-side-out vesicles. Ruthenium red inhibited by over 95% the potential-stimulated activity, supporting a uniport mechanism of $Ca^{++}$ transport. A small component of $Ca^{++}$ transport that was not potential-stimulated but was inhibited 85% by diltiazem indicates that there may also be a $Ca^{++}$ antiporter associated with this membrane. Roh *et al.* (1998) determined that the initial rate of calcium transport was 8.1 μmoles $min^{-1}mg^{-1}$ protein for the uniporter and 2.7 μmoles $min^{-1}mg^{-1}$ protein for the antiporter (Roh *et al.*, 1998).

### *B. $Ca^{++}$ Fluxes Across the Thylakoid Membrane*

Calcium is required in the thylakoid lumen for the assembly of the oxygen-evolving complex of photosystem II, where it directly participates with $Mn^{++}$ in the

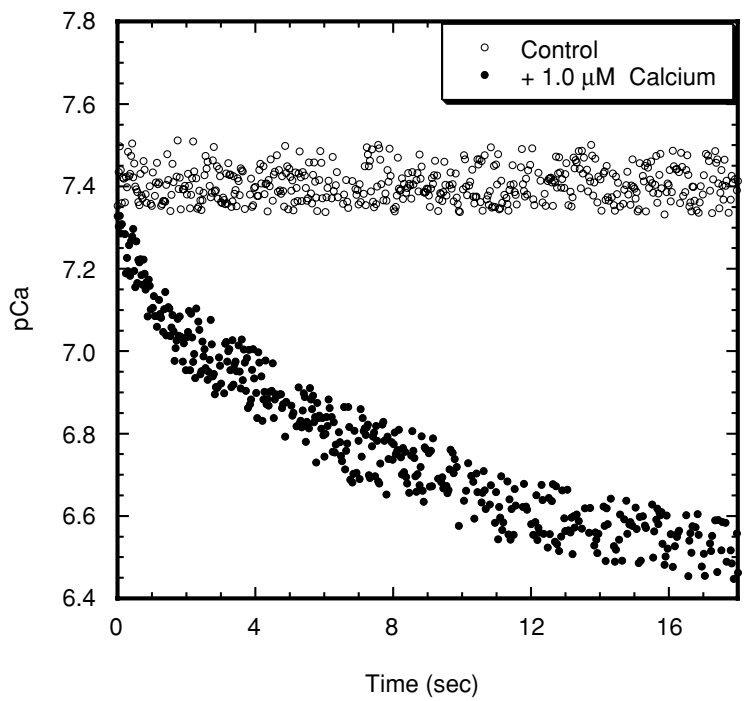


*Fig. 2.* Calcium transport across isolated chloroplast inner envelope vesicles. Inner envelope membranes (50 μg protein) were prepared by extrusion to load 1.2 μM fura-2 into vesicles. These vesicles were rapidly mixed in a stopped flow spectrofluorometer with $CaCl_2$ and fluorescence emission at 512 nm was monitored (excitation at 340 nm). "Control" (no added $Ca^{++}$) and "+1.0 μM $Ca^{++}$" conditions are shown. Total free $Ca^{++}$ was determined to be 0.35 μM after mixing. Adapted from Roh *et al.* (1998).

water splitting reaction (Vrettos *et al.*, 2001). While calcium transport across the thylakoid membrane may be dependent on simple diffusion from the stroma, the diffusion of a large divalent ion across the lipid bilayer of the thylakoid membrane could be a rate-limiting process of chloroplast biogenesis. The minimal concentration of $Ca^{++}$ required to support the assembly of the OEC *in vivo* has not been established. Numerous attempts have been made to determine the binding affinity of $Ca^{++}$ to the OEC using PSII preparations stripped of $Ca^{++}$ and/or the extrinsic polypeptides (OE33, OE23 or OE17). The number (n) of reported calcium binding sites and their affinity (K) for the OEC is dependent on the presence of the extrinsic polypeptides, the Mn redox state, the presence of light and the method used to deplete the OEC of calcium. Values of n ranging from 1–3 and of K ranging from approximately 0.0001 to 1.0 mM have been reported (Grove and Brudvig, 1998; Debus, 1992; Vander Meulen *et al.*, 2002). In spite of the uncertainty as to how much $Ca^{++}$ is required for the assembly of the OEC, $Ca^{++}$ must be continually supplied to the thylakoid lumen to support the light-dependent biogenesis of the OEC (Ghanotakis *et al.*, 1984; Becker *et al.*, 1985; Miller and Brudvig, 1989). The 0.1 to 0.2 μM free $Ca^{++}$ in the stroma (Johnson *et al.*, 1995; Kreimer *et al.*, 1988) is too low to continuously sustain OEC assembly in the light without the active transport of $Ca^{++}$ from the stroma to the thylakoid lumen.

In addition to the problem of providing $Ca^{++}$ to the thylakoid lumen to support assembly of the OEC, there is a discrepancy between the light-dependent uptake of $Ca^{++}$ by chloroplasts and the activation of Calvin-Benson cycle enzymes—both $Ca^{++}$ uptake and $CO_2$ fixation occur in the light. However, fructose-1,6-*bis*phosphatase, a key enzyme involved in the regulation of $CO_2$ fixation, is inhibited by excess $Ca^{++}$ (Hertig and Wolosiuk 1983), and elevated $Ca^{++}$ levels have a pronounced inhibitory effect on $CO_2$ fixation (Portis and Heldt, 1976; Demmig and Gimmler, 1979). Therefore, $Ca^{++}$ transported across the envelope membranes in the light must either be tightly bound in the stroma or sequestered in the thylakoid to prevent the inhibition of $CO_2$ fixation by $Ca^{++}$ (Wolosiuk *et al.*, 1993). The discovery of a $Ca^{++}/H^+$ antiporter in the thylakoid membrane provides a mechanism for the thylakoid to acquire essential $Ca^{++}$ to support OEC assembly and to sequester $Ca^{++}$ from the stroma in the light (Ettinger *et al.*, 1999).

Calcium import into the thylakoid lumen by the $Ca^{++}/H^+$ antiporter is not strictly dependent upon light exposure; ATP can support the reaction in the dark through the activity of the $H^+$–ATPase. $Ca^{++}$ uptake via the $Ca^{++}/H^+$ antiporter is insensitive to the presence of non-hydrolysable ATP analogs, and is highly sensitive to the ionophore nigericin that dissipates the trans-thylakoid ΔpH, but not the ΔV (Fig. 3) (Ettinger *et al.*, 1999). The trans-thylakoid proton motive force provides the energy to facilitate the accumulation of significant amounts of calcium in the thylakoid lumen. The ΔG of $Ca^{++}$ transport by the $Ca^{++}/H^+$ antiporter is dependent on the number of lumenal $H^+$ exchanged per $Ca^{++}$ imported. The plant vacuolar $Ca^{++}/H^+$ antiporter is reported to have a $3H^+/1Ca^{++}$ stoichiometry (Blackford *et al.*, 1990). Assuming that the thylakoid $Ca^{++}/H^+$ antiporter has the same stoichiometry, this would provide considerable driving force to support $Ca^{++}$ uptake into the lumen. A comparison of the measured rate of $Ca^{++}$ transport across intact isolated thylakoid membranes and the volume of the thylakoid lumen, 3.3 μL $mg^{-1}$ Chl (Heldt *et al.*, 1973), suggests that concentrations of $Ca^{++}$ to the millimolar level can accumulate in the thylakoid lumen. The antiporter requires a trans-thylakoid ΔpH to be active and is therefore active only in the light, or when there is an active $H^+$/ATP-synthase and sufficient stromal ATP in the dark. In effect, the thylakoid could be pumping $Ca^{++}$ out of the stroma from daybreak until a few minutes after dark when the thylakoid $H^+$/ATP-synthase is inhibited (Kramer and Crofts, 1989). After a transition to the dark, calcium would presumably be free to leave the thylakoid and signal the light-dark transition to the stroma and the rest of the cell. The rapid release of calcium from millimolar stores in the thylakoid lumen would result in dramatic changes in stromal $Ca^{++}$ concentrations such as those observed during the light to dark transition (see below).

The *Arabidopsis* genome sequence provides a look at the wide array of $Ca^{++}/H^+$ antiporter genes in higher plants. To date, eleven forms of the calcium antiporter have been identified and named *Atcax1-11* (Mäser *et al.*, 2001). A specific magnesium transporter (*mhx1*) that shares a great deal of similarity with the *cax* genes has also been identified (Shaul *et al.*, 1999). Of the *cax*-family gene products, CAX1 is localized to the vacuole and has been studied the most extensively. It was originally identified by its ability to suppress a $Ca^{++}$-hypersensitive mutant of *Saccharomyces cerevisiae* (Hirschi *et al.*, 1996). More recently, a full-length homolog of this gene has been shown to encode an additional 36 N-terminal amino acids that play a regulatory role in cytosolic $Ca^{++}$ homeostasis (Pittman and

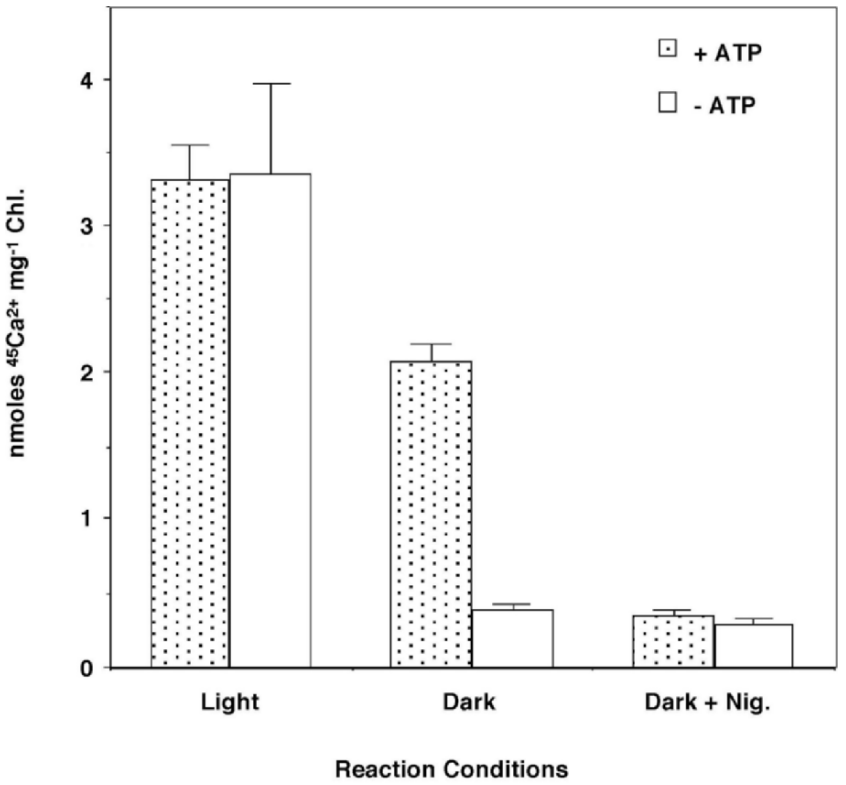


*Fig. 3.* A proton gradient is required for $^{45}Ca^{++}$ uptake by intact thylakoid membranes. Transport reactions were initiated by adding $^{45}Ca^{++}$ (1.5 μM final concentration) to thylakoid membranes suspended in import buffer containing 5 mM $MgCl_2$ and 3 mM ATP. Where indicated, 100 nM nigericin was added to the samples before incubation. Reactions were terminated after 15 min by centrifugation of the membranes through silicone oil. (Ettinger *et al.*, 1999 and C.H. Johnson, R. Shingles and W.F. Ettinger, unpublished data).

Hirschi, 2001; Pittman *et al.*, 2002) The N-terminal region of CAX1 interacts with a $Ca^{++}$-activated protein, SOS2, leading to the activation of CAX1 in the vacuolar membrane. Several other *cax* gene products (CAX2, CAX3 and CAX4) also have N-terminal extensions but do not interact with SOS2 (Cheng *et al.*, 2004). Interestingly the N-terminal extensions on CAX proteins also resemble chloroplast transit peptides, suggesting that some CAX homologs could be localized to the chloroplast thylakoid membrane (Table 2). Protein topology programs included in the ARAMEMNON analysis package (Schwacke *et al.*, 2003) indicate a consensus chloroplast targeting peptide in the protein products of *cax3* and *cax4*, while the Target P (Emanuelsson *et al.*, 2000) program predicts that CAX1 may also be targeted to the chloroplast. Obviously, different transit peptide prediction programs produce different results and may not accurately predict the localization of a protein *in vivo*. An antibody raised against the vacuolar $Ca^{++}/H^{+}$ antiporter from mung bean, VCAX1, was unable to detect the presence of protein in mung bean or *Arabidopsis* chloroplasts (Ueoka-Nakanishi *et al.*, 2000). For now the identity of the gene encoding the thylakoid $Ca^{++}/H^{+}$ antiporter is uncertain.

## III. Light/Dark Regulation of $Ca^{++}$ Fluxes in the Chloroplast

### *A. Light-Induced $Ca^{++}$ Uptake*

Isolated chloroplasts take up calcium upon illumination, as measured by the uptake of radioactive calcium from the medium (Muto *et al.*, 1982; Kreimer *et al.*, 1985a). This uptake process is probably mediated by $Ca^{++}$ transport across the inner-envelope membrane of the chloroplast (Roh *et al.*, 1998), and perhaps ultimately by $Ca^{++}$ transport across the thylakoid membrane (Ettinger *et al.*, 1999). Light-induced $Ca^{++}$ uptake into chloroplasts may also occur *in vivo*, based on measurements of cytosolic $Ca^{++}$ in characean algae. In the alga *Nitellopsis*, direct measurement using $Ca^{++}$-selective microelectrodes demonstrated that cytosolic $Ca^{++}$ decreased when plants were illuminated with

*Table 2.* The predicted subcellular location of identified *Arabidopsis* $Ca^{2+}/H^{+}$ antiporter/cation exchangers (the CAX subfamily of proteins). Protein sequences translated from the *Arabidopsis* genome were analyzed for chloroplast targeting domains using PSORT (Nakai and Horton, 1999) or TargetP, (Emanuelsson *et al.*, 2000) or for the most probable subcellular localization using ARAMEMNON (Schwacke *et al.*, 2003).

| | | Chloroplast targeting prediction | | |
|---|---|---|---|---|
| Type | Gene locus | Psort | TargetP | ARAMEMNON |
| (AtCAX1) | At2g38170 | 0.698 | 0.826 | no consensus |
| (AtCAX2) | At3g13320 | 0.937 | 0.108 | no consensus |
| (AtCAX3) | At3g51860 | 0.596 | 0.726 | chloroplast |
| (AtCAX4) | At5g01490 | 0.958 | 0.883 | chloroplast |
| (AtCAX5) | At1g55730 | 0.477 | 0.099 | no consensus |
| (AtCAX6) | At1g55720 | 0.511 | 0.032 | possibly mitochondrial |
| (AtCAX7) | At5g17860 | 0.000 | 0.078 | secretory pathway |
| (AtCAX8) | At5g17850 | 0.000 | 0.001 | secretory pathway |
| (AtCAX9) | At3g14070 | 0.000 | 0.028 | possibly mitochondrial |
| (AtCAX10) | At1g54115 | 0.581 | 0.019 | possibly mitochondrial |
| (AtCAX11) | At1g08960 | 0.000 | 0.104 | secretory pathway |

a strong light (Miller and Sanders, 1987). This phenomenon was dependent upon photosynthetic electron transport, because the inhibitor DCMU prevented the light-induced decrease of cytosolic $Ca^{++}$ (Miller and Sanders, 1987). These data implied that light causes a flux of $Ca^{++}$ from the cytosol to the chloroplast (thus, the decrease of cytosolic $Ca^{++}$ upon illumination) by a process that is ultimately dependent upon photosynthetic electron transport. Indirect measurements suggested that stromal $Ca^{++}$ levels may increase as a result of this light-induced $Ca^{++}$ uptake (Kreimer *et al.*, 1988), but direct measurements of stromal $Ca^{++}$ under these conditions using transgenic plants expressing the calcium-specific photoprotein aequorin (Johnson *et al.*, 1995; Sai and Johnson, 2002) contradicted that conclusion (next section).

## *B. Dark-Stimulated and Circadian $Ca^{++}$ Fluxes*

An obvious prediction from the data reported in the previous section would be that the $Ca^{++}$ concentration increases in the chloroplastidal stroma upon illumination. In fact, transgenic tobacco and *Arabidopsis* seedlings expressing aequorin that is targeted to the stroma show that stromal $Ca^{++}$ levels do not significantly increase during the light portion of light/dark cycles. In contrast, darkness elicits a profound transient increase of stromal $Ca^{++}$ levels (Johnson *et al.*, 1995; Sai and Johnson, 2002). These observations are depicted in Fig. 4 for seedlings in a 16 h light/8 h dark cycle. Does this mean calcium that is taken up into chloroplasts under illumination (Muto *et al.*, 1982) is immediately bound within the chloroplast or is transported into the thylakoids so that the stromal $Ca^{++}$ level is barely affected?

To make the situation even more complex, data from *Nitellopsis* indicated that DCMU should inhibit the light-induced uptake into chloroplasts (Miller and Sanders, 1987), but the data with transgenic tobacco showed the opposite—in the presence of DCMU, there is a measurable increase of stromal $Ca^{++}$ levels when seedlings are illuminated (Fig. 5). Therefore, the data from *Nitellopsis* and chloroplasts *in vitro* are not consistent with the data from transgenic plants in which aequorin is targeted to the stroma. Do the chloroplasts of *Nitellopsis* work differently from those of plants? Are short-term $Ca^{++}$ fluxes (as measured with $Ca^{++}$-sensitive microelectrodes in *Nitellopsis*) flowing

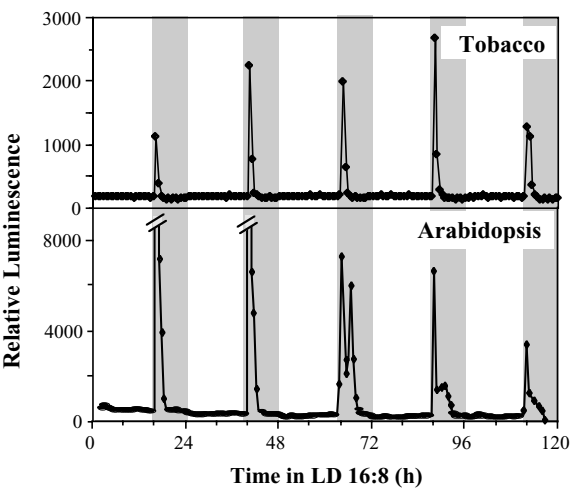


*Fig. 4.* Stromal $Ca^{++}$ spikes occur after lights-off on 24 h light/dark cycles in transgenic tobacco (upper panel) and *Arabidopsis* (lower panel) plants expressing the calcium photoprotein aequorin that has been targeted to the stroma. More luminescence equals higher $Ca^{++}$ level. The light cycle shown is a long day cycle (16 h light, 8 h dark = LD 16:8). White = light, grey = dark. Also see Sai and Johnson (2002).

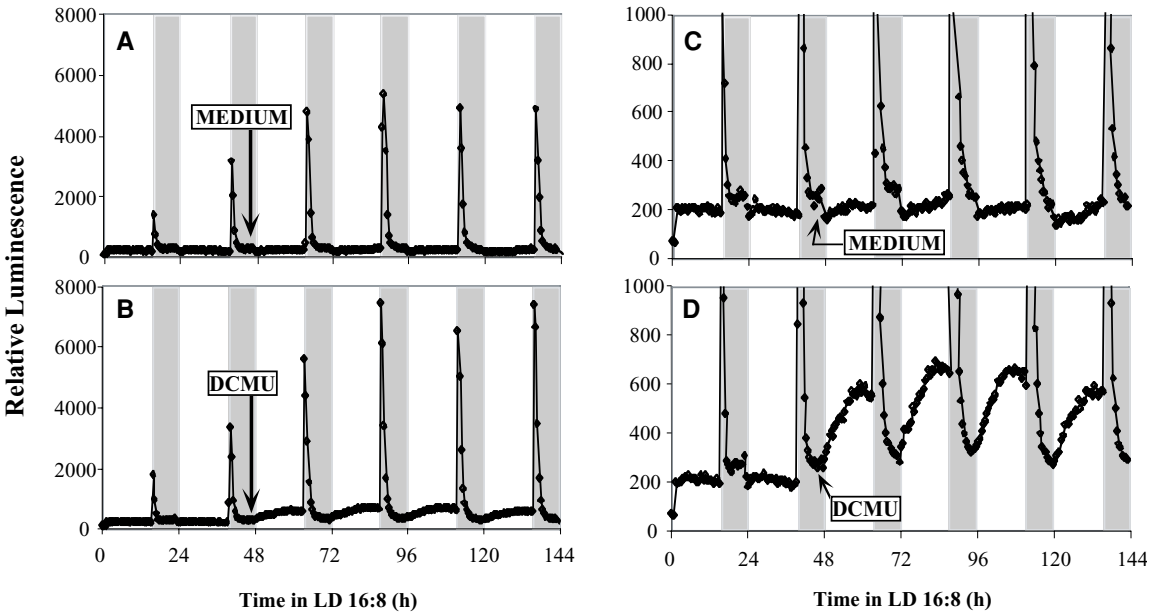


*Fig. 5.* DCMU promotes a light-dependent increase of $Ca^{++}$ in the stroma, but does not inhibit the dark-stimulated $Ca^{++}$ flux in LD 16:8. DCMU or medium was added to transgenic tobacco seedlings expressing the calcium photoprotein aequorin that has been targeted to the stroma at hour 44 of LD 16:8 (final DCMU concentration was 10 μM). Panels C and D are magnified versions of panels A and B (C is magnified from A; D is magnified from B). From Sai and Johnson (2002).

in opposite directions to the longer-term $Ca^{++}$ fluxes that are measured by aequorin luminescence? Is the light-induced calcium uptake into isolated chloroplasts an artifact of non-physiological conditions? Or is the answer that the calcium taken up from the cytosol by chloroplasts upon illumination is immediately bound and/or transported to the thylakoids? Our working hypothesis is that the latter explanation is correct. Obviously, these disparate observations concerning light-induced uptake of calcium into chloroplasts need to be reconciled.

Nonetheless, the data of Figs. 4 and 5 illustrate another fascinating observation—a large increase of stromal $Ca^{++}$ levels that is stimulated by darkness. While it clearly happens every night in a light/dark cycle (Fig. 4; Sai and Johnson, 2002), it was first observed after lights-off of an extended illumination (Fig. 6; Johnson *et al.*, 1995). As shown in Fig. 6A, there is no indication of daily $Ca^{++}$ oscillations in the stroma in constant light, but damped daily oscillations of $Ca^{++}$ appear after the transfer to darkness (Johnson *et al.*, 1995). (In contrast, there are robust daily oscillations of $Ca^{++}$ in the cytosol in constant light as reported by Johnson *et al.*, 1995.) The most prominent feature of stromal $Ca^{++}$, however, is the large spike of $Ca^{++}$ that occurs soon after the light-to-dark transition (Fig. 6). This $Ca^{++}$ flux results in surprisingly high $Ca^{++}$ concentrations in the stroma—perhaps as high as 1 to 5 μM (Johnson *et al.*, 1995). Given the potency of $Ca^{++}$ on cellular processes, this bolus of $Ca^{++}$ must have significant effects in the chloroplast. It might also have an impact on cytosolic $Ca^{++}$, as there appears to be a significant increase of cytosolic $Ca^{++}$ that occurs as the stromal $Ca^{++}$ spike is declining (Sai and Johnson, 2002). This observation implies that at least some of the stromal $Ca^{++}$ bolus is transported out of the stroma into the cytosol.

A curious feature of this $Ca^{++}$ spike is that it does not begin until more than 5 minutes after the transfer to darkness (Fig. 6B). This observation suggests that the $Ca^{++}$ increase in the stroma is not, therefore, due to a dark-stimulated dissipation of the proton gradient across the thylakoid membrane. Theoretically, protons leaking back into the stroma in the dark could displace bound $Ca^{++}$ ($Ca^{++}$ and protons often compete for the same binding sites), thereby increasing the level of free calcium ions in the stroma. If this were true, the time course of the dissipation of the proton electrochemical gradient across the thylakoid membrane should correlate with the kinetics of the $Ca^{++}$ spike within the stroma. However, it has long been known that the protonic electrochemical gradient across the thylakoid membrane in chloroplasts is dissipated completely within 30 sec of lights-off (Schuldiner *et al.*,

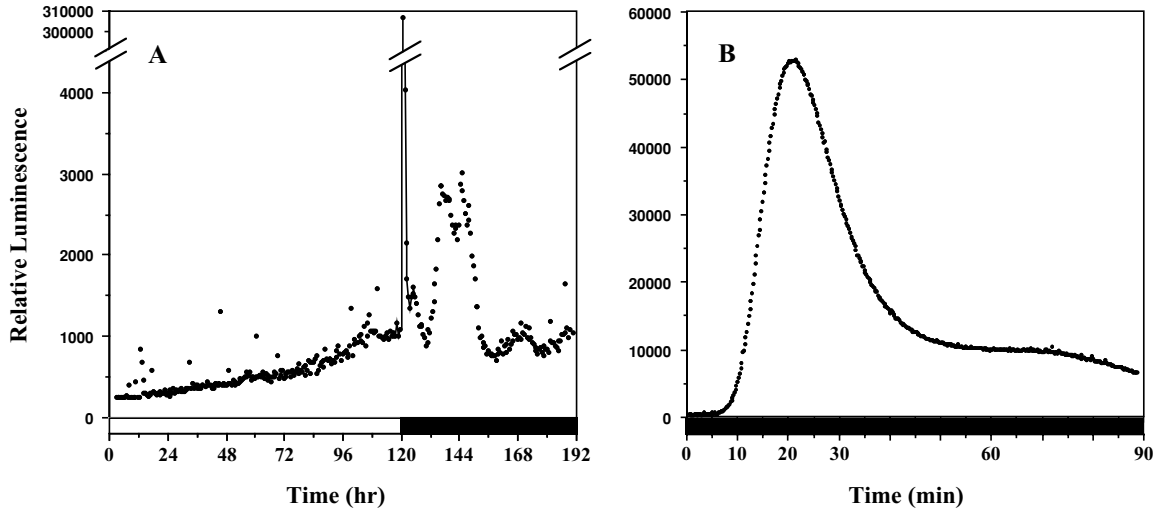


*Fig. 6.* Aequorin luminescence in tobacco seedlings expressing stromal aequorin. In panel A, seedlings were in constant light from time 0 to hour 120, thereafter in constant darkness. Abscissa for panel A is in hours. Panel B is a separate experiment in which the abscissa is expanded to minutes, and time "0" is the time of the light-to-dark transition. From Johnson *et al.* (1995).

1972). Because aequorin responds within milliseconds to changes of $Ca^{++}$ levels, it appears that the 5 to 10 min lag in the stromal $Ca^{++}$ spike (Fig. 6B) excludes the interpretation that the $Ca^{++}$ increase is a result of a dark-stimulated dissipation of the photosynthetically generated proton gradient (Sai and Johnson, 2002). The effect may be related to redox-mediated deactivation of the chloroplast ATP synthase that can function as a proton-pumping ATPase for a short period of time (5 to 10 minutes) after a light to dark transition (Kramer and Crofts, 1989).

## IV. Concluding Remarks

The source of the $Ca^{++}$ that appears in the stroma after lights-off is not known. On the basis of simultaneous measurements of cytosolic and stromal $Ca^{++}$ levels, Sai and Johnson (2002) presented a model for the dark-stimulated $Ca^{++}$ flux that suggested the $Ca^{++}$ in the flux comes largely from intrachloroplastidal stores (e.g. the thylakoids and other stores). Our current model is depicted in Fig. 7. We propose that in the middle of the night, the $Ca^{++}$ levels in the thylakoid lumen ("thy"), the stroma, and the cytosol are all low. At dawn, photosynthetic electron transport ("pet") pumps $H^+$ into the thylakoid lumen, generating a proton electrochemical gradient. This proton electrochemical gradient is used by the $Ca^{++}/H^+$ antiporter ("ap") to pump $Ca^{++}$ from stroma to lumen at the same time that the light-regulated $Ca^{++}$ uptake ("LR") proceeds. Therefore light-stimulated $Ca^{++}$ uptake does not result in a significant increase in stromal $Ca^{++}$, because that $Ca^{++}$ is transferred into the thylakoid lumen by the $Ca^{++}/H^+$ antiporter. The result of this transfer is to increase $Ca^{++}$ levels in the thylakoid lumen during the day. At dusk, the direction of the $Ca^{++}$ flux reverses so that stromal $Ca^{++}$ levels skyrocket. The reason for the delay from the onset of darkness to the beginning of the dark-stimulated $Ca^{++}$ flux is unknown, but it might be related to the redox status of proton pumping, the redox status of the chloroplast ATP synthase (Kramer and Crofts, 1989), and/or to $Ca^{++}$ transporting proteins.

Could the stromal $Ca^{++}$ spike play a signaling and/or regulatory role? Perhaps this massive $Ca^{++}$ flux into the stroma shuts down photosynthesis-related processes in the stroma. For example, stromal fructose-1,6-*bis*phosphatase, a key enzyme involved in the regulation of $CO_2$ fixation, is inhibited by excess $Ca^{++}$ (Hertig and Wolosiuk, 1983), and elevated $Ca^{++}$ levels have a pronounced inhibitory effect on $CO_2$ fixation. The dark-stimulated $Ca^{++}$ flux is not directly modulated by a circadian clock, because varying the circadian time of the lights-off stimulus does not significantly modify the amplitude of the $Ca^{++}$ spike (Sai and Johnson, 2002). On the other hand, the fact that the waveform of the $Ca^{++}$ spike is different on varying photoperiods—rapid on long photoperiods but prolonged on short photoperiods (Sai and Johnson, 2002) implies a sensitivity to environment that could have regulatory consequences. At the present time, it

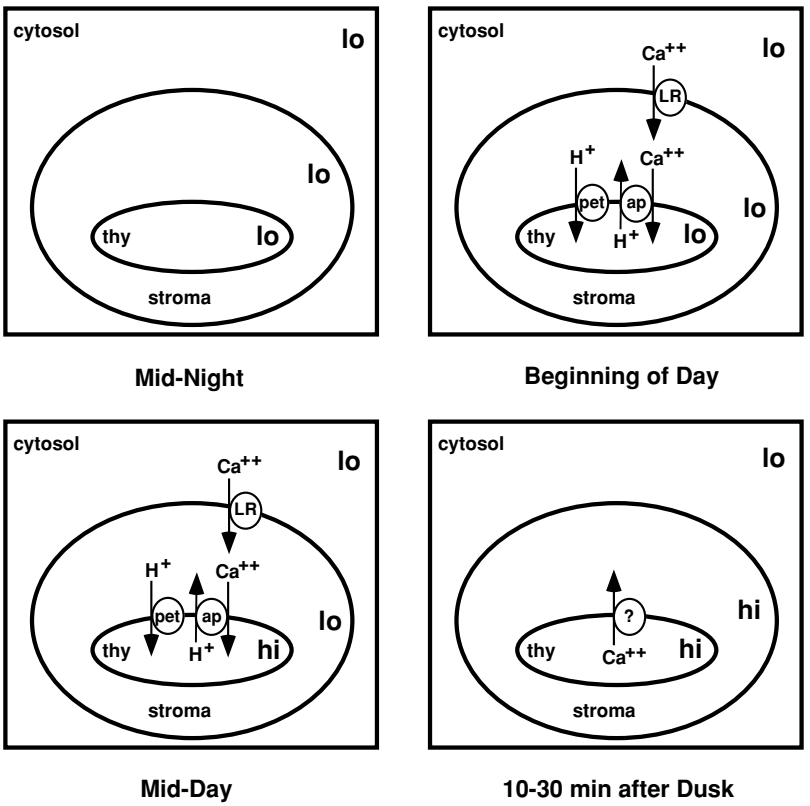


*Fig. 7.* Model for $Ca^{++}$ fluxes in and out of the chloroplast modulated by light and dark. Symbols: thy = thylakoid lumen; pet = photosynthetic electron transport; ap = $Ca^{++}$ /$H^+$ antiporter; LR = light-regulated $Ca^{++}$ uptake into chloroplasts. Relative levels of $Ca^{++}$ in the different compartments are indicated by "lo" and "hi."

must be admitted that the function of this intriguing dusk-stimulated stromal $Ca^{++}$ spike is unknown. In fact, as must be clear to the reader from this chapter, our understanding of the regulation of $Ca^{++}$ levels and $Ca^{++}$ fluxes in the chloroplast is in its infancy. Little is known and much remains to be discovered.

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# Section V

## Plastid Differentiation and Response to Environmental Factors

# Chapter 21

# The Role of Plastids in Ripening Fruits

Florence Bouvier and Bilal Camara*
*Institut de Biologie Moléculaire des Plantes du CNRS et Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France*

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## Summary

Although integrated into a sink tissue, fruit plastids play a key role in plant productivity because lowering fruit plastid metabolism decreases crop yield. Unlike leaf chloroplasts, the capacities of fruit plastids for photosynthetic electron transfer and carbon dioxide assimilation are low and decline dramatically during the ripening stage. However, during this developmental transition hexoses derived from plastid starch hydrolysis and metabolites imported from the cytosol are actively used for the biogenesis and accumulation of carotenoids, prenyl- and acyl- lipids and amino acids. These activities are sustained by non-photosynthetic generation of ATP and reducing power within the organelle. Here we summarize the function of plastids during fruit ripening in relation to recent advances in biochemistry and molecular biology.

## I. Introduction

One of the most prominent changes during fruit ripening is the breakdown of plastid thylakoids concomitantly to the degradation of chlorophylls and the down-regulation of photosynthetic gene expression (Piechulla *et al.*, 1985). Although these changes are reminiscent of senescent or aging processes (Rhodes, 1980), they are not deteriorative *per se*. Indeed, during fruit ripening, plastids gradually acquire new biosynthetic capabilities and in most cases the chloroplasts differentiate into non-photosynthetic chromoplasts.

Plastid starch present in unripe fruit is progressively converted to hexoses during the ripening period (Robinson *et al.*, 1988) and in parallel diverse metabolites are imported into the plastid or exported to the cytosol *via* specific plastid translocators (Fischer and Weber, 2002). The resulting carbon skeletons are used in the organelle for the generation of non-photosynthetic ATP and reducing power and for the biogenesis of diverse products. The latter includes carotenoids that give the yellow to orange colors characteristic of many fruits (Camara *et al.*, 1995). Although the biological significance of this phenomenon

*Author for correspondence, email: Bilal.Camara@ibmp-ulp.u-strasbg.fr

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is unknown, it is assumed that the accumulation of massive amount of carotenoids constitutes part of the signals favoring seed dispersal by animals (Goodwin, 1986). In a similar vein, in ripening fruit plastids play a key role in the *de novo* biosynthesis of acyllipids as shown by the fleshy oil palm mesocarp which produces palm oil, that ranks second among consumed vegetable oil (Salas *et al.*, 2000). Along with the utilization of the carbon skeleton, the plastidial glutamine synthetase-glutamate synthase, a main route for nitrogen assimilation, is subject to a ripening-specific regulation (Gallardo *et al.*, 1988, 1993). Beyond the fact that these events are developmentally regulated, little is known about the molecular mechanisms inducing these changes. This review focuses on the progress made using biochemical and molecular approaches to better understand the function of plastids during fruit ripening.

## II. Plastid Differentiation

### *A. Evolution of Photosynthetic Genes and Plastid Differentiation*

Fruits are largely considered as sink organs and as such, their photosynthetic capacity is usually low. In the absence or reduced presence of stomata (Willmer and Johnston, 1976; Blanke, 1986), $C0_2$ used for fruit photosynthesis is derived mainly from respiration (Blanke and Lenz, 1989). In tomato fruit, fruit photosynthesis contributes 10 to 15% of the fruit carbon gain (Tanaka *et al.*, 1974). On a protein basis ribulose-1,5,-*bis*phosphate carboxylase (Rubisco) of tomato pericarp is approximatively 35% that of tomato leaves (Piechulla *et al.*, 1987). In pepper fruit the Rubisco activity is about one-fifteenth the activity found in leaves (Steer and Pearson, 1976). This feature is obviously consistent with the sink characteristic of pepper fruit (Hall, 1977). Along with the steady decrease of Rubisco during fruit ripening, the 33-kD oxygen evolving protein, cytochrome b559, the chlorophyll a/b binding proteins the D1 protein of photosystem II also decline similarly to their corresponding transcripts (for a review see Camara *et al.*, 1995). These changes are generally followed by chloroplast to chromoplast differentiation which involves the disintegration of the chlorophyllous thylakoid and the appearance of new membrane or lipoprotein structures which sequester excess carotenoids and other lipophilic derivatives produced during the ripening process (Camara *et al.*, 1995).

### *B. Hormonal and Nutritional Control of Fruit Chromoplast Differentiation*

Attempts to understand the cellular mechanisms underlying differentiation of chloroplasts to chromoplasts have revealed hormonal (Coggins *et al.*, 1980; Gemmrich and Kayser, 1984; Goldschmidt, 1988; Trebitsh *et al.*, 1993; Alexander and Grierson, 2002) and nutritional determinants (Huff, 1984; Iglesias *et al.*, 2001).

In contrast to research on the hormonal effect, which has focussed largely on the predominant role of ethylene (Alexander and Grierson, 2002), the nutritional aspect has received limited attention. According to this hypothesis, the chloroplast to chromoplast development in *Citrus* fruit is regulated by the carbon to nitrogen ratio (C/N), i.e., a high ratio induces chromoplast differentiation while a low ratio favors the reversion process (Huff, 1983, 1984; Mayfield and Huff, 1986).

The glutamate-oxoglutarate aminotransferase (GOGAT) cycle, which represent the main route of nitrogen assimilation in plants, could play a key role in the C/N ratio. It has been shown that in tomato 70% of the total free amino acid of the pericarp belongs to the glutamate family (Valle *et al.*, 1998) especially glutamine and glutamate (Boggio *et al.*, 2000). It has also been established that tomato mutant *rin* (for ripening inhibitor) accumulates half the normal level of glutamate of wild fruit (Nagata and Saito, 1992). However, the contribution of tomato fruit plastids in the synthesis of glutamine and glutamate is limited because total GS and GOGAT activities are drastically

*Abbreviations*: ACCase – acetyl-CoA carboxylase; AOS – allene oxide synthase; Ccs – capsanthin-capsorubin synthase; CrtB – bacterial phytoene synthase; CrtHb – non-heme diiron monooxygenases; CrtIso – carotenoid isomerase; DMAPP – dimethylallyl diphosphate; FAS – fatty acid synthetase; FBPase – fructose-1,6-*bis*phosphatase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; GGPP – geranylgeranyl diphosphate; GGPPS – geranylgeranyl diphosphate synthase; GOGAT – glutamate-oxoglutarate aminotransferase cycle; GS– glutamine synthetase; G6PDH – glucose 6-phosphate dehydrogenase; Hggt – homogentisate geranylgeranyl diphosphate transferase; HPL – hydroperoxide lyase; IPP – isopentenyl diphosphate; KAS I, II, III – β-keto acyl-ACP synthases I, II, III; Lcyb – lycopene β-cyclase; Lcye – lycopene ε-cyclase; MACP – malonyl-acyl carrier protein; Nsy – neoxanthin synthase; OPP – oxidative pentose phosphate pathway; Pds – phytoene desaturase; pGlcT – plastid glucose transporter; PPO – polyphenol oxidase; PSY – phytoene synthase; PII – a signal transduction protein involved in monitoring cellular C and N status; Rubisco – ribulose-1,5,-*bis*phosphate carboxylase; TE – thioesterase; Zds – ζ-carotene desaturase; Zep – zeaxanthin epoxidase.

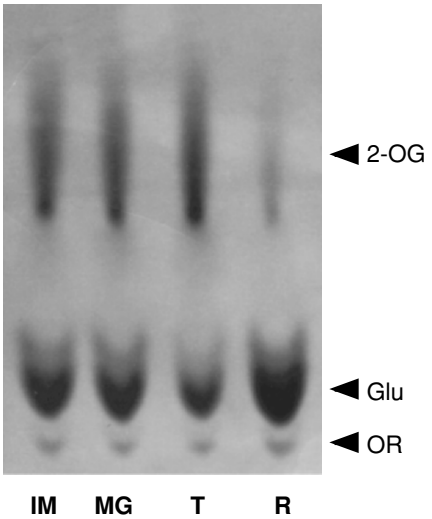



*Fig. 1.* Glutamic acid synthesis in isolated pepper plastids. Glutamate synthesis from 2-keto (1-$^{14}$C) glutaric acid in plastids isolated from pepper fruit at different stages (IM, immature green; MG, mature green; T, turning; R, red). The reaction products were separated using a Cellulose thin layer chromatography plate developed with butanol/formic acid/water: 70/12/10, v/v and visualized by autoradiography. The abbreviations refer to: Glu, Glutamic acid, 2-OG, 2-Oxoglutarate, OR, origin.

reduced during the ripening stage (Gallardo *et al.*, 1988, 1993; Boggio *et al.*, 2000). Therefore, these amino acids must be actively translocated from the leaves during the ripening period according to an unknown mechanism. Alternatively, the reversible reaction catalyzed by mitochondrial glutamate deshydrogenase which is induced during tomato ripening (Boggio *et al.*, 2000) could be involved. Clearly the down regulation of chromoplast GS and GOGAT during tomato ripening is a diagnostic feature of the elevation of the C/N ratio. Whether this mechanism could be generalized to other fruit plastids is debatable. Using an assay based on the use of radioactive 2-oxoglutarate, we observed in pepper fruit chromoplasts, a labeling pattern consistent with the synthesis of glutamic acid by transamination (Fig. 1). 2-Oxoglutarate is synthesized in the cytosol and mitochondria (Hodges, 2002) and is transported into the plastid by a malate-coupled, two-translocator system which involves a 2-oxoglutarate/malate translocator and a glutamate/malate translocator (Weber and Flugge, 2002; see Chapter 14). In addition to NADPH derived from the oxidation of glucose 6-phosphate via the plastidial pentose phosphate pathway, the redox equivalent for GOGAT could be provided by specific ferredoxin isoforms synthesized during chloroplast to chromoplast differentiation (Green *et al.*, 1991).

Obviously, further studies are required to test the C/N ratio and to analyze its potential relationship with the PII signal transduction protein which monitors the cellular status of C (oxoglutarate) and N (glutamine) and has been previously identified as a carbon/nitrogen sensor in bacteria (Stadtman, 2001) and plants (Hsieh *et al.*, 1998; Moorhead and Smith, 2003; Smith *et al.*, 2003).

## III. Plastid Biogenesis and Molecular Regulation

### *A. Carbohydrate Metabolism and Cytosolic Interactions*

During fruit ripening starch stored in plastids is totally or progressively transformed into hexoses by amylase, ADP Glucose pyrophosphorylase and phosphorylase (Fig. 2). In tomato fruits, the plastid starch content starts declining between 14 to 50 days after anthesis (Yelle *et al.*, 1988), according to a phosphorolytic pathway (Robinson *et al.*, 1988).

Although it has been shown that glucose resulting from the amylolytic degradation of starch is exported to the cytosol through a specific plastid glucose transporter (pGlcT) in leaves (Weber *et al.*, 2000), the situation in non-green tissue might different. This is based on the fact that the pGlcT transcript is highly expressed in the non-photosynthetic albedo tissue of *Citrus* fruit, apricot and tomato fruits (Fischer and Weber, 2002). A detailed analysis reveals that in tomato, a starch- and sugar-storing fruit and in olive, a lipid-storing fruit, the expression pattern of pGlcT is highest during the ripening period (Butowt *et al.*, 2003). This led to the suggestion that pGlcT could be involved in the plastidial import of glucose from the cytosol in non-green tissues and especially during chloroplast to chromoplast differentiation (Butowt *et al.*, 2003) (Fig. 2). This hypothesis is reinforced by the fact that unlike in leaves, the olive fruit pGlcT gene does not display diurnal expression (Butowt *et al.*, 2003) and also by the recent characterization of a plastid stromal hexokinase (Olsson *et al.*, 2003).

The role of plastid fructose-1,6-*bis*phosphatase (FBPase) could also be important for providing hexoses in non-green tissues. FBPase, a key enzyme of the Calvin-Benson cycle, is involved in the conversion of triose phosphates into hexose phosphates and is present in tomato leaves and green fruits, whereas

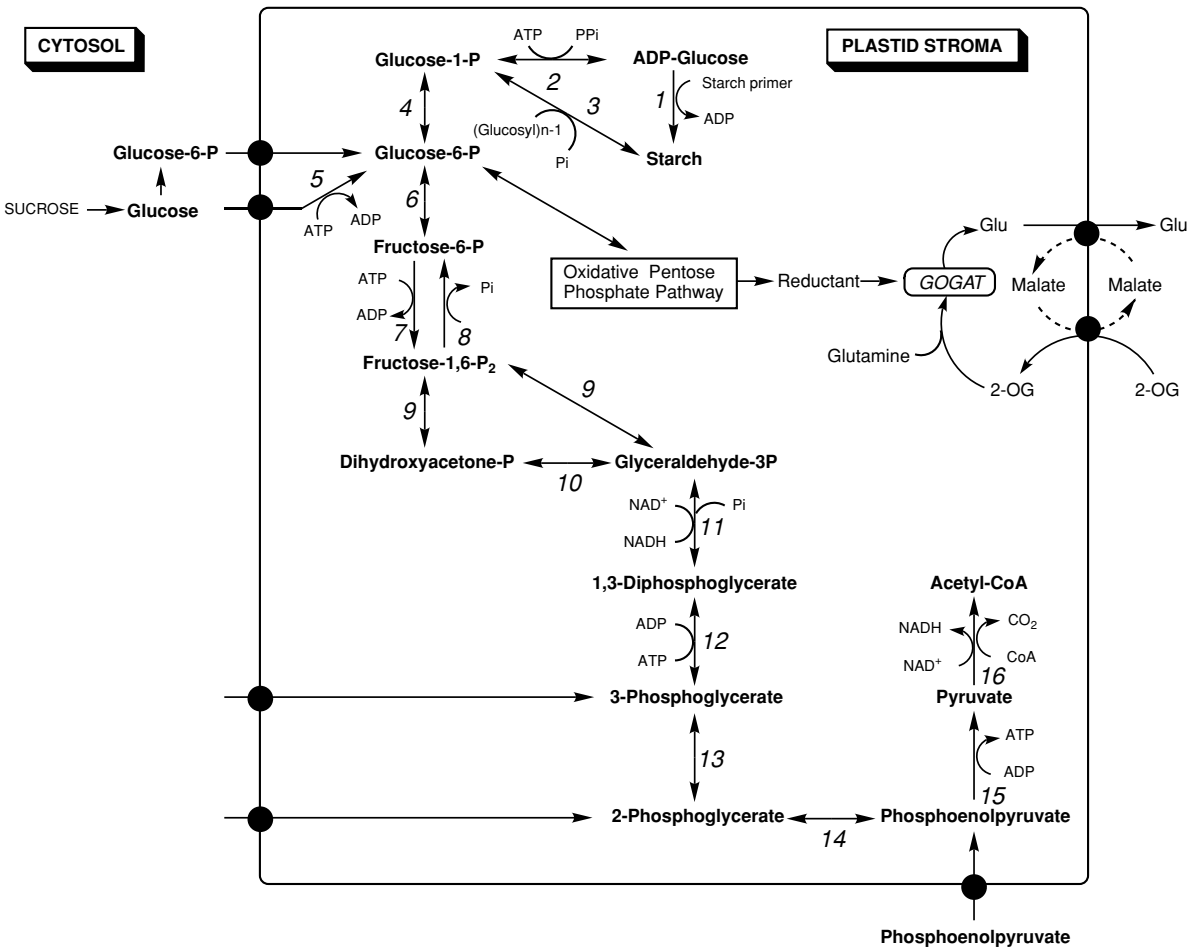



*Fig. 2.* Pathway of the utilization of sucrose-derived metabolites in plastids from ripening fruits. Numbers refer to the following enzymes : 1, starch synthase; 2, ADP-glucose pyrophosphorylase; 3, α-glucan phosphorylase; 4, phosphoglucomutase; 5, hexokinase; 6, hexose phosphate isomerase; 7, ATP-phosphofructokinase, 8; fructose,1, 6-*bis*phosphatase; 9, aldolase; 10, triose-P isomerase; 11, glyceraldehyde 3-P dehydrogenase; 12, phosphoglycerate kinase; 13, phosphoglycerate mutase; 14, enolase; 15, pyruvate kinase; 16, pyruvate dehydrogenase. Abbreviations refer to: GOGAT, glutamine-2-oxoglutarate aminotransferase; Glu, glutamic acid; 2-OG, 2-oxoglutarate. Plastid translocators are shown as solid, black circles.

red fruits contain only the cytosolic form (Büker *et al.*, 1998). This suggests that hexose phosphates are imported from the cytosol during chloroplast to chromoplast differentiation in tomato. In pepper fruits during the ripening process the decrease of Rubisco (Ziegler *et al.*, 1983) is paralleled by an increase in plastidial FBPase (Thom *et al.*, 1998).

Whatever their origin, the carbon skeletons are used in different biosynthetic pathways such as transient starch, fatty acids and isoprenoids. They are also used in the oxidative pentose phosphate (OPP) pathway which is a main source of reducing power in the absence of photosynthetic electron transport (Fig. 2). In this context, it is worth noting that tomato chromoplast glucose 6-phosphate dehydrogenase (G6PDH), a main enzyme of the OPP pathway, is 48.7 and 7.4 more active than leaf and green fruit chloroplast G6PDH from the same plant (Aoki *et al.*, 1998).

The capacity of fruit chromoplasts to oxidize hexoses to pyruvate (Fig. 2) has been reviewed previously (Camara *et al.*, 1995). In this context it is interesting to note that a chromoplast-specific, $NAD^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) named GapCp has been characterized recently from pepper fruits (Petersen *et al.*, 2003). The expression of *GapCp* is restricted to the ripe fruits and roots. On the other hand the chloroplast $NADP^+$-dependent GAPDHs, *GapA* and *GapB*,

are down-regulated during pepper fruit ripening. This suggests that in chromoplasts or in a more general context, non-green plastids, GapCp is specifically engaged in the production of energy in the absence of photosynthesis (Petersen *et al.*, 2003). In relation to the energy requirements in nongreen plastids, it is interesting to note that heterotropic daffodil chromoplasts can direct part of the NADP(H) generated by plastid glycolysis to the generation of ATP (Morstadt *et al.*, 2002).

The plastid glycolytic pathway is interconnected with the cytosolic glycolytic pathways by several membrane translocators (Fig. 2). It has been suggested that in relation to their metabolic activity plastid translocators could play specific roles (Heldt *et al.*, 1991). This contention is supported by the fact that the plastid triose phosphate translocator is more highly expressed in green tomato fruit than in the red fruit (Schünemann *et al.*, 1996), while the reverse situation is observed in red tomato fruits (Schünemann and Borchert, 1994). In a similar vein, the phosphoenolpyruvate transporter gene is more expressed in non-green tissue (Fischer *et al.*, 1997). Based on the fact that the initial phase of respiratory climacteric is correlated (at least in banana fruit) with a reduced level of PEP and an increased level of pyruvate (Beaudry *et al.*, 1989; Ball *et al.*, 1991) this suggests that during the ripening period fruit plastids may exert indirect control over cytosolic glycolytic flux.

## *B. Acyllipid Metabolism*

Although most commercial oils are derived from seeds, the ripe fleshy pericarp of oil palm and olive fruits represent an important source of vegetable oil. For instance, palm oil ranks second after soybean (Salas *et al.*, 2000). Palm oil contains about 45% palmitic acid and 40% oleic acid, while olive oil is 60 to 70% enriched in oleate (Salas *et al.*, 2000). In both fruits, the initial control of the lipid synthesis is exerted at the level of the *de novo* synthesis of fatty acid in the plastid, before the modification steps in the endoplasmic reticulum and eventual triacylglycerol accumulation (Daza and Donaire, 1982; Sambanthamurthi *et al.*, 2000) (Fig. 3).

Two routes have been established for the synthesis of the acetyl-CoA, the initial precursor (Ohlrogge and Browse, 1995). These include the plastid glycolytic degradation of hexoses via the pyruvate dehydrogenase complex. Alternatively, acetyl-CoA produced from the mitochondrial pyruvate dehydrogenase complex could be imported into the plastid. Both pathways have been demonstrated in olive fruit pericarp (Salas *et al.*, 2000). Acetyl-CoA is sequentially converted to malonyl-CoA by acetyl-CoA carboxylase (ACCase) which is transformed to malonyl-acyl carrier protein (MACP) before the formation of acyl-ACP by individual component enzymes of the fatty acid synthetase pathway (FAS)

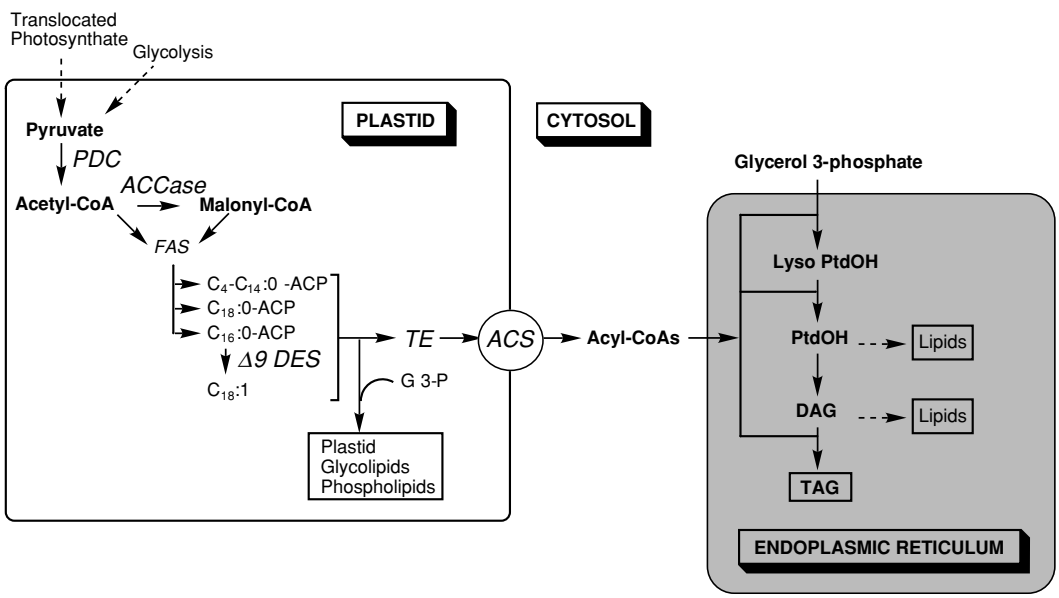



*Fig. 3. De novo* synthesis of lipids in plastids of ripening fruits and modification in the endoplasmic reticulum. Abbreviations refer to: PDC, pyruvate dehydrogenase complex; ACCase, acetyl-CoA carboxylase; FAS, fatty acid synthetase; ACP, acyl carrier protein; Δ9 DES, Δ9 desaturase; TE, thioesterase; ACS, acyl-CoA synthetase; PdtOH, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol.

(Ohlrogge and Jaworski, 1997) (Fig. 3). FAS comprises the condensing enzymes β-keto acyl-ACP synthases (KAS) I, II and III, β-ketoacyl-ACP reductase and β-hydroxyacyl-ACP dehydratase. The ratio between the final products 16-ACP and 18-ACP is specified by the plastid thioesterase (Fig. 3), which cleaves the acyl-ACP, and also by the KASII activity with elongates palmitoyl-ACP to steraroyl-ACP. Consistent with the regulatory role of plastid thioesterases and KASII, a specific palmitoyl-ACP thioesterase (Othman *et al.*, 2000) is induced during the ripening stage of oil palm fruit, while the KASII activity is decreased (Salas *et al.*, 2000; Sambanthamurthi *et al.*, 2000). Thus, these coupled activities contribute to the profuse plastidial synthesis and export of palmitate to the cytosol and account for the high palmitate content of oil palm fruit. On the other hand, in olive fruit the role of the plastidial $C_{18}$ acyl-ACP thioesterases seems to predominate (Harwood, 1996).

In some plants, specific plastidial KASs and acyl-thioesterases contribute to the synthesis of fatty acids having shorter carbon chains. The latter are probably exported to the cytosol and used for the synthesis of capsaicinoids during pepper fruit ripening (Aluru *et al.*, 2003) (Fig. 4). Alternatively, some early intermediates formed in the plastid could be exported to the cytosol and used for the biogenesis of acylglucose derivatives (van der Hoeven and Steffens, 2000) (Fig. 4).

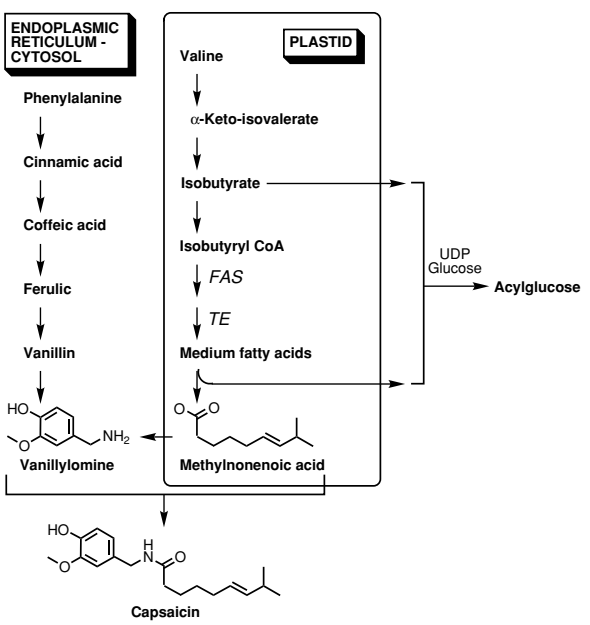



*Fig. 4.* Channeling of lipid precursors towards capsaicin and acylglucose synthesis. Abbreviations refer to: FAS, fatty acid synthetase; TE, thioesterase.

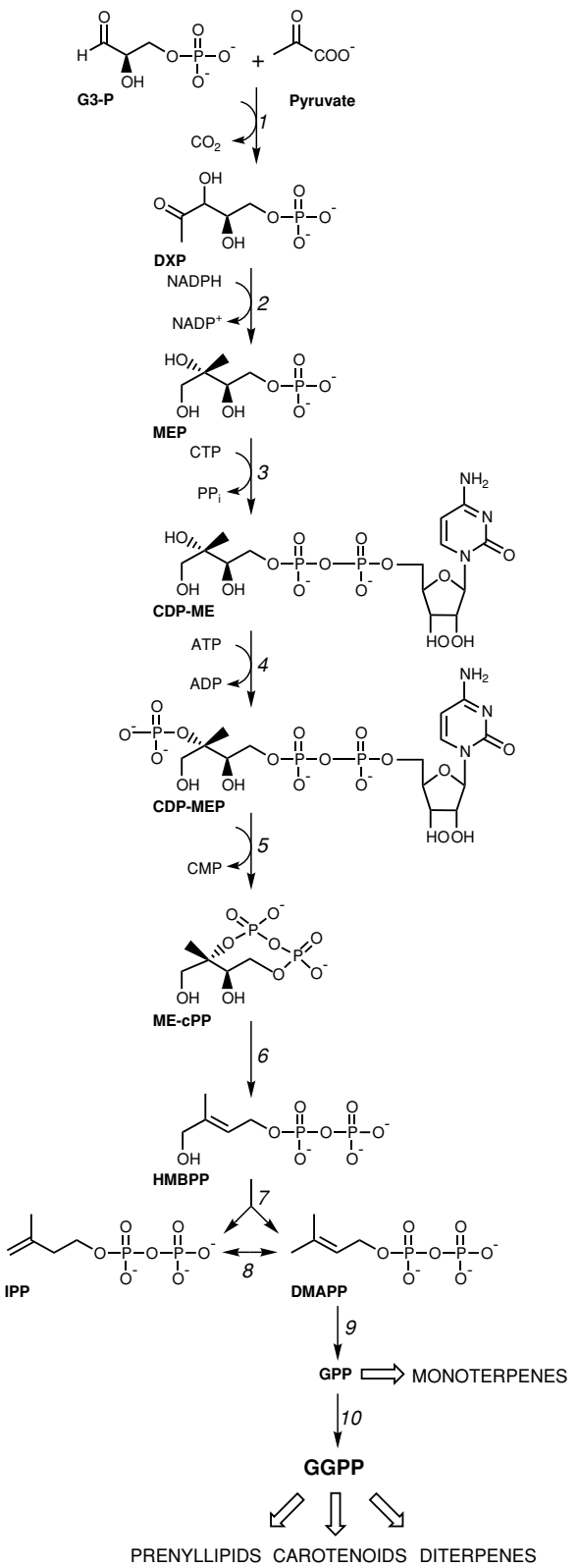
Several lines of evidence suggest the involvement of oxylipins derived from the lipoxygenase pathway in the ripening of fruits. Lipoxygenase catalyzed reactions produce 9- and 13-hydroperoxide derivatives. The 13-hydroperoxide is converted into several products including jasmonic acid and the volatile aldehyde hexenal (Mack *et al.*, 1987), while the 9-hydroperoxide product could be converted under constrained conditions into hexanal with low efficiency (Hatanaka *et al.*, 1992). In tomato, the hydroperoxide lyase (HPL) catalyzing the synthesis of hexenal and the allene oxide synthase (AOS) initiating the jasmonic acid pathway have been characterized and shown to be respectively located in the inner and the outer chloroplast membrane envelopes (Howe *et al.*, 2000; Feild *et al.*, 2001; Froehlich *et al.*, 2001). However, during the ripening period tomato fruit HPL and AOS genes are apparently not induced (Back *et al.*, 2000). A similar trend was observed during the ripening of pepper (Matsui *et al.*, 1997). Thus further studies are required to clarify the involvement of oxylipin metabolism during fruit ripening.

### *C. Carotenoid Metabolism*

The yellow, orange and red colors of many fruits are due to carotenoids which are classified into carotenes and their oxygenated derivatives, xanthophylls. Carotenoids are synthesized as $C_{40}$ isoprenoid derivatives in plastids. In green fruits, they accumulate in photosynthetic chloroplasts which differentiate into chromoplasts during the ripening process (Camara *et al.*, 1995).

Chromoplast-synthesized isoprenoids derive from deoxylulose phosphate (Fellermeier *et al.*, 2003) and not from mevalonate as envisioned previously (Fig. 5). The initial steps involve a transketolase reaction between pyruvate and glyceraldehyde 3-phosphate and the downstream steps lead to dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Fig. 5). This leaves unanswered the role of plastid IPP isomerase that catalyzes the isomerization of IPP to DMAPP (Dogbo and Camara, 1987). Further addition of three IPP to DMAPP by plastidial geranylgeranyl diphosphate synthase (GGPPS) (Dogbo and Camara, 1987) yields geranylgeranyl diphosphate (GGPP) (Fig. 5), the immediate precursor of carotenoids and other prenyllipids and diterpenes.

The first committed step of carotenoid biosynthesis is the dimerization of GGPP into phytoene by a bifunctional phytoene synthase (PSY) (Dogbo *et al.*, 1988) (Fig. 6). Phytoene is further desaturated by phytoene




desaturase (Pds) and ζ-carotene desaturase (Zds) to yield neurosporene and lycopene. Recently, the gene encoding carotenoid isomerase (CrtIso) has been characterized and shown to be involved in the isomerization of poly-*cis* carotenoids to all *trans* carotenoids (Isaacson *et al.*, 2002; Park *et al.*, 2002). The cyclization of lycopene catalyzed by lycopene β-cyclase (Lcyb) and/or lycopene ε-cyclase (Lcye), yields α-carotene and β-carotene (Fig. 6). The cyclase step represents a crucial branching point because only β-carotene is converted into zeaxanthin by non-heme diiron monooxygenases (CrtHb) (Bouvier *et al.*, 1998c), while α-carotene is converted to lutein by recently characterized cytochrome p450-type monooxygenases (Tian *et al.*, 2004). Zeaxanthin is further converted to violaxanthin via antheraxanthin by zeaxanthin epoxidase (Zep) and finally violaxanthin is converted to neoxanthin by neoxanthin synthase (Nsy) (Fig. 6). In ripening pepper fruit antheraxanthin and violaxanthin are further converted into the red ketocarotenoids, capsanthin and capsorubin by capsanthin-capsorubin synthase (Ccs) (Bouvier *et al.*, 1994) (Fig. 6).

Xanthophylls accumulating in ripening fruits are generally esterified by medium chain ($C_{12}$, $C_{14}$) fatty acids (Breithaupt and Bamedi, 2001) or even $C_4$ fatty acids (Pott *et al.*, 2003). The physiological significance of this phenomenon may be linked to the fact that acylation enhances the lipophilic character of the xanthophylls thus favoring their massive accumulation or sequestration in specialized chromoplast structures (Camara *et al.*, 1995). In addition to the esterification reaction, fruit carotenoids are cleaved by specific dioxygenases to yield diverse aroma compounds (Winterhalter and Rouseff, 2002).

*Fig. 5.* Overview of the MEP pathway in plastids. The numbers correspond to the following enzymes: 1, 1-deoxy-D-xylulose 5-phosphate synthase (DXS); 2, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR); 3, 2C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS); 4, 4-diphosphocytidyl-2 C-methyl-D-erythritol kinase (CMK); 5, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS); 6, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS); 7, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR); 8, IPP isomerase; 9, geranyl diphosphate synthase; 10, geranylgeranyl diphosphate synthase. Abbreviations refer to: DXP, 1-deoxy-D-xylulose 5-phosphate; MEP: methylerythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl ME; CDP-MEP, CDP ME 2-phosphate; ME-cPP, ME 2,4-cyclodiphosphate; HMBPP, hydroxymethylbutenyl 4-diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate.

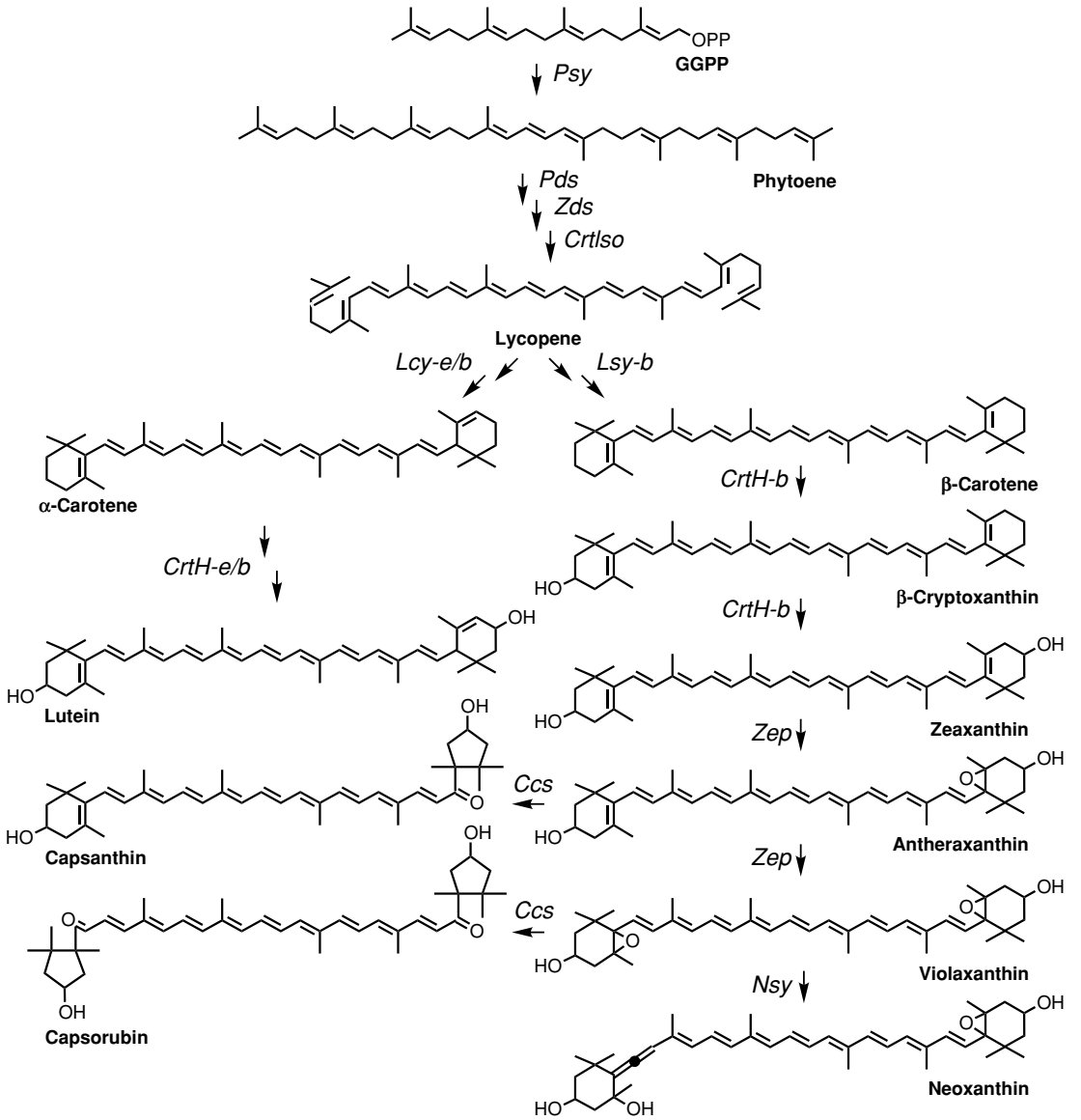



*Fig. 6.* Typical pathway leading to chromoplast specific carotenoids during fruit ripening. The enzymes are indicated in italics: Psy, phytoene synthase; Pds, phytoene desaturase; Zds, ζ-carotene desaturase; CrtIso, carotenoid isomerase; Lcy-b, lycopene β-cyclase; Lcy-e, lycopene ε-cyclase; CrtH-b, carotenoid β-hydoxylase; CrtH-e, carotenoid ε-hydoxylase; Zep, zeaxanthin epoxidase; Ccs, capsanthin-capsorubin synthase; Nsy, neoxanthin synthase.

Although carotenoid biosynthesis is a highly regulated process, its control is poorly understood. While many developmental and metabolic processes are light regulated in green tissues, this appears not to be the general rule for carotenogenic genes (Corona *et al.*, 1996; von Lintig *et al.*, 1997; Wetzel and Rodermel, 1998; Bugos *et al.*, 1999). In chromoplast-containing tissue the situation is less ambiguous since available data suggest that transcriptional regulation prevails. During tomato fruit ripening, the expression of *DXS* (Lois *et al.*, 2000; Bartley and Ishida, 2002), *Psy* and *Pds* (Giuliano *et al.*, 1993; Fraser *et al.*, 1994) increases, wheras the expression of *DXR* (Rodriguez-Concepcion *et al.*, 2001; Bartley and Ishida, 2002) and *HDS* (Rodriguez-Concepcion *et al.*, 2003) remain constant, while *Lcyb* (Pecker *et al.*, 1996) and *Lcye* (Ronen *et al.*, 1999) are down-regulated, thus leading to massive accumulation of lycopene sequestered in crystal structures. In pepper fruits the ripening is paralleled by an increased of the expression of *DXP*

(Bouvier *et al.*, 1998b), GGPPS (Kuntz *et al.*, 1992), *CrtHb* (Bouvier *et al.*, 1998c) and *Ccs* (Bouvier *et al.*, 1994). In Valencia orange and Satsuma mandarin the intense accumulation of α-cryptoxanthin, zeaxanthin and violaxanthin which occurs during the ripening period, is paralleled by a coordinated increased expression of *Psy*, *Pds*, *Zds*, *Lcyb*, *CrtHb* and *Zep* (Kato *et al.*, 2004).

In contrast to the situation prevailing in chloroplasts, the accumulation of carotenoids in chromoplasts is flexible and readily amenable to genetic manipulation. Transformation of tomato with the bacterial phytoene synthase (*CrtB*) from *Erwinia* increased 2- to 4-fold the total fruit carotenoid in the ripe fruits (Fraser *et al.*, 2002). As lycopene is an acylic precursor of β-carotene, introduction of heterologous *Lcyb* in tomato fruit via a specific promoter induces partial conversion of lycopene into β-carotene (Rosati *et al.*, 2000). In a similar vein, the introduction of *Lcyb* and *CrtHb* in tomato resulted in the accumulation of β-cryptoxanthin and zeaxanthin during the ripening stage (Dharmapuri *et al.*, 2002). This flexibility of fruit chromoplasts has also been observed by introducing the multifunctional bacterial phytoene desaturase in tomato fruit. Under these conditions, β-carotene represented 45% of the total carotenoid content (Romer *et al.*, 2000). Finally, the loss of function of *CrtIso* leads to the accumulation of prolycopene (Isaacson *et al.*, 2002) which is characteristic of tangerine tomato fruits.

Based on the above evidence one can suggest that the transition of chloroplasts to carotenogenic chromoplasts in ripening fruits involves up-regulation of specific genes in the pathway. How these changed are triggered is presently unknown. Efforts directed toward unraveling the mechanism inducing these changes revealed three facts. First, in tomato, the high pigment-2 (*hp-2*) locus which affects carotene accumulation, is involved in photomorphogenesis signalling (Mustilli *et al.*, 1999). Second, reactive oxygen species act as secondary messengers during the strong induction of carotenoid biosynthesis in pepper chromoplasts (Bouvier *et al.*, 1998a). Third, in nonphotosynthetic tissue, the accumulation of carotenoids is indirectly regulated by the sequestration of excess carotenoid in deposit structures (Deruère *et al.*, 1994; Vishnevetsky *et al.*, 1999). In this context, it is interesting to note that in cauliflower, the *Or* gene, which does not encode a carotenoid biosynthetic enzyme, induces the accumulation of massive amounts of β-carotene in the normally uncolored tissue of cauliflower (Li *et al.*, 2001; Li and Garvin, 2003).

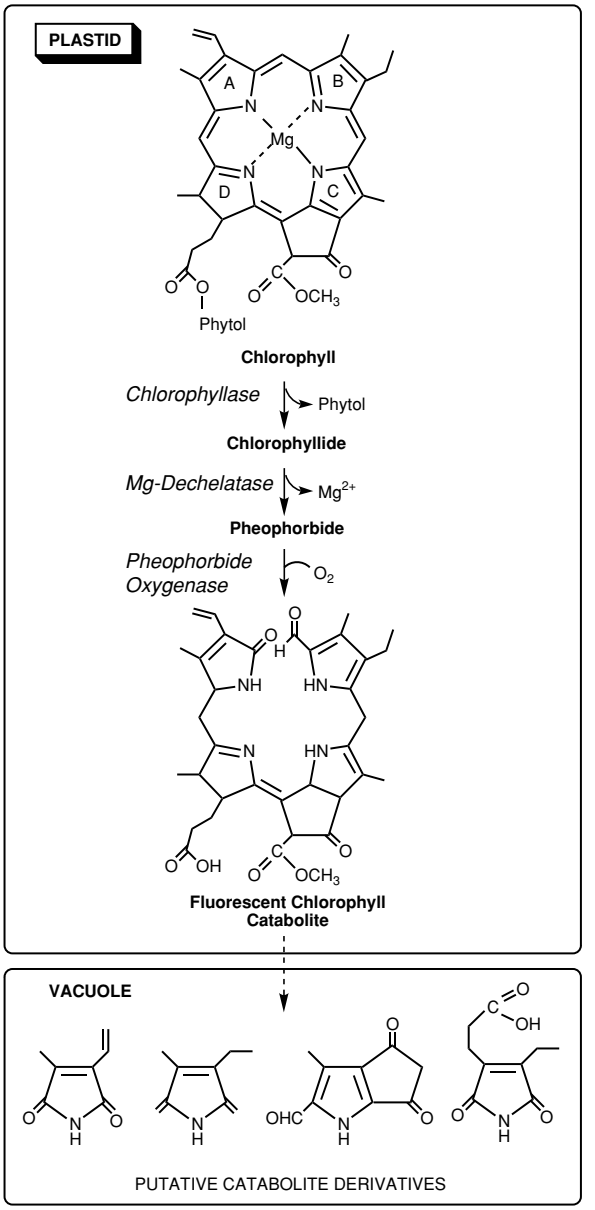



*Fig. 7.* Chlorophyll catabolism and vacuolar sequestration of final products during fruit ripening.

## *D. Prenyllipid Metabolism*

The breakdown of chlorophyll is a characteristic phenomenon associated with fruit ripening. As such, it is stimulated by ethylene, a regulator of ripening in many fruits (Shimokawa *et al.*, 1978). However, in some tomato mutants (*Green flesh*) and pepper cultivars (Mulato), chlorophyll content is preserved during the ripening process without affecting the accumulation of lycopene in tomato or capsanthin and capsorubin in

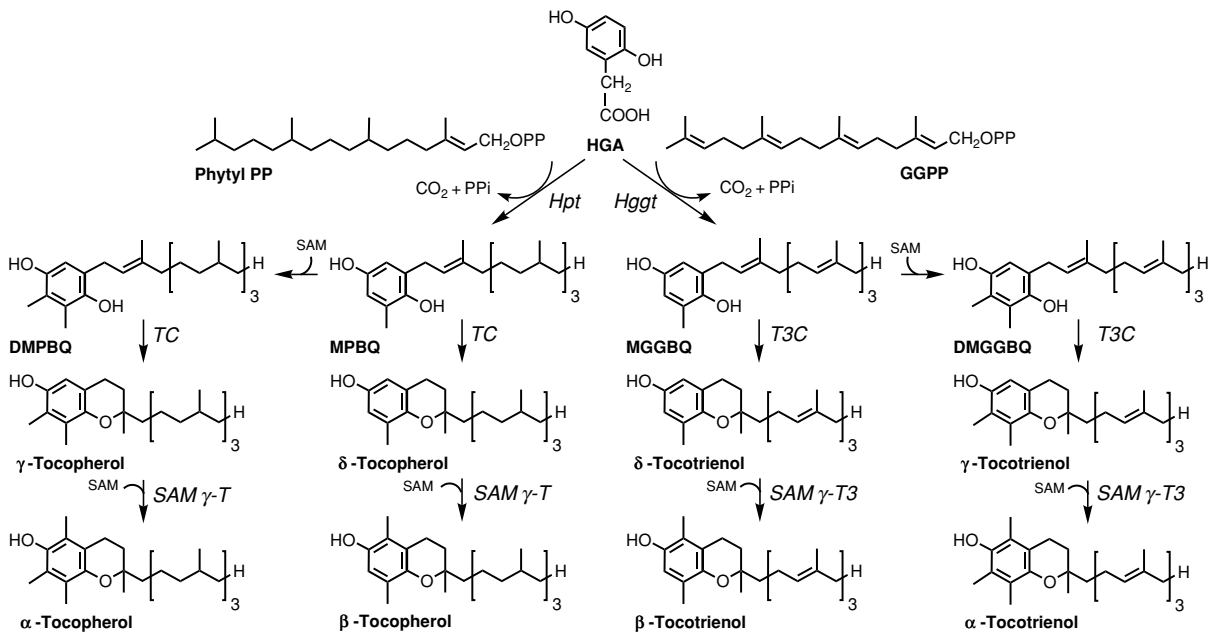



*Fig. 8.* Tocopherol and tocotrienol biosynthetic pathways. Abbreviations refer to: HGA, homogentisic acid, Hpt, homogentisate phytyl transferase; Hggt, homogentisate geranylgeranyl transferase; GGPP, geranylgeranyl diphosphate; MPBQ, 2-methyl-6-phytylbenzoquinol; DMPBQ, 2,3-dimethyl-5-phytylbenzoquinol; MGGBQ, 2-methyl-6-geranylgeranylbenzoquinol; DMGGBQ, 2,3-dimethyl-5-geranylgeranylbenzoquinol; TC, tocopherol cyclase, T3C, tocotrienol cyclase, SAM, S-adenosyl-L-methionine; SAM γ-T, S-adenosyl-L-methionine γ-tocopherol methyltransferase; SAM γ-T3, S-adenosyl-L-methionine γ-tocotrienol methyltransferase.

pepper. The enzymic breakdown of chlorophyll is initiated in the plastid by chlorophyllase (Jacob-Wilk *et al.*, 1999) which catalyzes the cleavage of cholorophyll into phytol and chlorophyllide (Matile *et al.*, 1999) (Fig. 7). While the fate of phytol is unknown, the chlorophyllide moiety is enzymatically converted into fluorescent catabolites which are further degraded and sequestered in the vacuole as colorless derivatives (Matile *et al.*, 1999; Suzuki and Shioi, 1999) (Fig. 7). The degradation of chlorophylls in ripening fruits is not irreversible. Gibberellin treatments are able to induce the regreening of chlorophyll-free fruits (Devidé and Ljubesic, 1974; Coggins *et al.*, 1980). This is probably linked to the fact that chlorophyll-free chromoplasts maintain the potential for chlorophyll biosynthesis as shown by chlorophyll synthesis during *in vitro* incubation with exogenous chlorophyll precursors (Dogbo *et al.*, 1984; Kreuz and Kleinig, 1984; Lutzow and Kleinig, 1990).

In contrast to chlorophylls, tocopherols are generally actively synthesized in plastids during fruit ripening (Camara *et al.*, 1982; Burns *et al.*, 2003). The pathway involves the prenylation of homogentesic by homogentisate phytyl transferase (Hpt) followed by cyclization (Arango and Heise, 1998) and methylation (d'Harlingue and Camara, 1985) in a reaction sequence probably similar to that operating in leaf chloroplasts (Cheng *et al.*, 2003) (Fig. 8).

With regard to tocotrienols, the initial step involves a homogentisate geranylgeranyl diphosphate transferase (Hggt) (Fig. 8). Usually this enzyme is not active in leaves (Cahoon *et al.*, 2003), but in fruits (Silva *et al.*, 2001; Kallio *et al.*, 2002) and especially the mesocarp of ripening oil palm fruit, the presence of an active chromoplast Hggt leads to an accumulation of α-, β-, δ-, γ-tocotrienols (Sambanthamurthi *et al.*, 2000).

## *E. Polyphenol Oxidase Activity*

It has been established that the browning coloration of several fruits is induced by polyphenol oxidase (PPO) which oxidizes phenolic substrates into reactive quinones that are prone to polymerization and to give brown covalent adduct with reactive amino acids. This visually limits both consumer acceptance and the nutritional quality of fruits. Therefore, effort has been invested to down regulate PPO activity by genetic engineering.

PPOs are nuclear-encoded, plastid-destined proteins associated with thylakoid membranes (Vaughn *et al.*,

1988). In general PPO activity is highest in growing fruits and decreases in ripening fruits (Vamos-Vigyazo, 1981). In some plants like apple (Boss *et al.*, 1995) and pineapple (Stewart *et al.*, 2001), the activation of PPO by mechanical wounding is exerted via transcriptional mechanism. Alternatively, the browning mechanism may be explained or enhanced by the fact that PPO is latent and stable (Dry and Robinson, 1994) and thus any loss of cellular compartmentation due to mechanical disruption may give PPO access to phenolic substrates (Walker and Ferrar, 1998).

## IV. Conclusions

Plastids constitute a large family of interconvertible organelles among which fruit plastids reveal high structural and metabolic flexibility. This phenomenon is particularly evident during the ripening process which is paralleled by the mobilization of starch, the import of cytosolic metabolites and the generation of non-photosynthetic ATP or reducing power that are used for organelle biogenesis. This offers the unique opportunity to study the biochemical and genetic function of plastids in the absence of photosynthesis.

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# Chapter 22

# Fate and Activities of Plastids During Leaf Senescence

Karin Krupinska*
*Institute of Botany, University of Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany*

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## Summary

During leaf senescence, chloroplasts are transformed into gerontoplasts. This process is characterized by changes in the ultrastructure and in the biochemical and functional properties of the plastids. Concomitantly, a decline in the cellular population of chloroplasts/gerontoplasts may occur. While gerontoplast differentiation to a certain extent is a reversible process, a reactivation of plastid division does not occur. A model depicting three different pathways by which chloroplasts may be degraded in senescing mesophyll cells is presented. Plastids may be engulfed in the central vacuole by phagocytosis or by membrane fusion of plastid containing autophagosomes with the vacuole. Alternatively, or in addition, plastids may release vesicles containing stromal material and globules consisting of thylakoid-derived material into the cytoplasm. The release of globules appears to occur at a later stage of senescence when the plastid envelope is ruptured. There is evidence that Rubisco and other stromal proteins are at least partly degraded outside the plastid. Degradation of chlorophylls is a prerequisite for degradation of the apoproteins of the pigment-protein complexes. After cleavage of chlorophyll *a* by chlorophyllase, the resulting chlorophyllide is

*Author for correspondence, email: kkrupinska@bot.uni-kiel.de

*Robert R. Wise and J. Kenneth Hoober (eds.), The Structure and Function of Plastids,* 433–449.

further degraded via pheophorbide to fluorescent catabolites. Two different chlorophyllases have been characterized, one targeted to the plastid and the other seems to be synthesized at the endoplasmic reticulum. It is speculated that the initial steps of chlorophyll catabolism are not restricted to the plastid but may also occur in the vacuole. The lipid moiety of thylakoid membranes, mainly galactolipids, is probably degraded by an inverse pathway to their biosynthesis that finally leads to gluconeogenesis. In parallel with degradation of the photosynthetic apparatus, levels of water-soluble antioxidants decline, and the level of lipophilic tocopherols increases during senescence. It is proposed that reactive oxygen species and tocopherols accumulating in gerontoplasts may be involved in signalling during senescence.

## I. Introduction

During leaf senescence, chloroplasts are transformed into so-called gerontoplasts (Sitte, 1977) resembling the chromoplasts of reproductive organs (Thomas *et al.*, 2003). Gerontoplasts and chromoplasts have in common a reduced thylakoid system and many plastoglobuli. In contrast to chromoplasts, gerontoplasts are unable to divide (Matile, 1992). The development of gerontoplasts is under genetic control and is reversible up to a certain time point, which has been termed "point of no return" (Noodén and Leopold, 1978). When reversibility of senescence is lost, cells enter the terminal phase of senescence leading to cell death (Noodén *et al.*, 1997; Dangl *et al.*, 2000). It is yet not known what causes the loss of senescence reversibility.

Though senescence-associated changes in plastid activities during differentiation of chloroplasts into gerontoplasts have been characterized in detail (for recent reviews see Feller, 2004; Krupinska and Humbeck, 2004; Mae, 2004), several fundamental questions concerning the fate and activities of plastids have not been answered so far. In principle, there are two ways to explain the senescence-related decrease in photosynthesis, biosynthetic activities and plastid constituents, which become rich sources for nitrogen and phosphorous after degradation. (1) Either whole chloroplasts are removed from the mesophyll cells by intracellular digestion of the entire organelles, or (2) persisting plastids gradually undergo transformation to non-photosynthetic gerontoplasts. Because the chloroplast components are not broken down in a parallel manner (Krupinska and Humbeck, 2004; Mae, 2004), changes in persisting plastids are obviously significantly contributing to the decline of chloroplast constituents and function. Nevertheless, a decline in plastid population has been frequently observed, and under these circumstances it surely contributes to the loss of plastid components. Ford and Shibles (1988) suggested that the decline in chloroplast function is a two-stage process: after a transition of persisting plastids into gerontoplasts, the plastid population declines during a brief terminal phase.

Neither the mechanisms of the changes in chloroplast population nor the mechanisms underlying the remobilization of nutrients from chloroplasts have been clarified. Most of the proteases and lipases encoded by genes with enhanced expression during senescence are not targeted to the plastid (Buchanan-Wollaston, 1997; Guiamet *et al.*, 1999). Likewise, the ubiquitin-dependent proteolytic pathway that seems to play an important role during senescence (Yoshida *et al.*, 2002) does not operate in chloroplasts (Vierstra, 1996).

The cytological sequence of senescence related events was investigated comprehensively in rice coleoptiles by fluorescence and electron microscopy (Inada *et al.*, 1998a,b). The findings confirmed earlier results on the sequence of degradation events, as reported by Butler and Simon (1971). Degradation of DNA precedes the shrinkage of the chloroplasts, the degradation of Rubisco, the dilation of thylakoid membranes, and the increase in number and size of osmiophilic globules.

Despite numerous studies reported in the literature, discrepancies still exist regarding the sequence of the breakdown of the thylakoid membrane system and of the components constituting the photosynthetic apparatus (Ghosh *et al.*, 2001; Krupinska and Humbeck, 2004). The discrepancies are probably due to the different plant materials used for senescence analyses. Besides naturally senescing leaves, leaves induced to senesce by darkness and by different other stress treatments have been used. Comprehensive studies on the same material employing ultrastructural methods as well as physiological, biochemical and molecular analyses are lacking.

*Abbreviations:* FCC – fluorescent chlorophyll catabolite; LHC – light harvesting complex; PaO – pheophorbide *a* oxidase; POR – NADPH-protochlorophyllide oxidoreductase; RCB – Rubisco-containing bodies; RCCR – red chlorophyll catabolite reductase; Rubisco – ribulose-1,5-*bis*phosphate carboxylase/oxygenase; TAG – triacylglycerol.

## II. Decline in Plastid Population of Mesophyll Cells During Senescence

Several ultrastructural studies showed that the number of chloroplasts is higher in mesophyll cells of younger leaves in comparison to mesophyll cells from senescing leaves of the same plant (Wittenbach *et al.*, 1980, 1982; Hashimoto *et al.*, 1989; Kura-Hotta *et al.*, 1990; Ono *et al.*, 1995; Yamasaki *et al.*, 1996; Zavaleta-Mancera *et al.*, 1999a; Minamikawa *et al.*, 2001). For instance, in senescing wheat leaves about 20% of the chloroplasts disappeared slowly in parallel with chlorophyll degradation until the final stage of senescence, when rapid degradation of the plastids took place (Ono *et al.*, 1995). Investigations on plastid numbers during leaf senescence in barley, however, revealed conflicting results. According to Martinoia *et al.* (1983) and Matile (1992), the entire population of plastids does persist in senescing mesophyll cells. Chloroplasts undergo specific changes in structure and composition leading to the decrease in photosynthetic capacity as reported also by Mae *et al.* (1984), Gepstein (1988) and Grover (1993). When plastids of senescing barley leaves, on the other hand, were counted morphometrically in a defined subpopulation of mesophyll cells located at a certain distance from the vascular tissue, an early decrease in plastid number per cell area in parallel to the decrease in chlorophyll content of the leaves was observed (S. Pfeiffer and K. Krupinska, unpublished results).

It is likely that the contradictions concerning the decline in chloroplast number are due to differences in the various materials used to study senescence and in the methods used for counting the plastids, respectively. In fast senescing organs such as the cotyledons, the change in the number of chloroplasts may be observed rather early (Minamikawa *et al.*, 2001), while it occurs at a late stage in other situations of senescence. Analyses of plastid numbers in protoplasts and in separated cells may lead to other results than analysis of plastid numbers of specific mesophyll cells within intact leaf tissue (Wittenbach *et al.*, 1982; Wardley *et al.*, 1984). In the latter case, it is possible to analyze only a specific subpopulation of mesophyll cells, which are known to vary considerably in the degree of senescence related degradation (Hurkman, 1979).

It is likely, therefore, that both the degradation of components in persisting plastids and a destruction of whole plastids occur during senescence. In fact, in a vertical gradient of leaves of *Chenopodium album* both mechanisms could be demonstrated (Yamasaki *et al.*, 1996). Taken together the studies on plastid population during senescence make it rather likely that mechanisms for degradation of whole plastids do exist. Wittenbach (1982) presented ultrastructural evidence indicating that chloroplasts during senescence of wheat leaves are associated with invaginations of the vacuole and may move into the vacuole. Phagocytosis of whole plastids by the vacuole has been also observed in senescing cells of other plants (Noodén, 1988a). Minamikami *et al.* (2001) provided ultrastructural evidence for an association of chloroplasts with the vacuole. They further observed that chloroplasts after invagination by the vacuole had a rough surface indicating that the outer membrane had been subjected to degradation by vacuolar enzymes.

Other studies indicate that plastids may be degraded by an autophagic cell death mechanism that was observed in plants in the endosperm and suspensor tissue (Wredle *et al.*, 2001). It has been also presumed that plastids in the suspensor tissue may turn themselves autophagic. Such elements with lytic activity have been postulated before and have been termed plastolysomes (Nagl, 1977). However, no evidence for such an autophagic capacity of plastids during leaf senescence has been provided so far. It is possible that plastids containing autophagosomes may fuse with the central vacuole. Such an autophagic mechanism has been proposed for the degeneration of mitochondria and cytoplasm during senescence of cotyledons of mung bean (*Vigna mungo*) seedlings (Toyooka *et al.*, 2001).

## III. Reversibility of Gerontoplast Differentiation and Loss of Plastid DNA

Regreening of senescing leaves had been studied already very early (Mothes and Baudisch, 1958). Until recently it has not been clearly shown whether gerontoplasts are capable of redifferentiation into chloroplasts or whether new chloroplasts develop from proplastids during regreening. By ultrastructural analyses of senescing tobacco leaves, Zavaleta-Mancera *et al.* (1999b) clearly showed that the chloroplasts of regreened tobacco leaves were redifferentiated gerontoplasts. Regreening coincides with an increase in the level of NADPH-protochlorophyllide oxidoreductase (POR) required for chlorophyll biosynthesis, and a progressive recovery of the lamellar system (Zavaleta-Mancera *et al.*, 1999a,b). Though chlorophyll and the photosynthetic machinery are resynthesized during regreening of tobacco leaves, the number of plastids does not increase again in the mesophyll cells of these leaves, which during senescence already underwent a

reduction in chloroplast population (Zavaleta-Mancera *et al.*, 1999b). The regreening capacity declined with age of the gerontoplasts, indicating that the point of no return (Noodén and Leopold, 1978) had been approached.

The presence of intact plastid DNA is an irrevocable prerequisite for regreening. It has been reported, however, that the DNA of plastids is lost early during senescence (Sodmergen *et al.*, 1989, 1991). Recent analysis of the sequence of senescence-associated events in the rice coleoptile showed that plastid DNA degradation indeed precedes the degradation of Rubisco and of thylakoid membranes (Inada *et al.*, 1998a,b). An early degradation of plastid DNA before yellowing was also observed in second leaves of rice (Sodmergen *et al.*, 1991). Inada *et al.* (1998a,b) deduce from their findings on DNA content of chloroplasts during coleoptile development that the "chloroplast genome already releases all of the information needed for construction of the chloroplast" before chloroplast maturation is completed.

In contrast, other studies have shown that plastid DNA is preserved during barley leaf senescence and even is transcribed as revealed by run-on transcription analysis with isolated plastids (Krause *et al.*, 1998). Among an overall increase in transcriptional activity during light induced reversion of senescence induced by darkness, the transcription of individual genes encoding components of the photosynthetic apparatus is preferentially activated (Krause *et al.*, 1998). The regreening capacity of senescing chloroplasts clearly indicates that the whole genetic machinery of the plastids is still intact. If DNA degradation is indeed an early event of leaf senescence, a decline in DNA copy number and not a complete loss of DNA is likely to occur. It is well known that plastids are highly polyploid and show developmentally related changes in DNA copy number. With cereal primary foliage leaves it has been shown that the DNA copy number, after a transient increase during early leaf development, steadily declines (Baumgartner *et al.*, 1989; Hashimoto and Possingham, 1989).

By staining DNA with DAPI and by immunohistochemistry on ultrathin sections, Inada *et al.* (1998a,b) showed that plastid DNA is first digested to DNA-fragments that are dispersed throughout the chloroplast and the cytoplasm. Eventually these fragments are broken down completely. So far, it is not known whether the final degradation of DNA occurs within the plastid. The possible involvement of a $Zn^{2+}$-dependent nuclease has been discussed by Sodmergen (1991). However, this and other deoxyribonucleases involved in degradation of plastid DNA during senescence have not been identified.

Though it is well known that during leaf senescence total RNA content decreases (Brady, 1988; Krause *et al.*, 1998) and total ribonuclease activity increases (Blank and McKeon, 1991; Taylor *et al.*, 1993; Lers *et al.*, 1998), no clear-cut picture exists about senescence-related RNA degradation within the plastid. Earlier studies reported a preferential degradation of ribosomal plastid RNA, but usually both cytoplasmic and plastid ribosomal RNA were observed to decrease in parallel during senescence (Brady, 1988). At least a few ribosomes seem to persist in senescing plastids.

## IV. Senescence-Related Changes in the Ultrastructure of Plastids

Ultrastructural changes in senescing chloroplasts are well documented by conventional electron microscopy techniques (Butler and Simon, 1971; for reviews see Biswal and Biswal, 1988; Gepstein, 1988). They comprise a reduction in the thylakoid membrane system, a loosening of the grana stacks, a swelling of intrathylakoidal space, a shrinkage of the size of the organelle with a transition from ellipsoid to circular shape and an increase in size and number of plastoglobuli (Butler and Simon, 1971; Noodén, 1988a; Guiamet *et al.*, 1999; Kura-Hotta *et al.*, 1990; Matile, 1992; Inada *et al.*, 1998b).

### *A. The Degeneration of the Thylakoid Membrane System*

In most studies it has been observed that stromal thylakoids loose their integrity earlier than granal thylakoids (Hurkman, 1979; Gepstein, 1988). Hashimoto *et al.* (1989) observed that in senescing chloroplasts the organization of stromal and granal thylakoids does change, and granal stacks with a large number of thylakoids similar to those of shade chloroplasts become prevalent. This observation is consistent with studies reporting a decrease in the chlorophyll *a/b* ratio of senescing rice leaves (Kura-Hotta *et al.*, 1987) and a high stability of LHC during barley leaf senescence under field conditions (Humbeck *et al.*, 1996). In a recent investigation with barley leaf segments kept in the light, however, grana disintegrated before stromal thylakoids (Spundova *et al.*, 2003). The authors suggested that under these specific conditions the LHC is degraded to lessen the risk of photooxidative damage. Recent work on cucumber cotyledons indicates

that the granal stacks may get reduced by lateral movement of the light-harvesting complex to Photosystem I (Prakrash *et al.*, 2001, 2003, see also section IV.B).

At later stages of senescence, thylakoids become distended and dissociate into distinct vesicles (Hurkman, 1979). In the studies with rice coleoptiles, intergranal lamellae began to swell already at the onset of senescence (Inada *et al.*, 1998b).

## B. Plastoglobuli

### 1. Increase in Size and Number

One of the most obvious changes occurring in chloroplasts during senescence is the increase in number and size of plastoglobuli (Butler and Simon, 1971; Biswal and Biswal, 1988; Gepstein, 1988; Guiamet *et al.*, 1999; Gosh *et al.*, 2001). During leaf senescence of rape (*Brassica napus*) aggregation and enlargement of plastoglobuli was accompanied by the loss of photosystem II activity and grana compactness (Gosh *et al.*, 2001).

It is common opinion that these senescence-associated plastoglobuli contain the breakdown products of the thylakoid membranes (Tevini and Steinmüller, 1985; Matile, 1992). Indeed, the composition of plastoglobuli changes during senescence (Lichtenthaler, 1969). In chloroplasts of rice coleoptiles the plastoglobuli were observed to increase in size and number just at the time when intergranal membranes began to swell (Inada *et al.*, 1998b). An association of osmiophilic globules with thylakoids and vesicle formation from the distentions of the thylakoids was observed by electron microscopic investigations (Butler and Simon, 1971; Hurkman, 1979).

### 2. Blebbing of Lipid-Protein Particles from Thylakoids

The mechanism of plastoglobuli formation may be similar to the formation of lipid-protein particles, which are continuously produced from thylakoids to allow for maintenance of an intact photosynthetic apparatus, parts of which are destroyed during their function (Melis, 1999). Such lipid-protein particles, which were first isolated from the stroma of intact chloroplasts, contain thylakoid lipids, free fatty acids, thylakoid proteins and catabolites of these proteins (Gosh *et al.*, 1994). Similar lipid-protein particles were generated *in vitro* from isolated light-stressed thylakoids. They were isolated from cytosolic fractions and were called "deteriosomes" indicating that they serve as a vehicle for removing catabolites from membranes that would otherwise destabilize the membranes (Yao *et al.*, 1991). It is therefore likely that blebbing of lipid-protein particles is a means to remove catabolites from thylakoid membranes. Though these particles are usually smaller than plastoglobuli, their composition resembles the composition of plastoglobuli (Steinmüller and Tevini, 1985). It is therefore feasible that plastoglobuli are formed by fusion of such thylakoid-derived lipid-protein particles.

### 3. Blebbing of Plastoglobuli into the Cytosol

Rupture of chloroplast envelopes and release of plastoglobuli to the cytoplasm at late stages of leaf senescence was described early by electron microscopy (Butler and Simon, 1971; Hurkman, 1979). Indeed, osmiophilic deposits resembling plastoglobuli have often been reported to occur in the cytoplasm of senescing leaves (reviewed in Guiamét *et al.*, 1999). Until recently, it has been assumed that these lipids were derived from membranes outside the plastid. The remarkable ultrastructural study on soybean leaf senescence of Guiamét *et al.* (1999), however, provided evidence that these lipids are derived from senescing chloroplasts. In this report it was shown that globules specific for senescing cells protrude through the chloroplast envelope and emerge into the cytoplasm. Judging from the protrusions in the chloroplast envelope, the authors postulated that the plastoglobuli press against and squeeze through the outer membrane of the chloroplasts. The fluorescence characteristics of the senescence-associated globules indicated that they contain chlorophyll or chlorophyll degradation products. This new mechanism of blebbing from the chloroplast may also be used for the secretion of thylakoid membrane breakdown products–lipids, proteins and pigments. Immunological analyses clearly showed the presence of the thylakoid membrane protein CP47 within these globules. The globules were observed to accumulate transiently before leaf abscission. Some of the senescence-associated globules in the cytoplasm were surrounded by a coat with a pattern resembling the polygonal pattern of clathrin coats. Since these coats were not detectable at the sites of globule emergence from the chloroplast, they seem not to be involved in the blebbing process itself. The authors assume that during senescence the final degradation of these globule-associated thylakoid membrane components occurs outside the plastids. Lipids are supposed to be degraded in glyoxysomes, and proteins by proteases in the cytoplasm and in the vacuole. Recently, it was

suggested that plastoglobuli are analogous to the lipid bodies generated at the endoplasmic reticulum (Kaup *et al.*, 2002). From this point of view, similarities in the structure and the blebbing mechanism are likely. Indeed plastoglobuli were shown to be covered with the structural protein fibrillin, analogous to oleosin covering ER-derived oil bodies.

The fluorescing globules described by Guiamet *et al.* (1999) may be similar to the colored vesicles observed in the cytoplasm of senescing broccoli florets (Terai *et al.*, 2000). These vesicles are derived from globules inside the chloroplasts and become larger when the granal thylakoids degenerate. Both by electron microscopy and light microscopy the colored vesicles or globules derived from the senescing chloroplasts of broccoli were observed to emerge on the outside of the plastids and then to spread into the cytosol (Terai *et al.*, 2000). The authors discuss that the vesicles may be either transferred into the cytosol through the intact plastid envelope or may be exposed to the cytosol by destruction of the envelope. Since no information has been provided on membranes surrounding these structures, the term "vesicle" should be avoided in this case.

Although the mechanism of globule formation from the plastid is not known, the final destination of the globules in any case could be the vacuole. Indeed Inada *et al.* (1998b) in their comprehensive study on chloroplast senescence in rice coleoptiles observed oil drops sequestered in the vacuole.

## C. Extrusion of Stromal Material

### 1. Vesicle-Mediated Degradation

Vesicles in the cytoplasm that include Rubisco and/or Rubisco degradation products (Rubisco-containing bodies, RCB) (Chiba *et al.*, 2003) and other stromal proteins, e.g. glutamine synthase, were detected by ultrastructural analyses of wheat leaves either senescing naturally or induced to senesce by darkness. These RCBs were observed to be surrounded by a double membrane and by other membranous structures in the cytoplasm. In contrast to the colored globules observed during soybean leaf senescence (Guiamet *et al.*, 1999) and broccoli floret senescence (Terai *et al.*, 2000), these vesicles appeared very early in senescence when Rubisco degradation started. The authors postulated that RCBs are involved in degradation of stromal proteins at the onset of senescence. Using high pressure freezing in combination with freeze substitution and immunogold labelling, S. Pfeiffer and K. Krupinska (unpublished results) observed that

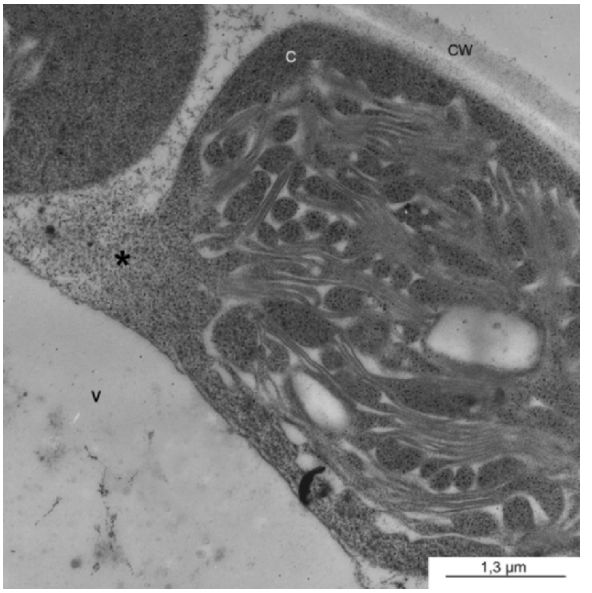


*Fig. 1.* Transmission electron microscopic image of a thin section derived from a cryofixed senescing barley primary foliage leaf 17 days after sowing. Cryofixation was performed as described (Pfeiffer *et al.* 2003). At this stage of development, chlorophyll content of the leaves has declined by 10% (S. Pfeiffer and K. Krupinska, unpublished results). Rubisco was immunolabelled by an antiserum directed towards large subunit of Rubisco (LSU) and a goat anti-mouse immunoglobulin conjugated to 10- nm gold particles. Rubisco-containing stroma material is released by the chloroplasts (C) into the cytoplasm. The region of Rubisco release into the cytoplasm is indicated by an asterisk. In addition to the chloroplast (C), the vacuole (V) and the cell wall (CW) are labeled.

Rubisco-containing material may be directly released into the cytoplasm at an early stage of senescence of barley primary foliage leaves (Fig. 1).

As described below, indeed different pathways for degradation of stromal proteins seem to exist.

### 2. Involvement of Autophagic Processes

The ultrastructural analysis of RCB indicates that these bodies may be degraded by autophagocytosis similar to autophagy in yeast, which is a process of vacuolar or lysosomal degradation of cytoplasmic components including organelles under nutrient-deprived conditions (Klionsky and Oshumi, 1999) and has been also proposed as one of several mechanisms for plant cell death (Noodén, 2004). Membrane whorls, which are typical for autophagy, were indeed often observed by ultrastructural analyses around vesicles found in senescing cells (Noodén, 1988). Autophagic vacuoles are generally believed to originate from concentric cisternae of the endoplasmic reticulum, which coalesce

to enclose cytoplasmic space possibly containing organelles (Matile, 1975; Dunn, 1990). Another type of autophagy is the invagination of the tonoplast of ordinary vacuoles, which results in the sequestration of cytoplasmic material and its eventual digestion (Matile, 1975). It seems that autophagic cell death is activated when massive removal of cells is demanded by developmental programs (Clarke, 1990). Inada *et al.* (1998b) observed membranous structures that enclosed cytoplasmic material within the vacuole during senescence of the rice coleoptile. The authors postulated that the material was taken up by inclusion of the tonoplast and that it was finally degraded inside the vacuole.

In plant cells, autophagy has been observed to occur under nutrient-starved conditions (Aubert *et al.*, 1996; Moriyasu and Ohsumi, 1996). Recently, it was reported that autophagy under these circumstances involves the formation of autolysosomes in the cytoplasm. These lysosomes are separated from the central vacuole and accumulate in the cytoplasm in the presence of an autophagy inhibitor (Takatsuka *et al.*, 2004). During tracheary element formation, partial autolytic processes in the cytoplasm precede the final comprehensive autolysis of the cell, which is caused by a collapse of the lytic vacuole (Ye *et al.*, 2002). Plastid-derived starch granules in cotyledons of mung bean that are senescing during germination seem to be degraded by autophagic forms of lytic vacuoles, which also contain α-amylase required for starch degradation (Toyooka *et al.*, 2001).

Findings with *Arabidopsis* mutants suggest that the autophagic pathway is important for senescence. Mutants carrying T-DNA insertions within autophagy genes *apg7* and *apg9* (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002) show accelerated senescence, suggesting that the autophagic proteins are required for maintenance of cellular activities during leaf senescence, possibly by the removal of damaged proteins.

In summary, besides the transformation of chloroplasts into gerontoplasts, a decline in the population of plastids may occur during leaf senescence. Loss of plastids may occur by engulfment in the central vacuole or by the formation of autophagosomes and their fusion with the vacuole. In addition, different pathways for exclusion of plastid material into the cytoplasm and the vacuole seem to exist (Fig. 2). It is possible that certain pathways are dominant under specific conditions of senescence. Future investigations are necessary to elucidate the factors determining the contribution of the different pathways to the decline in plastid population, and how the plastids engulfed by the central vacuole are further degraded. Moreover, further investigations should clarify under which conditions plastids release material into the cytoplasm. Is the material released by the plastids engulfed by the central vacuole or is it remobilized outside the vacuole? Are the lipids included in the globules reused for gluconeogenesis? It is possible that there is a relation between the demand for gluconeogenesis and the release of lipid containing material to the cytoplasm. Finally, it will be interesting to see whether the plastids that release globules and vesicles into the cytoplasm are finally engulfed by the vacuole.

## V. Degradation of Thylakoid Membrane Lipids

Thylakoid membranes have a characteristic lipid composition. The galactolipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol, collectively comprise approximately 80% of the thylakoid lipid content (Lee, 2000). Their predominant acyl residues are polyunsaturated fatty acids of the 18:3 and 16:3 types (Matile, 1992), which in theory could be easily oxidized in the course of free-radical reactions (Leshem, 1992). In contrast to the cytoplasmic membranes, which are rather prone to lipid peroxidation and concomitant rigidification, thylakoids do not undergo phase changes or alterations in lipid composition during senescence (Thompson *et al.*, 1998). Though thylakoid membrane lipids have a high content of polyunsaturated fatty acids, the proportion of these fatty acids remains essentially unchanged over the period when photosynthetic activity declines by approximately 80% and chlorophyll levels are reduced by 75% (summarized by Thompson *et al.*, 1998).

During thylakoid breakdown, galactolipids do not accumulate in plastoglobuli as do other lipid metabolites originating from thylakoids (Tevini and Steinmüller, 1985). From this finding it was concluded that these lipids may be utilized as a source of carbon and energy (Matile, 1992). It has been proposed that galactolipids are degraded by a pathway that is the reverse of their biosynthesis. Such a pathway would involve the activities of α-galactosidases, ß-galactosidases and galactolipases (Matile, 1992). There is evidence for the involvement of peroxisomes, which during senescence are transformed into glyoxysomes with increasing levels of the key enzymes of the glyoxylate cycle (del Rio *et al.*, 1998). This development is important to provide a pathway for gluconeogenesis.

Senescence-related up-regulation of a ß-galactosidase gene has been shown in wheat leaves (Bhalla and Dalling, 1984) and in asparagus (King *et al.*, 1995), and

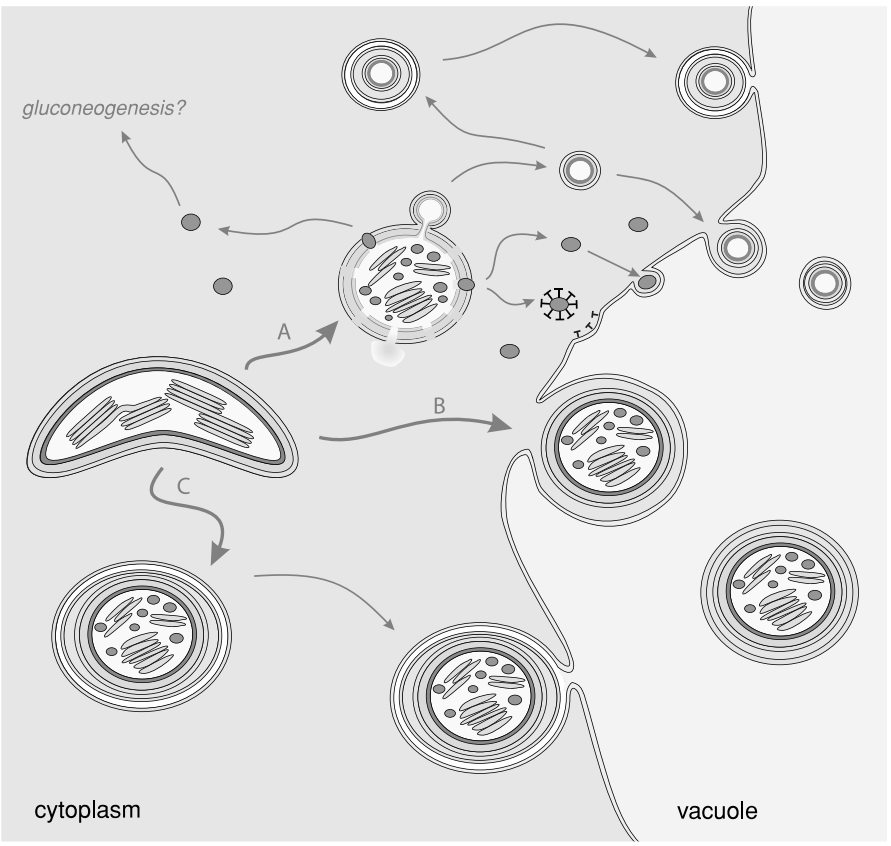


*Fig. 2.* (See also Color Plate 5, p. xxxviii.) Model of three different pathways by which chloroplasts may be degraded in senescing mesophyll cells. Chloroplasts are getting smaller and change from an ellipsoid to a round shape. Thylakoids get swollen and plastoglobuli with thylakoid-derived material are accumulating. Senescing chloroplasts may release material into the cytoplasm (A), may be engulfed by the central vacuole (B) or may be included in autophagosomes which may fuse with the vacuole (C). A. Early during senescence, vesicles including stromal material are formed. These vesicles are called Rubisco-containing bodies (RCB). The vesicles may be engulfed directly by the central vacuole or may give rise to the formation of autophagosomes, which are incorporated into the central vacuole by membrane fusion. Later on, during senescence the envelope ruptures and releases globules containing thylakoid-derived material including chlorophyll. The lipids included in the globules may be used for gluconeogenesis. Other components may be further degraded in the cytoplasm. A part of the globules is covered by a coat of proteins resembling clathrin. A receptor- mediated endocytosis by the vacuole may occur. B. A part of the population of chloroplasts is under certain conditions engulfed by the central vacuole. The material is degraded by enzymes of the vacuole. C. Another part of the chloroplast population may be surrounded by ER membranes resulting in the formation of autophagosomes. These structures may be incorporated into the vacuole by membrane fusion.

senescence-associated regulation of an α-galactosidase gene has been shown in senescing barley leaves by Chrost and Krupinska (2000). So far, it is unknown in which cell compartments these enzymes are operating. De-esterification of galactolipid fatty acids may be mediated by one or more senescence-induced galactolipases (Kim *et al.*, 2001). The pool of free fatty acids is, however, rather low in chloroplasts and even decreases further during senescence (Gut and Matile, 1989).

Recently, Kaup *et al.* (2002) showed that triacylglycerols (TAG) accumulate in rosette leaves of *Arabidopsis thaliana* with advancing senescence, coincident with the increase in the abundance and size of plastoglobuli. Since these TAGs of senescing rosette leaves are enriched in hexadecatrienoic acid (16:3) and linolenic acid (18:3), it is likely that the TAGs derive from thylakoid galatactolipids inside the plastid. A diacylglycerol acyltransferase enzyme whose RNA and protein levels increase during senescence was found in the chloroplast membrane fraction (Kaup *et al.*, 2002). Activity measurements suggest that the enzyme is associated with the envelope (Martin and Wilson, 1984). TAGs had been detected already earlier in plastoglobuli of spinach chloroplasts (Steinmüller and Tevini, 1985), where they seem to accumulate transiently during dismantling of the thylakoids. The study of Kaup *et al.*

(2002) suggests that TAG formation during senescence is an intermediate step in the conversion of thylakoid fatty acids to sucrose, which is finally transported by the phloem to other parts of the plant. As described above, plastoglobuli of senescing chloroplasts may be extruded through the envelope into the cytoplasm (Guiamét *et al.*, 1999). By this way the fatty acids bound in TAG may gain access to glyoxysomes for ß-oxidation and may be subsequently used for gluconeogenesis.

## VI. Degradation and Mobilization of Proteins

General protein degradation during senescence was reviewed comprehensively by Hörtensteiner and Feller (2002) and Feller (2004). In this review only plastid proteins are considered.

Chloroplasts contain 70 to 80% of the total nitrogen of mature leaves (Mae *et al.*, 1993). This nitrogen is mainly bound in protein components of the chloroplasts, which are either carbon reduction cycle enzymes of the stroma or proteins of the photosynthetic apparatus located in the thylakoid membrane. Investigations on senescent primary foliage leaves of wheat led to the conclusion that chloroplast proteins are mobilized in two different ways during senescence: by gradual degradation of the proteins inside the chloroplast, and by the successive disappearance of whole chloroplasts (Ono *et al.*, 1995). Experiments performed by Mae *et al.* (1993) with shaded and unshaded leaves of *Lolium tremulentum* showed that the senescence-related decrease of Rubisco was hardly affected by the light intensity, whereas the senescence associated decrease in the levels of other proteins of the photosynthetic apparatus (e.g. LHCII and cytochrome *f*) was clearly retarded under low light condition. These results indicate that stromal and thylakoid membrane proteins are degraded by different pathways.

### *A. Rubisco and Other Stromal Proteins*

Degradation of the most abundant protein compound, the stroma-localized ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), has been studied intensively (see also Gepstein, 1988). In some studies it was observed that the decrease in Rubisco was much faster than the decline in the chloroplast population (Martinoia *et al.*, 1983, Ono *et al.*, 1995). A close correlation between the decline in Rubisco activity and content on one hand and the decline in photosynthetic function on the other hand (Mae *et al.*, 1993; Jiang *et al.*, 1993; Ono *et al.*, 1995; Mae, 2004) indicates that Rubisco degradation is the key factor responsible for the decline in photosynthetic capacity during senescence. Other stromal enzymes, which are much less abundant than Rubisco, are degraded with kinetics similar to Rubisco. This was shown for Rubisco activase and phosphoribulokinase (Crafts-Brandner *et al.*, 1996).

Several studies suggest that oxidation may be a trigger for degradation of Rubisco during stress and senescence (Mehta *et al.*, 1992; Desimone *et al.*, 1998). To exclude the possibility of Rubisco oxidation, Simova-Stoilova *et al.* (2002) chose dark-induced senescence of barley primary foliage leaves to study the degradation pathway of Rubisco. By incubation of biotinylated Rubisco with plastid extracts *in vitro* they showed that under this condition distinct plastid-associated proteolytic activities contribute to Rubisco degradation, of which one activity was induced early during senescence and required ATP and divalent metal ions, while the activity measured at a later stage of senescence was ATP-independent.

Indeed, several ways of Rubisco degradation may occur in senescing chloroplasts. There are reports that indicate involvement of vacuolar proteases in degradation of Rubisco after engulfment of a part of the chloroplast population by the vacuole (see sections II and IV, and Fig. 2). By immunogold labeling, Minamikawa *et al.* (2001) investigated the possible involvement of vacuolar cysteine proteinases in degradation of Rubisco. As a consequence of the engulfment of chloroplasts by the vacuole, at least a fraction of Rubisco is degraded by vacuolar proteinases (Minamikawa, 2001). Alternatively, Rubisco could be degraded in the cytoplasm after release of stromal material by early degrading chloroplasts as observed by high pressure freezing and freeze substitution of the samples (Fig. 1).

A so far unknown pathway for the extrusion of chloroplast components into the cytoplasm and/or the vacuole by outer envelope protrusions has been proposed on the basis of ultrastructural studies with *Chlamydomonas* cells during photoautotrophic growth conditions (Park *et al.*, 1999). The authors detected chloroplast proteins synthesized on plastid ribosomes, e.g., the large subunit of Rubisco, within dense granules in certain vacuoles of the cells. Ultrastructural analysis of cryofixed algal cells revealed protrusions of the outer membrane of the chloroplast envelope, which occasionally were found to enclose stromal material with particles similar in size to chloroplast ribosomes. As also reported for stromules that connect plastids (Köhler *et al.*, 1997), the protrusions regularly seem to

contain no thylakoid membranes and no chlorophyll. It is, however, not very likely that the protrusions are analogous to stromules, which are formed in interaction with microfilaments in the cytoplasm for exchange of protein molecules between plastids and which are probably not related to degradative processes (Kwok and Hanson, 2004).

Cryofixation as used by Park *et al.* (1999) is a technique that preserves native structures to a greater extent than conventional chemical fixation. Recently, stroma-containing, plastid protuberances were also described for higher plant chloroplasts by ultrastructural analysis employing high-pressure freezing and freeze-substitution (Bourett *et al.*, 1999). A comprehensive re-examination of senescence-associated ultrastructural changes in plastids by using high-pressure freezing in combination with freeze-substitution will provide new insights into the process of chloroplast degradation.

Though thylakoid membranes and chlorophyll were not found in the protrusions (Park *et al.*, 1999), light-harvesting complex proteins were detected immunologically in vacuoles in a previous study on photoautotrophically grown *Chlamydomonas* cells (Wolfe *et al.*, 1997). The accumulation of these proteins in the vacuole was enhanced in a mutant impaired in thylakoid formation, suggesting that these light-harvesting complex proteins were imported and processed within the plastid but not inserted in a membrane.

### *B. Thylakoid Membrane Proteins*

Pigment-protein complexes in thylakoid membranes account for more than 30% of the total protein of chloroplasts (Matile, 1992). The loss of the apoproteins is well coordinated with degradation of the chlorophylls, which are non-covalently bound to these apoproteins (Okada and Katoh, 1998). Chlorophyll breakdown is actually a prerequisite for the remobilization of these compounds (see section VII, A).

While the chlorophyll *a/b* ratio stays constant during dark-induced senescence, under natural senescence this ratio was observed to decline. This indicates that the light-harvesting system, harbouring all the chlorophyll *b*, has an enhanced stability in comparison to the reaction center complexes, which exclusively contain chlorophyll *a* (Kura-Hotta *et al.*, 1987; Humbeck *et al.*, 1996; Miersch *et al.*, 2000). In a comparison of pine needles over a three-year period, a significant reduction in the levels of photosystem I and cytochrome $b_6/f$ complexes was observed in second- and third-year needles (Shinohara and Murakami, 1996). Other complexes of the photosynthetic apparatus, including LHCII, decayed later in senescence. Recently, Humbeck and Krupinska (2003) showed that the decline in photosystem II efficiency in barley collected in a field coincided with the decline in the levels of the inner light-harvesting complexes of photosystem II (CP29) and photosystem I (LHCI), which are supposed to play an important role in energy transfer within the photosynthetic units (Bassi *et al.*, 1993). A comprehensive study on the kinetics of disappearance of six light-harvesting proteins during dark-induced senescence clearly confirmed their differential stability (Rosiak-Figielek and Jackowski, 2000).

Specific alterations in the organization of chlorophylls within the light-harvesting complexes, leading to possible changes in energy transfer within the complexes, were deduced from visible circular dichrosim spectra (Prakash *et al.*, 2003). In a previous study it was suggested that destacking and flattening of grana thylakoids are accompanied by a migration of LHCII to photosystem I in senescing cucumber cotyledons (Prakash *et al.*, 2001) as shown by protein and pigment analysis of photosystem I particles. Confirmative immunological analyses with antibodies specific for photosystem I and LHCII are unfortunately lacking. It was proposed that migration of LHCII could result from irreversible phosphorylation (Prakash *et al.*, 2001), although evidence for senescence-related changes in activity of the LHC-specific phosphatase is lacking.

## VII. Pigment Catabolism

### *A. Chlorophylls*

Chlorophyll degradation is a prerequisite for degradation of the protein moiety of pigment protein complexes. Chlorophyll is degraded to nonfluorescent catabolites (NCC), which finally accumulate in the vacuole (for reviews, see Matile *et al.*, 1988; Takamiya *et al.*, 2000; Kräutler, 2003; Hörtensteiner and Matile, 2004).

Chlorophyllase catalyzes the hydrolysis of chlorophyll to yield chlorophyllide and phytol and is thought to be the first enzyme in the degradation pathway (Matile *et al.*, 1999). Chlorophyllase activity has been found to be associated with the chloroplast envelope (Matile, 1997). Chlorophyllase specific cDNAs have been cloned from Valencia Orange (*Citrus sinensis*) (Jacob-Wilk *et al.*, 1999) and from leaves of *Chenopodium rubrum* (Tsuchiya *et al.*, 1999). While the amino acid sequence of the chlorophyllase cloned from Valencia Orange has a N-terminal transit

sequence for the plastid, the sequence of the chlorophyllase from *Chenopodium* (CrCHL) has a N-terminal signal sequence indicating that it is synthesized on ribosomes associated with the endoplasmic reticulum. The chlorophyllase of *Chenopodium* may be glycosylated and has in addition to the N-terminal signal sequence a NPIR motif, which is a vacuolar sorting determinant. This suggests that the chlorophyllase is transported to the vacuole via the ER (Tsuchiya *et al.*, 1999). Database analysis revealed that *Arabidopsis thaliana* has two genes that supposedly encode chlorophyllases. While *AtCHL1* has a signal sequence, *AtCHL2* has a typical transit sequence for the plastid (Tsuchiya *et al.*, 1999). Interestingly, the *AtCHL1* gene has been cloned previously as a gene induced by coronatine and jasmonate, two compounds known to promote chlorosis and senescence, respectively (Benedetti *et al.*, 1998).

By the action of chlorophyllase and magnesium chelatase, chlorophyll is converted to pheophorbide and then later to red and fluorescent chlorophyll catabolites (RCC, FCC). Pheophorbide is degraded by oxygenolytic opening of the porphyrin ring system and reduction of the double bond in the α-methine bridge (Pruzinská *et al.*, 2003). There is evidence for an export of fluorescent catabolites to the cytoplasm. After modifications, they are finally imported into the vacuole (Matile *et al.*, 1999; Hörtensteiner and Matile, 2004). Though chlorophyll *b* also disappears during senescence, breakdown products have only been identified from chlorophyll *a*. Indeed, chlorophyll *b* is reduced to chlorophyll *a* before degradation. Highest activity of chlorophyll *b* reductase has been found to precede the activity increase of chlorophyllase during dark-induced senescence of barley leaves (Scheumann *et al.*, 1999).

It has been proposed that the early steps of chlorophyll degradation take place in chloroplasts (Matile *et al.*, 1999; Hörtensteiner and Matile, 2004). Considering, however, that in addition to a plastid-targeted chlorophyllase and a second enzyme located in the vacuole, two different subcellular sites of chlorophyll degradation have to be envisaged. Perhaps chlorophyll is degraded in the chloroplast continuously during repair of the photosynthetic apparatus and is, in addition, degraded in the vacuole during certain conditions, e.g. during chlorosis induced by coronatine, a toxin produced by *Pseudomonas syringae* (Benedetti *et al.*, 1998), and during senescence induced by jasmonate and its derivates (Tsuchiya *et al.*, 1999). In accordance with these findings, it has been observed that during senescence chlorophyll containing plastid material may be enclosed by the vacuole (Guiamét *et al.*, 1999).

Chlorophyll is a photodynamic molecule that must be degraded to prevent oxidative stress (Hörtensteiner, 2004). In this regard the most important reaction in detoxification of chlorophyll is the oxygenolytic opening of the porphyrin ring system by pheophorbide a oxygenase (PaO), followed by reduction to FCC by red chlorophyll catabolite reductase (RCCR) (Hörtensteiner and Matile, 2004). The gene encoding PaO is identical with the *Accelerated cell death 1 gene* (*Acd1*) of *A. thaliana* (Pruzinská *et al.*, 2003). RCCR has been purified from barley and a partial gene sequence was cloned (Wüthrich *et al.*, 2000). Heterologous expression of the corresponding gene of *A. thaliana* yielded a protein active in reducing RCC to FCC in a reaction that required ferredoxin (Wüthrich *et al.*, 2000). *Arabidopsis* mutants defective in the gene encoding RCCR have spontaneously spreading cell death lesions that are probably caused by the accumulation of intermediates in the chlorophyll breakdown pathway (Mach *et al.*, 2001). PaO is only active during leaf senescence, whereas the activities of other enzymes of the pathway such as RCCR and chlorophyllase are expressed constitutively (Hörtensteiner *et al.*, 1995; Jakob-Wilk *et al.*, 1999). Because PaO mRNA and protein are present in non-senescent tissue, it has been concluded that PaO is regulated at the posttranslational level (Pruzinská*et al.*, 2003; Hörtensteiner, 2004).

## *B. Carotenoids*

The relative stability of carotenoids as compared to chlorophylls might be explained in terms of their association with plastoglobuli formed during senescence. They accumulate in the plastoglobuli at later stages of senescence (Lichtenthaler, 1969). It was suggested that fatty acids released from thylakoids interact with carotenoids to form stable esters that accumulate in plastoglobuli (Tevini and Steinmüller, 1985). Another explanation for the relative stability of carotenoids over chlorophylls would be a continued biosynthesis throughout the phase of senescence comparable to the situation during fruit ripening (Fraser *et al.*, 1994). During senescence of wheat leaves under field conditions, changes in the stoichiometry of carotenoids have been observed. While neoxanthin and ß-carotene decrease in parallel with chlorophylls, the xanthophyll cycle pigments are less affected (Lu *et al.*, 2001). Compositional changes and specific degradation pathways for carotenoids are still obscure, although some experimental data for the contribution of enzymes have been reported. So far, neither specific enzymes nor

degradation products have been identified. At least during the final stage of senescence, free-radicals seem to be involved in carotenoid breakdown (Biswal, 1995).

## VIII. Formation of Reactive Oxygen Species and Changes in Antioxidative Systems

A well-known feature of senescence is the decline in photosynthesis. As a consequence of the declining rate of photosynthetic activity, reactive oxygen species may accumulate and damage the photosynthetic apparatus (Krupinska and Humbeck, 2004). It is supposed that the oxidative damages accumulate over time as many anti oxidative systems decline with age (Munné-Bosch *et al.*, 2001). An imbalance of antioxidative systems on one hand and of reactive oxygen species on the other may be the cause of increasing oxidative stress during senescence.

A recent report on a plastid-located peroxiredoxin suggests that senescence is indeed accompanied by an increase in oxidative stress in plastids (König *et al.*, 2003). As a result of over-oxidation and thereby inactivation, the peroxiredoxin oligomerizes, and oligomers attach to the thylakoid membrane in leaves subjected to drought stress and during senescence. The authors propose that binding of the oligomers to the membrane could provide a long-term memory for oxidative stress.

Among the antioxidative systems that decline during senescence is vitamin C (Kunert and Ederer, 1985). In a study with *Cistus* plants, it was observed that plastid-located antioxidants (ß-carotene, α-tocopherol) have lower levels in old plants compared to young plants. In parallel, the level of malondialdehyde, which is a marker for lipid peroxidation, is enhanced in leaves of old *Cistus* plants (Munné-Bosch and Alegre, 2002b). This suggests that oxidative stress associated with aging in plants occurs at least partly in chloroplasts (Munné-Bosch and Alegre, 2002a). In contrast to the study of Munné-Bosch and Alegre (2002b), other studies have shown that the amount of tocopherols increases in plastids during senescence (Lichtenthaler, 1969; Peisker *et al.*, 1989; Rise *et al.*, 1989). In barley flag leaves senescing under field conditions, a transient increase in the level of tocopherols was observed around the onset of senescence (Chrost *et al.*, 1999). Since expression of the gene encoding 4-hydroxyphenylpyruvate dioxygenase, a key enzyme of tocopherol biosynthesis, is also enhanced during senescence (Kleber-Janke and Krupinska, 1997; Falk *et al.*, 2002), the increase in tocopherols is at least in part regulated at the transcriptional level. Rise *et al.* (1989) suggested that the phytol released by chlorophyllase activity may be reused for tocopherol biosynthesis during senescence. However, the amount of chlorophyll broken down was much higher than the amount of tocopherols that accumulated during senescence. Taken into account that a considerable part of the chlorophyll may be degraded outside of the plastid (see Section IV and Fig. 2), it is rather unlikely that tocopherol accumulation is a consequence of chlorophyll catabolism. Recently, it was reported that tocopherols also accumulated during development of leaves of transgenic plants showing delayed senescence. The delay of senescence was achieved by accumulation of cytokinins due to the over-expression of the gene encoding isopentenyl transferase (*ipt)* under control of a senescence-regulated promoter (Dertinger *et al.*, 2003). Tocopherol accumulation during senescence may be related to modulation of gene expression, by controlling the extent of lipid peroxidation and thereby the production of the signalling compound jasmonic acids and its derivatives (Chrost *et al.*, 1999; Munné-Bosch and Alegre, 2002a; Munné-Bosch and Falk, 2004).

## IX. Plastid Function in Relation to Senescence Signalling

The senescence-associated decline in photosynthetic activity has been proposed to initiate the senescence program (Hensel *et al.*, 1993). It has been further suggested that the mechanism underlying the induction of senescence in response to declining rates of photosynthesis may involve sugars or other photosynthetic metabolites as signalling compounds (Wingler *et al.*, 1998). Alternatively, or in addition, reactive oxygen species that accumulate as by-products of the photosynthetic electron transport in old leaves and under stress situations may be involved in triggering senescence. As a consequence of impaired photosystem I and reduced chlorophyll content, the *Arabidopsis* mutant *ore4-1* with a T-DNA inserted in the gene encoding plastid ribosomal small subunit 17 (*PRPS17*) (Woo *et al.*, 2002) shows delayed senescence. Likewise, Rubisco antisense plants (Miller *et al.*, 2000), which have reduced photosynthetic capability, have an extended leaf longevity.

Plastid derived reactive oxygen species may indeed act as signalling molecules in initiation of senescence. Among the reactive oxygen species, hydrogen peroxide is rather stable and may be considered as signalling molecule (Neill *et al.*, 2002). Recently it

was demonstrated that hydrogen peroxide accumulates transiently before the onset of senescence in *A. thaliana* (U. Zentgraf, personal communication). Whether the burst in production of hydrogen peroxide before senescence onset indeed occurs in chloroplasts as a consequence of declining photosynthesis (Krupinska *et al.*, 2003) has still to be investigated.

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# Chapter 23

# The Kleptoplast

Mary E. Rumpho* and Farahad P. Dastoor
*Department of Biochemistry, Microbiology and Molecular Biology, 5735 Hitchner Hall, University of Maine, Orono, ME 04469, U.S.A.*

James R. Manhart
*Department of Biology, Texas A&M University, College Station, TX 77843, U.S.A.*

Jungho Lee
*School of Biological Sciences, Seoul National University, Shillim, Kwanak, Seoul, South Korea 151-747*

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*Author for correspondence, email: mrumpho@umit.maine.edu

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## Summary

Kleptoplasty, the process by which a typically heterotrophic organism acquires and retains chloroplasts from a photosynthetic organism, is quite widespread in ciliates, foraminifera and sacoglossans and variable in terms of longevity and functionality. The sacoglossans are the only group of metazoans which have been shown to harbor functional plastids intracellularly. The ability of the sea slug *Elysia chlorotica* to "steal" algal chloroplasts, retain them intracellularly, and then integrate the foreign organelles with the sea slug's metabolism allowing the animal to survive photoautotrophically for months, is unprecedented and for the most part, currently unexplainable. Equally remarkable is the stability and adaptability of the chloroplasts; they not only survive the ingestion process and resist digestion by the host, but they also adjust osmotically and metabolically to the entirely new cellular environment devoid of any new protective membrane. The biochemistry of such an association is intriguing because of the evidence supporting the reliance of normal chloroplasts on the nucleus to encode the great majority of their proteins and regulate the expression of chloroplast encoded proteins. There appear to be no algal nuclei in *E. chlorotica* and the chloroplast genome of *V. litorea* does not have an unusual coding capacity to account for all of the nuclear encoded chloroplast targeted proteins necessary to sustain the observed chloroplast activity. Preliminary results supporting lateral gene transfer are encouraging and exciting. It is likely that a combination of organelle/protein stability and lateral gene transfer play key roles in sustaining this fascinating association in sacoglossan molluscs.

## I. Introduction

Chloroplast symbiosis (Hinde and Smith 1974; R. Trench, 1975), chloroplast retention (Marín and Ros, 1993), chloroplast farming (Hinde, 1983), and kleptoplasty (Clark *et al.*, 1990) are all terms which have been used to describe in Trench's (1975) words, "the phenomenon where, under natural circumstances an animal acquires intracellularly and retains undamaged plant chloroplasts, free from other associated plant organelles and cytoplasm. Such chloroplasts show sustained active photosynthesis, and photosynthetic products become available to and are utilized by the animal host." The first report on the presence of "green bodies" in sacoglossan molluscs was in 1904 by Brüel (as reported by Clark *et al.*, 1990), but additional reports did not appear until Kawaguti and Yamasu's observation of chloroplasts in *Elysia atroviridis* in 1965. Following these initial observations, Trench, Muscatine, Clark, Jensen and colleagues pioneered the field of functional kleptoplasty in sea slugs with their many ecological and physiological studies (R. Trench, 1969, 1975; Greene and Muscatine, 1972; R. Trench *et al.*, 1973a,b; Muscatine *et al.*, 1975; Clark and Busacca, 1978; Jensen, 1980; to list a few).

The fact that chloroplasts have lost their ability to live independently following their endosymbiogenic origins (reviewed in Douglas and Raven, 2003; Yoon *et al.*, 2002; Palmer, 2003; see Chapter 4) has only heightened interest among scientists to better understand the degree of genetic and functional autonomy among the different plastid types. This curiosity led scientists early on to various attempts at transferring isolated chloroplasts to other cell types and studying their survival. Mouse fibroblasts (Nass, 1969), hen's eggs (Giles and Sarafis, 1971), and carrot protoplasts (Bonnett, 1976) were all employed with varying success. The most promising results were seen with chloroplasts isolated from the green, siphonaceous marine alga *Caulerpa sedoides* implanted into hen's eggs. There, the plastids remained intact for 27 days, but exhibited sustained Hill activity for only five days (Giles and Sarafis, 1971). In contrast, where scientists have tried and achieved limited success, nature has been more successful. Chloroplasts as foreign "endosymbionts" (kleptoplasts) have now been reported in several different sacoglossan molluscs (sea slugs) (see Williams and Walker, 1999 and Marín and Ros, 2004 for reviews), in marine ciliates (Stoecker, 1991), in at least three families of *Foraminifera* (J. Lee, 1998; Chai and Lee, 2000; Bernhard, 2003), and in one limited report in rotifer stomach wall cells (Taylor, 1970). The longevity and functionality of the kleptoplasts vary widely, but in some sea slugs the plastids remain intact and functional for several months, as described below.

The elysiid sea slugs, in general, and *Elysia chlorotica*, in particular, have attracted considerable attention due in part to their brilliant green color and uncanny resemblance to a dicot leaf (Fig. 1a to c), but more so due to the longevity of their functional kleptoplastic associations. *E. chlorotica* harbors chloroplasts from the siphonaceous heterokont alga *Vaucheria litorea*

*Abbreviations:* ctER – chloroplast endoplasmic reticulum; Lhcp – light harvesting complex proteins; PRK – phosphoribulokinase; Rubisco – ribulose bisphosphate carboxylase/oxygenase.

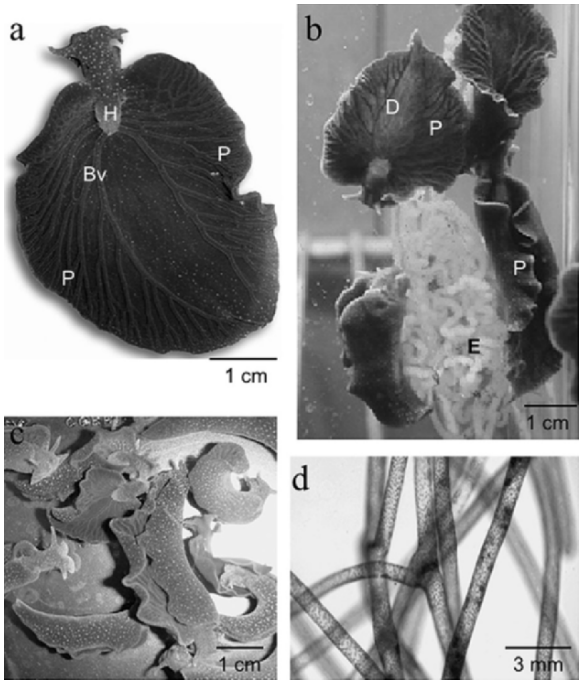



*Fig. 1.* (See also Color Plate 5, p. xxxviii.) Images of *Elysia chlorotica* and *Vaucheria litorea*. (a) Dorsal view of *E. chlorotica* with extended wing-like parapodia and a highly branched blood vascular system emanating from the lighter-colored pericardial mass containing the heart. (b) *E. chlorotica* specimens cultured for 6 months in a saltwater aquarium showing parapodia furled and unfurled and non-chlorophyllous eggs. The more ventrally located digestive gland can be better seen in this panel as a lighter colored network that branches off from the stomach (not visible). It is in this extensively branched system that the chloroplasts pass and get phagocytotically absorbed, ultimately distributing the chloroplasts throughout the entire surface of the animal. As a result of the uniform pigmentation and the two highly branched systems (vascular and digestive), the sea slugs appear much like a dicot leaf. (c) Specimens of freshly collected sea slugs demonstrating variation in size and morphology. (d) Filaments of coenocytic *V. litorea* cultured in a modified f/2 quarter-strength artificial seawater medium. Images "a", "c" and "d" are reprinted with permission of the *Journal of Plant Physiology*. Image "b" is reprinted with permission of the journal *Zoology*. Bv, blood vessels; D, digestive gland; E, egg; H, heart; P, parapodia.

(Fig. 1d) (see reviews by R. Trench, 1975; Pierce *et al.*, 1996; Rumpho *et al.*, 2000); a product itself of secondary endosymbiosis of red algal origin (reviewed in R. Lee, 1989; Palmer, 2003). What is so remarkable in this case, is that the original acquired kleptoplasts sustain the starved animals photoautotrophically for their entire life-span (at least nine months in nature or in the lab) (Pierce *et al.*, 1996, Green *et al.*, 2000; Rumpho *et al.*, 2001). Knowing that plastids are under the genetic and regulatory control of their own nucleo-cytosol and also observing that the organelle-host relationships do not form randomly among just any alga and mollusc, suggests that these kleptoplasts are either unusually stable in all aspects of their structure and function and/or that the animal host is directly or indirectly participating in sustaining the organelles.

The advent of molecular tools leading to the publication of a variety of chloroplast and whole genome sequences, the availability of more federal support to study symbiosis and evolution, and the prospect of documenting lateral or horizontal gene transfer all occurrences of "horizontal gene transfer" or "lateral gene transfer" between organisms of two different kingdoms, has fueled the current interest and excitement in kleptoplasty research. Here, we briefly review the distribution of kleptoplasty in nature and the variability in longevity and functionality of kleptoplasts. More extensive reviews of the distribution, ecology, feeding preferences, etc., of sacoglossan/algal kleptoplast associations can be found in the following publications and references therein: Clark *et al.* (1990), Jensen (1993), Williams and Walker (1999), and Marín and Ros (2004).

## II. Evidence for Kleptoplasty

### *A. Protists*

#### *1. Ciliates*

Most ciliates are heterotrophs, obtaining nutrition by phagotrophic ingestion, and have been studied extensively due to their association with red-tide phenomena. However, recently many ciliates have been observed with sequestered chloroplasts and chlorophyll content comparable, on a unit volume basis, to that found in diatoms and dinoflagellates, and therefore, are potentially photosynthetic (Rogerson *et al.*, 1989; Dolan and Perez, 2000). Plastid-retaining ciliates comprise about 30% of the total ciliate population and appear to be limited to habitats receiving sufficient illumination and a constant supply of algal prey (Stoecker, 1991; Dolan and Perez, 2000). Plastid retention has been most extensively studied in the ciliates *Mesodinium rubrum* and *Strombidium capitatum*, as detailed below. Ciliates represented by the taxa *Coleps*, *Euplotes* and *Paramecium* also retain chloroplasts (Jones, 2000), but only in conjunction with intact autonomous algal cells as symbionts and will not be discussed herein.

The presence of chloroplasts in *M. rubrum* and *S. capitatum* affords an opportunity for multiple modes of metabolism. Although the exact function of

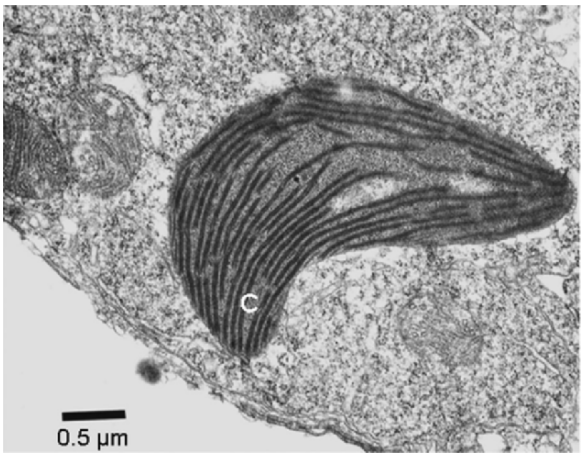



*Fig. 2.* Cryptophyte (*Rhodomonas salina*) chloroplast just under the cell surface of the ciliate *Strombidium capitatum* 16 h after feeding. (Image kindly provided by D. Stoecker and M. Silver.) C, chloroplast.

mixotrophy amongst ciliates remains elusive, it has been proposed that it provides the host organism with the advantage of being able to exploit or survive nutrient poor environments (Dolan, 1992). Most plastidic ciliates retain chloroplasts from a wide range of algal taxa; some *Strombidium sp.* appear to selectively digest or retain chloroplasts from cryptophytes (Fig. 2) while others show a preference for chlorophytic plastids (Stoecker, 1991).

One of the most common plastidic ciliates in temperate neritic waters is *S. capitatum* (Laval-Peuto and Rassoulzadegan, 1988; Montagnes *et al.*, 1988; Stoecker *et al.*, 1988/1989). Sequestered plastids in *S. capitatum* are surrounded by three membranes, two of plastidic origin and one periplastidal membrane believed to be derived from the ciliate endoplasmic reticulum (Laval-Peuto, 1991). The plastids are also functional; tracer studies indicated that photosynthetic rates in ciliates are sufficient to satisfy the cells' basal metabolic requirements (Stoecker and Michaels, 1991). The carbon fixed by these plastids is found in the ciliate's polysaccharide pool (Putt, 1990b). Although cryptophyte chloroplasts contain a nucleomorph, the presence of cryptophyte DNA within *S. capitatum* chloroplasts remains controversial. Chloroplasts within ciliates do not replicate and thus must be replenished regularly (Stoecker and Silver, 1990).

*S. capitatum* can sequester plastids quite rapidly; plastids within digestive vacuoles were detected by epifluorescent microscopy within 15 minutes of being exposed to cryptophytes. Within 1 h of exposure, free cryptophyte plastids were observed within the ciliate cytoplasm and this rate of accumulation remained linear for 4 h (Stoecker and Silver, 1990). Uptake of chloroplasts by *S. capitatum* differs from uptake by sacoglossan molluscs. The latter suck up the algal cytoplasm and individual chloroplasts are then sequestered by phagocytosis into digestive cells (R. Trench, 1975). *S. capitatum*, instead, phagocytes entire algal cells. How the chloroplasts are removed from the algal cells and moved into the ciliate cytoplasm remains unknown. Transmission electron microscopy analyses by Stoecker and Silver (1990) localized intact cryptophytes within digestive vacuoles as well as free chloroplasts within the ciliate cytoplasm. However, the intermediate steps have never been observed, suggesting a very rapid process. Interestingly, only some chloroplasts are sequestered while others are digested along with the rest of the algal cell. Chloroplasts, mitochondria, and starch grains persist longer in digestive vacuoles than other organelles, but the process of uptake remains unexplained as only chloroplasts, and not mitochondria, are sequestered.

When *S. capitatum* is removed from a cryptophyte-rich medium, at least 84% of the plastids are lost within 9 h (Stoecker and Silver, 1990). This is greater than could be explained by culture dilution. It is not known whether these chloroplasts are lost through digestion or expulsion. When *S. capitatum* cultures were transferred from a cryptophyte-rich medium to a starvation medium (either on a diurnal light-dark cycle or in darkness), the chloroplast-clearing rates were lower than those in ciliate cultures transferred to nutrient-rich medium. Additionally, cryptophyte chloroplasts persisted for longer periods of time than chloroplasts of other origin. Chloroplasts sequestered within cells of *Laboea strobila* or *Strombidium* sp. survive and remain functional for at least 6 d and 14 d, respectively (Stoecker, 1991; Putt, 1990a).

The common marine ciliate *M. rubrum* also contains functional cryptophyte chloroplasts as well as non-ciliate mitochondria (Lindholm *et al.*, 1988). Plastidic ciliates appear to adjust their position in the water column in order to maintain maximum photosynthetic rates via an unknown mechanism that is not thought to be phototaxis. *M. rubrum* isolated from McMurdo Sound, Antarctica, was culturable at 2 to 6° C in the light, but only if provided with a source of algal prey (the polar cryptophyte *Teleaulax acuta*) (Gustafson *et al.*, 2000). The majority (80%) of added cryptophytes was consumed within 48 h with a concomitant increase in chlorophyll a and phycoerythrin fluorescence of the host, which was maintained for 14 d post-feeding. At the same time, the average size of the *M. rubrum* cell

decreased by 20%. Fed cultures were noticeably bright pink whereas unfed cultures were colorless.

Thus, it appears that the ciliates *M. rubrum* and *S. capitatum* are able to sequester plastids from a variety of algal types and these plastids remain functional in their new environment. However, the mechanism of sequestration and what biological role the plastids play remains elusive and the focus of ongoing research.

### 2. Foraminifera

Foraminifera are almost all marine and dwell either in sand, attached to rocks and algae, or as planktonic individuals. Most foraminifera are heterotrophs, however, some contain chloroplasts, either in symbiotic algae that live within their shells or as free chloroplasts maintained within the cytoplasm. Plastid-containing foraminifera that have been studied belong to the Protist families Nonionidae, Elphiidae and Rotaliellidae (J. Lee and Anderson, 1991; J. Lee, 1998).

The plastid-containing foraminifera *Nonionella stella* is one of the most intensively studied. *N. stella* is a benthic organism and has been found in the upper three centimeters of sediments of the Santa Barbara Basin, CA (Bernhard *et al.*, 2000; Grzymski *et al.*, 2002). Its kleptoplasts appear evenly distributed throughout the cytoplasm and contain a centrally localized pyrenoid with a single lamella passing through the center, a three-layer lamella along the periphery, and interconnections between the lamellae (Grzymski *et al.*, 2002). Recently, photosynthetic pigments have been reported for another benthic foraminifera, *Virgulinella fragilis*, from the Cariaco Basin, Venezuela (Bernhard, 2003). Although the origin of the *V. fragilis* plastids is not known, they appear morphologically similar to those found in *N. stella* and may have originated from either *Skeletonema costatum* or *Odontella sinensis* as determined by 16S rDNA gene sequence similarity (Grzymski *et al.*, 2002).

Kleptoplasts in *N. stella* remain functional for up to one year (Grzymski *et al.*, 2002). Additionally, Western blot analysis revealed three major chloroplast proteins: ribulose *bis*phosphate carboxylase/oxygenase (Rubisco) (both subunits are chloroplast encoded in diatoms), the photosystem II D1 protein (chloroplast encoded in diatoms), and the fucoxanthin chlorophyll a/c protein complex (nuclear encoded in diatoms) were maintained for over nine months in these foraminifera. Grzymski and co-authors (2002) suggest that, since some of these chloroplast-localized proteins are encoded by nuclear genes and since the diatom nucleus has not been detected in foraminifera, the turnover of the plastid machinery in the foraminifera must be very slow. Interestingly, the *N. stella* isolates found in the Santa Barbara Basin are benthic organisms located 600 m below the sea surface and they have retained an ability to feed and digest food. Therefore, it is not clear what role the sequestered chloroplasts are playing in such a low light environment.

Although foraminifera appear to prefer the plastids of diatoms, some hosts have been found associated with plastids from rhodophytes, chlorophytes, chrysophytes, and dinoflagellates. How the plastids are identified and retained are as yet unknown. Within the host cell, extremely dense populations of identical appearing plastids accumulate. While *N. stella* from the Santa Barbara Basin harbors plastids related to *S. costatum* and *O. sinensis*, these two diatom species do not represent the predominant phytoplankton in this environment (Reimers *et al.*, 1990). Both diatoms have a specific glycoprotein on their surface that is lacking in diatoms which do not serve as plastid donors (Chai and Lee, 2000). It seems that the presence of a unique cell-surface marker may serve as a target for the identification of suitable kleptoplasts for foraminifera.

## B. Sacoglossans

The first solid evidence for kleptoplasty in sacoglossans originated with Kawaguti and Yamasu's (1965) electron microscope observations of "green bodies" (*Codium fragile* chloroplasts) in the digestive gland of *Elysia atroviridis*. Shortly thereafter, Taylor (1968) used autoradiography to demonstrate a functional relationship between algal chloroplasts (*C. tomentosum*) and *E. viridis*, one of the earliest well-studied sea slugs. These reports were followed by one of many from Trench's lab in which he elegantly detailed the presence and functional capabilities of kleptoplasts in three different sacoglossans (R. Trench *et al.*, 1969). We now know that kleptoplasty is wide-spread in sea slugs of the order Sacoglossa (phylum Mollusca, subclass Opisthobranchia). Members of this order are exclusively herbivorous (Williams and Walker, 1999) and are divided into two major suborders: the more primitive shelled species (suborder Oxynoacea) and the non-shelled species (suborder Plakobranchacea) (taxonomy based on the phylogenetic system of Jensen, 1997a, 1997b; and as reviewed by Williams and Walker, 1999 and Marín and Ros, 2004).

Noting the wide variability in retention and functionality of kleptoplasts among the sacoglossans, Clark *et al.* (1990) proposed a 6-level system to describe kleptoplast retention and function. His system also allowed

for a co-evolutionary study of host and algal chloroplast source. The levels ranged from: Level 1) feeding on algae but no plastid retention to Level 6) long-term functional retention, i.e., starved animals which photosynthesize for more than one week. At the time, functional retention of one week was considered "long-term" and no higher levels were considered necessary.

### 1. Shelled Sacoglossans (Suborder Oxynoacea)

All of the shelled sacoglossans that have been characterized in the field or in the lab feed on the green alga *Caulerpa*, but do not exhibit functional kleptoplasty (Jensen, 1980; Clark *et al.*, 1990; Williams and Walker, 1999; Marín and Ros, 2004). This may be due to an inability to retain plastids (Level 1) as seen in *Volvatella* and *Ascobulla* or due to the retention of non-functional plastids (Level 3) as exhibited by *Oxynoe* species. In the latter case, *Caulerpa* chloroplasts are retained for a few hours to a few days, but no net fixation of $CO_2$ is observed (Clark and Busacca, 1978; Hinde, 1980; reviewed in Williams and Walker, 1999). The ability to retain chloroplasts or at least their pigments may be a primitive trait that allows for cryptic camouflage and a precursor to functional kleptoplasty in the more advanced and less protected, shell-less sacoglossans (Clark *et al.*, 1990).

### 2. Non-Shelled Sacoglossans (Suborder Plakobranchacea)

The majority of Sacoglossans is shell-less (suborder Plakobranchacea) and divided into two superfamilies: the Plakobranchoidea (a.k.a. Elysioidea) and the Limapontioidea (Polybranchioidea or Stiligeroidea). The Plakobranchoidea is the largest superfamily and contains the parapodia-bearing Elysiids, including the genus *Elysia* (Jensen 1997a, 1997b). The parapodia are the wing-like lateral extensions of the body that the animals can unfurl, increasing the exposure of their body surface to sunlight and exchange of $CO_2$ and $O_2$(see Fig. 1a to c; Fig. 5a) (Rahat and Monselise, 1979). The largest family in the Limapontioidea is the Stiligeridae, characterized by having dorsal extensions or cerata into which the sea slug's digestive gland extends and then branches (see Rudman, 1998 for images of other elysiid and stiligerid species). Lining the digestive diverticula in the cerata and throughout the sea slug body are specific cells containing the kleptoplasts. Like the parapodia of the elysiids, the cerata increase the body area available for storing chloroplasts and surface area for light absorption and gas exchange (Clark *et al.*, 1981). Since neither the elysiids nor stiligerids possess a protective shell, they must rely on a defense system of camouflage and chemical synthesis and emission to escape predation. The presence of kleptoplasts contributes to both of these defenses in many of the shell-less sea slugs.

#### a. Stiligerids

Originally, it was reported that functional kleptoplasty was limited to the shell-less elysiid species (R. Trench, 1975; Graves *et al.*, 1979), but short- (photosynthesize less than 24 h after removal from the field) and long-term kleptoplasty in the stiligerids has now been documented (Clark *et al.*, 1981, 1990; Marín and Ross, 2004). One of the longest associations among the stiligerids is that of *Costasiella ocillata* (=*Costasiella lilianae*) and chloroplasts derived from the green alga *Avrainvillea nigricans*. These sea slugs display net carbon fixation through 65 d starvation with a light to dark ratio ranging from 18 to 90 and initial assimilation rates of 200 to 300 μg C $mg^{-1}$Chl $h^{-1}$. However, these high rates drop off rapidly after 7 d, despite chlorophyll levels remaining fairly level (Clark *et al.*, 1981).

#### b. Elysiids

In contrast to the stiligerids, long-term (Level 6) functional kleptoplasty is widespread among the elysiids, especially in the genus *Elysia* (Williams and Walker, 1999). Here, functional kleptoplasty ranges from non-existent (Clark *et al.*, 1990), to a few days (less than ten for *E. hedgpethi* (Greene, 1970), to a few weeks (e.g. about six for *E.* [=*Tridachia*] *crispata, E.* [=*Tridachiella*]*diomedea* and *Placobranchus ianthobapsus*) (R. Trench, 1969; Greene, 1970), to a few months (three for *E. viridis*) (Hinde and Smith, 1972), to several months (at least nine for *E. chlorotica*) (Pierce *et al.*, 1996; Rumpho *et al.*, 2001; Mondy and Pierce, 2003). *E. viridis* was one of the first species studied in-depth because of the unusual longevity (3 months) of the functional kleptoplasts (*Codium fragile*) in the sea slug (discussed below) (Hinde and Smith, 1972; R. Trench *et al.*, 1973a,b).

Compilations of several sacoglossan associations, their algal food source(s), and kleptoplast functional longevity can be found in Williams and Walker (1999) and Marín and Ros (2004). Of seventy species of sacoglossans tested (mostly by Jensen as summarized by Williams and Walker, 1999), 60 feed on green

algae and 24 feed specifically on *Caulerpa*, but kleptoplasts also originate from non-green algae including rhodophytes and heterokonts.

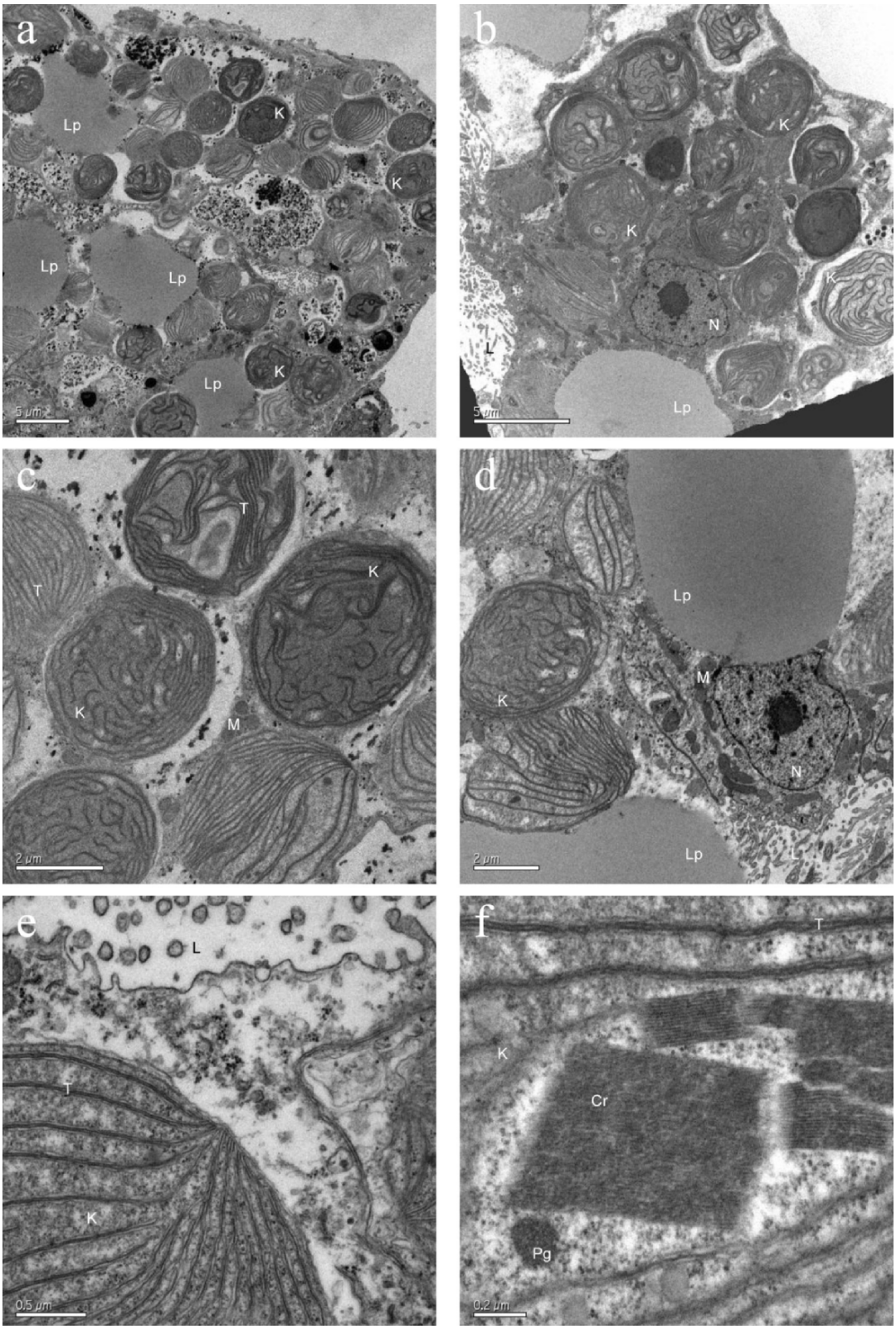
*Elysia timida* retains functional chloroplasts from the green alga *Acetabularia acetabulum* for about 40 d (Marín and Ros, 1993), but it is the feeding ecology of this sea slug that is especially interesting. *E. timida* graze along the stalks of *Acetabularia* just ahead of ensuing seasonal calcification of the algae, at which time feeding declines and dependence on the kleptoplasts increases. The ability to acquire and then harbor functional kleptoplasts prior to times of food shortage (typically no longer than two months) may be an evolutionary advantage for *E. timida*.

The ability to switch between algae would also be an obvious advantage ensuring a greater likelihood of finding a suitable food source to sustain the sea slugs through their annual life-cycle. The green, brown and red specimens of *E. furvacauda* found in nature reflect this animal's seasonal changes in retained kleptoplasts. At any given time, *E. furvacauda* cells may harbor at least three different types of plastids (Brandley, 1984). These are believed to have originated from the algae found associated with the sea slugs at different times of the year, more specifically the green algae *Codium* and *Microdictyon* and the red alga *Sargassum*. During the winter months, *E. furvacauda* favors *Sargassum* over the green algae and red algal kleptoplasts predominate in the red and brown pigmented sea slugs; photosynthetic rates are very low. In the spring and summer, animals move to *Codium,* acquiring more green algal kleptoplasts, a green pigmentation, and high rates of photosynthesis (especially in the summer). *E. furvacauda* survives for only two to three weeks in the light if starved of all algae.

The longest functional kleptoplasty reported for a sacoglossan is that of the host *E. chlorotica* and plastids of the chromophytic alga *Vaucheria litorea*. Chloroplasts are typically retained by starved animals for the animal's entire life (∼9 months) (Pierce *et al.*, 1996; Green *et al.*, 2000); the record in the laboratory for a single animal has been 14 months (M.E. Rumpho, personal observation). *E. chlorotica* is a relatively large emerald green sea slug found in brackish water from southern Florida to Nova Scotia. It can tolerate varying environmental factors including salinities of 3 to 32% and temperatures of 4° to 24° C (Harrigan and Alkon, 1978; West *et al.*, 1984). Animals range in size from the smallest of about 3 mm to the largest at about 6 cm (Fig. 1a to c; Fig. 5a). There is no evidence of chlorophyll, plastids or chloroplast DNA in *E. chlorotica* eggs (Fig. 1b) (R. Trench, 1975; Mujer *et al.*, 1996; Rumpho *et al.*, 2000, 2001), thus, the plastids must be reacquired with each new generation.

The life-cycle of *E. chlorotica* is completed in approximately 10 to 11 months whether the animals are left in their native environment or maintained in the laboratory with or without an algal food source (West, 1979; West *et al.* 1984; Pierce *et al.*, 1996). The animals exhibit planktotrophic development producing many small fertilized eggs that hatch prior to metamorphosis. Adults are hermaphroditic but cross-fertilize. The life cycle begins with adults laying eggs in late spring. The larval veliger stage develops after approximately 3 d. Veligers develop a black pigmented band on the dorsal surface behind the velum before they hatch (about 9 d from egg stage). Once hatched, they feed on unicellular algae (at least *Monochrysis lutherie* or *Rhodomonas salina*) for about two weeks (Harrigan and Alkon, 1978; West *et al.*, 1984). Towards the end of this period, veligers develop eyespots and propodium. The black pigmented band seen before they hatch spreads to most of the dorsal surface of the animal so that the entire veliger appears black. The pigmented veligers then feed on algal filaments, principally *Vaucheria* sp. (West, 1979). Under laboratory conditions, no veligers were observed to undergo metamorphosis before pigmentation of the entire body (Harrigan and Alkon, 1978; West, 1979; West *et al.*, 1984). Metamorphosis takes place in about 1 to 2 d, during which time the velum is resorbed and the shell and operculum are cast. The juvenile sea slugs continue feeding on algal filaments and incorporating chloroplasts. The resultant uniformly dark green color gives elysiid sea slugs their "leaves that crawl" appearance (R. Trench, 1975; Fig. 1a to c). West *et al.* (1984) observed that metamorphosis of the veligers to juvenile sea slugs occurred only in the presence of either *V. litorea* or *V. compacta*. Other algae tested in the laboratory included *Cladophora, Bryopsis carticulans*, *B. plumosa*, *Codium fragile* and isolated chloroplasts from *C. fragile* and *Spongomorpha*. In its natural environment near Halifax, Nova Scotia, and Martha's Vineyard, MA, *E. chlorotica* is typically found grazing on mats of *V. litorea* (Rumpho *et al.*, 2000).

The stramenopile *V. litorea* is the major source of kleptoplasts for *E. chlorotica*. The siphonaceous alga belongs to the family Vaucheriaceae, order Vaucheriales and phylum Xanthophyta. Vaucher first described the genus *Vaucheria* in 1803; the only genus in the Vaucheriaceae which includes multinucleate, siphonaceous xanthophytes (Cox, 1980; R. Lee, 1989). Species in this genus, like other xanthophytes, appear yellowish-green in color. *Vaucheria* is a filamentous,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branched coenocytic alga (Fig. 1d) that is ubiquitously present in freshwater, on land, and occasionally in marine habitats. *V. litorea* is found in brackish marine waters (R. Lee, 1989). The thin cell walls of *Vaucheria* are composed of 90% cellulose and the center of the cell is occupied by a large vacuole containing lipids and degenerated chloroplasts. The cytoplasm is confined to the periphery of the filamentous cell with the nuclei towards the center and the chloroplasts parietal. Reflective of their red algal secondary endosymbiotic evolutionary history, chloroplasts of Xanthophytes are surrounded by four membranes: the inner and outer envelopes of the chloroplast, the periplastid membrane (a remnant of the plasma membrane of the engulfed alga) and the outermost chloroplast endoplasmic reticulum (ctER) membrane (R. Lee, 1989; McFadden, 1999, 2001; Ishida *et al.*, 2000; Rumpho *et al.*, 2001; Bhattacharya *et al.*, 2004). Interestingly, *V. litorea* kleptoplasts in *E. chlorotica* appear in electron micrographs to be surrounded by only two membranes, with the periplastid and ctER membranes apparently having been stripped off during the uptake process (Fig. 3 and 4; Rumpho *et al.*, 2000). The implications for protein targeting and import into the algal chloroplasts vs. the animal kleptoplasts are significant.

## III. Selection and Uptake Processes

It is still unclear what controls or influences the specificity of feeding, uptake and maintenance of the kleptoplasts. Early reports suggested that the only suitable source of chloroplasts for kleptoplastic associations were green algae of the order Siphonales. The reduced cell wall and large cytoplasmic volume facilitate acquisition of a large number of plastids with one puncture of the filament. While shelled sea slugs have been observed to feed only on the siphonale *Caulerpa*, shell-less sea slugs exhibit a much broader diet including *Caulerpa*, but also other green siphonaceous and filamentous algae, as well as red algae and secondarily derived chromists (some with a siphonaceous habit) (see West, 1979; Williams and Walker, 1999; Yoon *et al.*, 2002). Although as a group the diet is much broader for shell-less sea slugs, the feeding of an individual species is very narrow, to the extent that individual diets have been examined (Clark and Busacca, 1978; Gallop *et al.*, 1980; West, 1979; Trowbridge, 1998). For example, when *E. viridis* was fed five different algae for four weeks, only those specimens fed the siphonale *Codium* maintained or gained mass. In a separate experiment, specimens of *E. viridis* were observed to only associate with filaments of *Codium* in preference to five other algae offered in the same container (Gallop *et al.*, 1980).

The general process of acquiring chloroplasts by sea slugs is similar as far as it is understood. All of the sacoglossans (except *Olea hansineensis* which feeds on eggs of other opisthobranchs and does not acquire plastids) feed suctorially on algae (Greene, 1974). The sea slug's feeding apparatus, including its recessed mouth (Fig. 5) and digestive gland (Fig. 1b) aid in establishing the chloroplast symbiosis. The feeding apparatus is a highly modified uniserate radula which helps in puncturing algal cells and sucking out the fluid contents (Jensen, 1980, 1993; Williams and Walker, 1999; Marín and Ros, 2004). Following the very detailed examination of radular teeth in 55 species of sacoglossans, Jensen (1993) correlated three different types of tooth morphology with cell wall structure and composition of the consumed alga. Triangular or blade-shaped teeth, both with lateral denticles, are found in the shelled sacoglossans associated with diets of *Caulerpa* and other calcified algae. The triangular teeth are adapted for rasping and are used to remove enough of the xylan-containing cell wall to allow penetration of the cytosol. Although siphonaceous, *Caulerpa* stalks are characterized by a very rigid internal structure of

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*Fig. 3.* Electron micrographs of kleptoplasts in digestive tubule cells of *E. chlorotica* cultured in the lab in the absence of algae for 1 month. (a) Section of a young, healthy sea slug with a high density of kleptoplasts (about 5 μm in diameter) per mollusc cell and large and numerous lipid deposits. *V. litorea*, the algal source of the kleptoplasts, produces lipids as its major photosynthetic product. (b) The lumen of the digestive tubule with its microvilli and associated small vesicles can be seen adjacent to the mollusc cell harboring the kleptoplasts. A mollusc nucleus is also present. (c) Higher magnification of kleptoplasts illustrating the somewhat disorganized nature of the thylakoids. No obvious encapsulating envelopes are seen with the kleptoplasts. (d) Large lipid deposits in cells containing kleptoplasts as well as a mollusc nucleus and numerous mitochondria near the lumen. (e) High magnification of a kleptoplast adjacent to the lumen of the digestive tubule. The fine structure of the trilamellar thylakoids is evident in this section. (f) Crystalline arrays are frequently seen in the kleptoplasts, but not in chloroplasts in algal cells (Rumpho *et al.*, 2000; 2001). Thylakoid fine structure and a plastoglobuli are also evident. Cr, crystalline array; K, kleptoplast; Lp, lipid deposit; L, lumen of digestive tubule; M, mitochondria; N, nucleus; Pg, plastoglobuli; T, thylakoids.

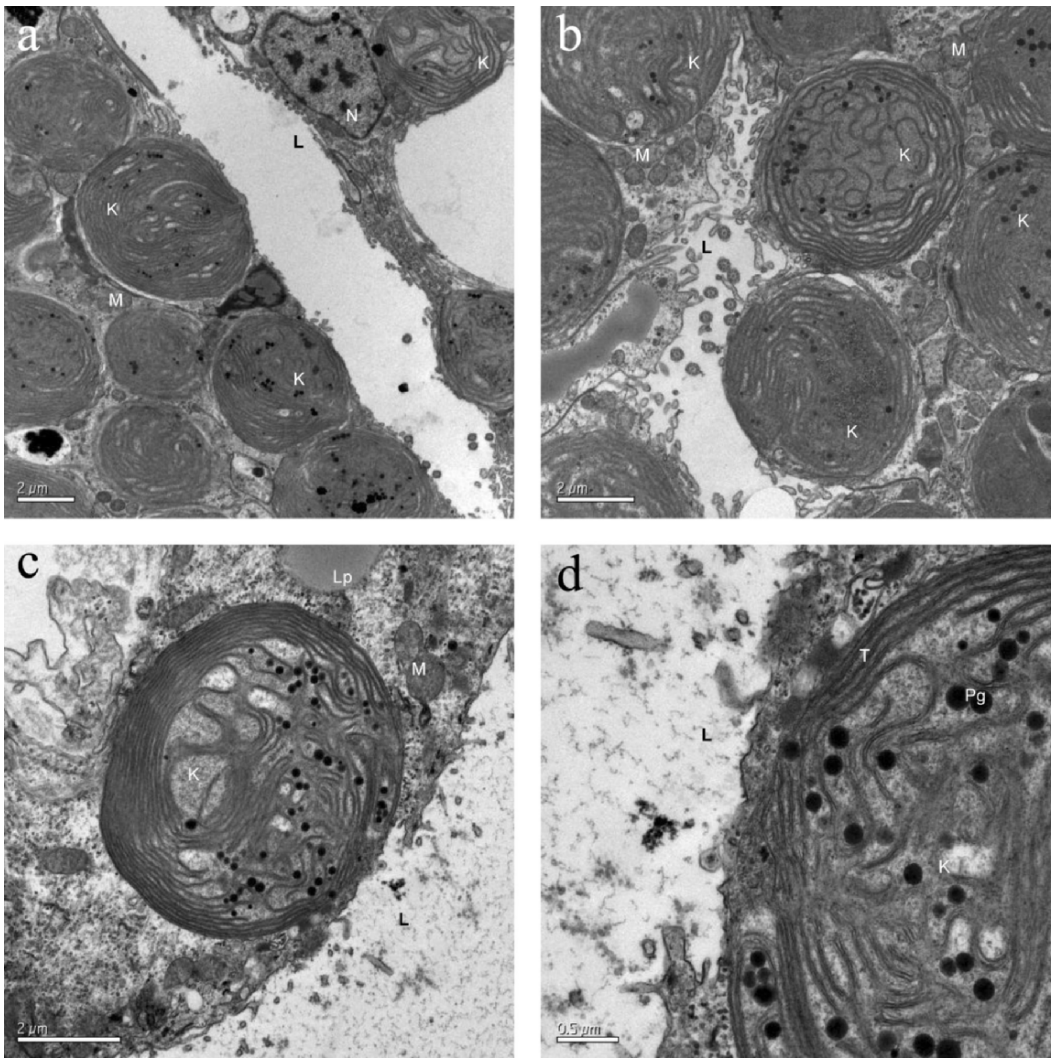


*Fig. 4.* Electron micrographs of kleptoplasts immediately after uptake into cells of the digestive tubules of *Elysia*. After starving the sea slugs for four months, the animals were exposed to high light to disrupt their kleptoplasts. The resultant chlorotic sea slugs were then supplied with fresh *V. litorea* filaments and allowed to feed for 10 d before being fixed in 2% glutaraldehyde and processed for transmission electron microscopy. Panels a–d illustrate increasing magnification of kleptoplasts adjacent to the lumen of the tubules. Although the cell membrane cannot be followed completely, several kleptoplasts appear to be in the process of phagocytosis. Numerous plastoglobuli are seen in the freshly acquired kleptoplasts and few lipid deposits. K, kleptoplast; Lp, lipid deposit; L, lumen of digestive tubule; M, mitochondria; N, nucleus; T, thylakoids.

trabeculae requiring considerable force to suck out the cellular contents. The majority of elysiids have blade-shaped teeth adapted for puncturing the cell wall (usually cellulose-containing) and then opening a hole in the cell membrane by pulling from the inside out (R. Trench, 1975; Jensen, 1993). Evolution of this feeding mechanism along with a change in the pharyngeal musculature is believed to have aided in expanding the algal diet among the non-shelled sacoglossans. Within a group of sacoglossans or even within one species, e.g., *E. viridis*, the shape of the teeth can change with a change in diet. While a study like Jensen's (1993) of the radular teeth can tell us much about which algae individual sea slugs can most likely successfully feed on, it still does not answer the question of what attracts the sea slug to a specific alga to begin with. Several feeding studies have shown that most sea slugs do not "try out" a variety of algal choices before settling on the one they can successfully penetrate. Rather, the sea slugs go directly to their preferred food source, in most cases totally ignoring all the other choices even if it means starvation.

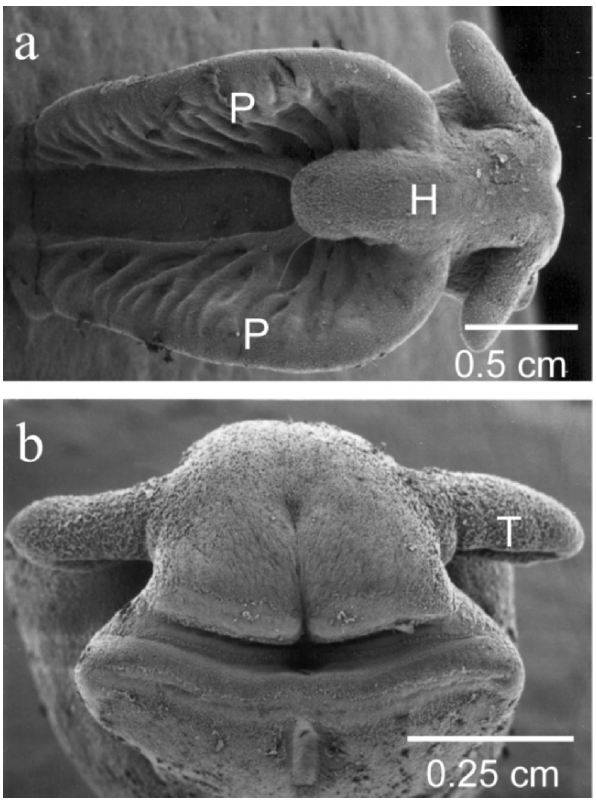


*Fig. 5.* Scanning electron micrographs of the mollusc *E. chlorotica*. Young, small (about 3 to 4 mm in length) animals were fixed in 2% glutaraldehyde, processed and their external structure examined with an AMRay 1000 scanning electron microscope. (a) Dorsal image illustrating the extensive vascular system which branches from the heart as two major ducts and spreads throughout the animal. The raised pericardium which houses the heart is very obvious in this image. (b) Head image illustrating the sensory tentacles and recessed, sucking mouth. Within the mouth structure are the uniserate radular teeth used to puncture the algal filaments prior to sucking out the chloroplasts. H, heart; P, parapodia; T, tentacle.

Once ingested, all of the algal cell contents are digested or excreted except the chloroplasts which pass through the sea slug digestive tract before being phagocytized and sequestered intracellularly in cells of the digestive epithelium (McLean, 1976, 1978; Green *et al.*, 2000; Rumpho *et al.*, 2000, 2001; Mondy and Pierce, 2003). The mechanism by which the animal differentiates between organelles is not understood. Importantly, the digestive gland is believed to not initiate intracellular digestion immediately after ingestion, thereby retaining chloroplasts intact. The highly branched digestive gland extends to much of the sea slug's surface and helps to distribute chloroplasts over the surface of the animal in the digestive epithelium, increasing exposure to sunlight (Fig. 1a to c). Once sequestered, the chloroplasts in most cases exist in direct contact with the sea slug's cytoplasm (Fig. 3 and 4) (Brandley, 1981; Hinde, 1983; Marín and Ros, 1993; Mujer *et al.*, 1996; Rumpho *et al.*, 2000, 2001), but in some cases the kleptoplasts may be sealed off by a vacuolar-type membrane (Clark *et al.*, 1981; R. Trench, 1975; Mondy and Pierce, 2003).

In many associations between marine invertebrates and whole algae, the algae are passed on from one generation to the next via maternal inheritance (Davy and Turner, 2003; Habetha *et al.*, 2003). This has not yet occurred for kleptoplasts in the sacoglossans; kleptoplasts must be established with each generation since they have not yet entered the germ cell line in any cases studied (Greene, 1968; R. Trench *et al.*, 1969). The survival of the animal hinges on the availability of a suitable chloroplast source and accessibility to the chloroplasts, undoubtedly leading to a co-evolution of the animal (and its feeding apparatus) and alga in their natural habitat.

## IV. Functional Capacity of Sacoglossan Kleptoplasts

Interpreting published results of functional kleptoplasty is complicated by several, oftentimes uncontrollable, variables. For example, to date it has been virtually impossible to raise large numbers of kleptoplastic sacoglossans in the laboratory to controllably study algal food specificity or uptake, establishment, and longevity of the kleptoplasts. Thus, the researcher is dependent on collecting animals from the sea, noting what algae they are associated with, and maintaining them in laboratory conditions under artificial lighting, usually constant temperatures, and with or without the presumed kleptoplast source (assuming one can collect or culture the alga). In many cases it is difficult to determine how old the kleptoplasts or animals are at the time of collection or how uniform the kleptoplast population is in terms of age and origin. Some functional experiments are carried out immediately after collection from the environment, others are carried out on animals cultured in the presence of their food alga, still others use animals that have been starved for a short period (h) prior to measuring photosynthetic activity. For all of these reasons, it is difficult to directly or quantitatively compare functional measurements among a number of sacoglossans and the reader should carefully evaluate the experimental conditions when interpreting the results. With these caveats in mind, biochemical evidence supporting functional kleptoplasty in a number

of sacoglossans is presented below and in more detail in the references cited.

### A. Oxygen Evolution and $CO_2$ Fixation

The most common ways to demonstrate that captured plastids are capable of carrying out photosynthesis are to measure light- and $CO_2$-dependent oxygen evolution and light-dependent incorporation of radiolabeled $CO_2$. Greater oxygen evolution in the light relative to uptake in the dark has been demonstrated in a number of elysiid species including *E. crispata* (R. Trench *et al.*, 1969) and *Tridachiella diomedea* (R. Trench, 1975), both harboring *Caulerpa* chloroplasts.

Clark *et al.* (1990) measured light vs. dark $^{14}CO_2$ fixation rates for 19 different Atlantic sacoglossans within a maximum of one week from collection. No net fixation was recorded for shelled species, but non-shelled stiligerid species exhibited greater fixation in the light compared to the dark, ranging from a low of 0.18 (*Ercolania coerulea*) to a high of 2.88 (*Alderia modesta*). Of the elysiid species in this experiment, *Bosellia mimetica* (feeds on *Halimeda tuna*) exhibited the highest ratio (29.2). In an entirely separate experiment, other elysiids have yielded ratios up to 100 (*E. tuca*) and incorporation rates of 60 μg C $mg^{-1}$Chl $h^{-1}$ (Stirts and Clark, 1980). Marín and Ros (1989) recorded net carbon fixation in four Mediterranean elysiid species including *E. timida* (feeds on *Acetabularia acetabulum*) with the highest rate equaling 144 μg C $mg^{-1}$Chl $h^{-1}$. High temperatures (>35°C) and high light (400 μmol $m^{-2}$ $s^{-1}$) inhibited photosynthesis in *E. timida*. While many of the sea slugs examined quickly lost their ability to assimilate carbon, *E. timida* continued to fix carbon after 40 d starvation, but the rate dropped to about 60%, with the most rapid decrease occurring in the first few days.

*E. viridis*, *E. hedgpethi* and *Placobranchus ianthobapsus* all acquire kleptoplasts from *Codium* and display greater levels of $^{14}CO_2$ fixation in the light relative to the dark (Taylor, 1968; R. Trench, 1969; Greene and Muscatine, 1972; R. Trench *et al.*, 1973b). Many studies have taken the radiolabeling experiments one step further to demonstrate that kleptoplast-generated $^{14}$C-photosynthates are utilized by the host animal. After 1 h of incubation in the light, $^{14}$C was detected in *E. crispata* tissues devoid of plastids (R. Trench, 1969). The amount of newly-fixed photosynthetic-carbon contributed to the animals varied from 21% in *P. ianthobapsus* (Greene, 1970) to 36 to 40% in *E. viridis* (R. Trench *et al.*, 1973a) to 50% in *T. crispata* (M. Trench *et al.*, 1972). In one representative experiment, Kremer (1976) demonstrated that the initial products of kleptoplastic $^{14}CO_2$ incorporation were not only transferred to the host tissue, but also metabolized. Radiolabeled products were compared for the host *E. viridis* and kleptoplast provider, *C. fragile*. On a qualitative basis, virtually identical soluble products were labeled in both organisms, except the sea slugs did not yield any radiolabeled sucrose. In *Codium*, $^{14}$C-sucrose represented 7% of the total soluble pool. Quantitatively, sugar-phosphates, galactose, asparagine and glutamate, and citric acid cycle intermediates represented a larger percentage of the total labeled soluble pool in the sea slugs vs. the alga. In turn, lipids and the free amino acids glycine and serine were more heavily labeled in the alga.

*V. litorea* chloroplasts in *E. chlorotica* also carry out net photoautotrophic fixation of $CO_2$ and light-driven $O_2$ evolution for several months (West, 1979; Pierce *et al.*, 1996; Green *et al.*, 2000; Rumpho *et al.*, 2000; 2001). Gibson *et al.* (1986) demonstrated a correlation between chlorophyll content and net $O_2$ evolution by analyzing natural populations of *E. chlorotca* varying from dark green to non-green. No additional reports on this non-green population of sea slugs have appeared in the literature. Whole chain photosynthetic electron transport (PET) rates in the thylakoids of kleptoplasts isolated from starved *E. chlorotica* were comparable to those of the respective algal thylakoids for the first six months of starvation. Kleptoplasts were also capable of splitting water through seven months although a decline in oxygen evolution and PET rates were observed after five and six months, respectively. A measurable decrease in chlorophyll concentration was also observed after seven months; chlorophyll c decreased 80% while chlorophyll a levels decreased 45% on a fresh weight basis (Green *et al.*, 2000). *E. chlorotica* starved for four months incorporated [$^{14}$C]$NaHCO_3$ in the light into acid-stable products at rates comparable to that of *V. litorea* filaments (Rumpho *et al.*, 2001). Although *E. chlorotica* generally exudes copious amounts of mucus, very little of the radiolabel was found in the mucus, rather, the major products were water soluble metabolites.

### B. $\delta^{13}C$ Values

Although oxygen evolution and $CO_2$ incorporation studies in most cases estimate gross and net photosynthetic rates, they do not quantitatively measure the contribution of kleptoplastic photosynthesis to the total carbon intake of the animal in cases where the sacoglossans are not starved. Raven *et al.* (2001) attempted to quantify the minimal contribution of kleptoplastic

photosynthesis in natural populations by comparing $^{13}C/^{12}C$ ratios in several Australian sea slugs with their green algal food sources. They obtained values ranging from 16% (*E. australis* associated with *Cladophora*) to 60% (*Oxynoe viridis* associated with *Caulerpa longifolia*) of the total carbon input coming from kleptoplastic $CO_2$ assimilation. The remainder of the carbon was assumed to come from direct ingestion of the alga on which the sea slugs feed. These are fairly gross values considering the many assumptions that were necessary in the calculations and the variability in feeding of the sacoglossans in nature. The authors could not definitively conclude that the alga the sea slugs were found associated with was also the source of the kleptoplasts in the sea slug. Furthermore, $\delta^{13}C$ values were determined on the entire algal sample, but the sea slugs do not ingest and retain carbon from the whole algal cell. It is likely that the $\delta^{13}C$ values would be slightly different for the algal cell wall vs. the algal cytosol. Other assumptions are discussed in more detail in their study. Interestingly, the authors found that the C/N ratio was considerably lower in the sea slugs than in the alga with which the sea slugs were associated. They attributed this in part to the higher C/N ratio in the cell wall of the alga that is not ingested by the sea slugs. In addition, DeFreese and Clark (1991) demonstrated that sacoglossans can directly absorb dissolved nitrogen-rich amino acids from the sea water and this may contribute to the higher N-values observed in the sea slugs. Unfortunately, similar $\delta^{13}C$ analyses have not been carried out on cultured sea slugs starved for several months in the laboratory in artificial seawater where some of the variables encountered in nature could be more controlled.

### C. Synthesis of Macromolecules

The ability of kleptoplasts to synthesize photosynthetic pigments, RNA, DNA and various proteins in their foreign host has been investigated in a variety of organisms. Initial attempts to study protein synthesis by kleptoplasts in sea slugs were focused on Rubisco. Trench was unable to detect the synthesis of this protein by kleptoplasts in *E. crispata* or *E. viridis* using $^{3}$[H]Leu or $^{14}CO_2$ as substrates (R. Trench and Gooday, 1973; R. Trench, 1975). In contrast, Pierce *et al.* (1996) demonstrated incorporation of $^{35}$[S]Met/Cys into Rubisco, the photosystem II protein D1, and several other unidentified proteins in *E. chlorotica* kleptoplasts. This incorporation was also shown to be sensitive to chloramphenicol, an inhibitor of plastid-directed protein synthesis. Mujer *et al.* (1996) also demonstrated *de novo* synthesis of many plastid-encoded thylakoid membrane proteins at eight months starvation indicating active translation in kleptoplasts of *E. chlorotica*.

The entire pathways of chlorophyll and carotenoid biosynthesis are found in plant plastids, but the majority of enzymes essential for these pathways are nuclear encoded. The ability of kleptoplasts to synthesize pigments was first studied by M. Trench *et al.* (1970) in *E. viridis* and *E. crispata* by measuring the amount of $^{14}C$ incorporated into pigments after 10 h of photosynthesis in the presence of [$^{14}$C]$NaHCO_3$. Large quantities of $^{14}C$ were incorporated into $\alpha$- and $\beta$-carotenes, but no label was detected in chlorophylls. They concluded that either the kleptoplasts had lost the ability to synthesize pigments or the carbon substrate used for synthesis did not originate from photosynthetic fixation of carbon. No studies have been carried out in *E. chlorotica* with respect to pigment biosynthesis.

The ability of kleptoplasts to replicate their DNA is of significance in order to understand if they divide in the animal cytoplasm, although there is no visual support for kleptoplast division. Greene (as cited by R. Trench, 1975) investigated the possibility of DNA synthesis using [$^{3}$H]thymidine and $^{32}P$ in *P. ianthobapsus* and found no evidence to support it. Trench and Taylor (R. Trench, 1975) carried out similar experiments in *E. viridis* and made similar negative observations. No further studies have been reported on DNA synthesis in kleptoplasts of any other sea slug and no evidence supporting chloroplast division has been reported.

Mujer *et al.* (1996) studied the ability of kleptoplasts to synthesize RNA in *E. chlorotica*. The levels of 16S rRNA transcripts remained constant throughout an eight month starvation period, whereas, levels of the plastid encoded *psb*A transcript were constant for the first two to three months but decreased gradually over the next five months. Levels of *psb*A transcripts were also shown to be sensitive to the RNA synthesis inhibitor 6-methyl purine indicating active transcription.

### D. Synthesis of Defense Compounds and Anti-Cancer Agents

While much of the focus on kleptoplasty has been on the theft and maintenance of the chloroplasts and detailing their biochemical functioning, it appears that these fascinating associations have led to other unexpected products. Scheuer and colleagues (Hamann and Scheuer, 1993; Becerro *et al.*, 2001) isolated the anticancer agent Kahalaide F from *E. rufescens* (*Bryopsis* kleptoplasts) and this compound is now being tested on human cancer patients in phase II clinical trials (PharmaMar, 2003).

Marín and Ros (2004) have also shown that many kleptoplastic sacoglossans ingest and in some cases, modify algal chemicals for their own defense; a process they refer to as "kleptochemistry." Some sea slugs (*E. timida* and *E. viridis*) can synthesize their own biodeterrents (polypropionate metabolites) (Gavagnin *et al.*, 1994a,b), but others use the ingested chemicals directly (several of the shelled sacoglossans and the elysiids *E. translucens* and *Bosellia mimetica*) or after chemical modification (some shelled sacoglossans and the elysiid *Thuridilla hopei* that feeds on *Derbesia*) (Paul and Van Alstyne, 1988). Several species of *Caulerpa* produce toxic compounds that serve as feeding deterrents to most generalist herbivores, but not the primitive sacoglossans which have adapted to feed on these algae and at the same time be "protected" (Marín and Ros, 2004).

## V. What Sustains the Longevity of the *Elysia chlorotica/Vaucheria litorea* Kleptoplast Association?

It is now quite well accepted that chloroplasts originated when a free-living cyanobacterium was engulfed by a protoeukaryotic organism. During evolution, the cyanobacterium was reduced to a semi-autonomous organelle, the chloroplast, as a result of the transfer of a majority of its cyanobacterial genes to the nucleus of the host (Martin and Herrmann, 1998). The largest number of protein-coding genes in the genome of photosynthetic chloroplasts sequenced to date is 243 in *Cyanidioschyzon merolae* (Ohta *et al.*, 2003). The smallest genome of a free-living cyanobacterium, *Prochlorococcus* MED4 strain, codes for 1761 proteins (Rocap *et al.*, 2003) while the largest cyanobacterial genome, *Nostoc punctiforme*, codes for 7,432 proteins (Meeks *et al.*, 2001). Due to the massive reduction of its genome, the chloroplast is largely dependent upon the nucleo-cytosol for protein synthesis, targeting and delivery to sustain it biochemically. Products necessary for the chloroplast's own expression system, such as the bacteriophage-type nuclear encoded plastid RNA polymerase, sigma–like factors that associate with the plastid-encoded RNA polymerase (Gray and Lang, 1998; Maliga, 1998), most of the 70S ribosomal proteins including the plastid specific ribosomal proteins of the 30S subunit (Yamaguchi and Subramanian, 2003), transacting translation factors, and some tRNA synthetase enzymes, are all encoded by the nucleus and targeted to the chloroplast. The majority of chloroplast proteins involved in photosynthesis or subunits of such proteins are also encoded by the nucleus (reviewed in Raghavendra, 1998).

The ability of kleptoplasts to function in sea slugs implies that essential plastid enzymes and regulatory proteins, whether encoded by the nuclear or chloroplast genome of the alga, are present throughout the functional life-span of the kleptoplasts. Active transcription and translation have been demonstrated in kleptoplasts of *E. chlorotica* (Mujer *et al.*, 1996), but very little is known about the origin and maintenance of the essential nuclear-encoded chloroplast proteins. Either all of the essential kleptoplast proteins are incredibly stable (up to nine months in one case) or they are synthesized de novo from within or outside of the kleptoplast. De novo synthesis could result from an autonomous kleptoplast genome, "contaminating" algal nuclei and/or algal RNA species which remain very stable in the sea slugs, homologous animal gene products redirected to the foreign organelle, and/or horizontal gene transfer of algal nuclear genes to the animal nuclear genome. These possibilities are discussed below relative to the *E. chlorotica*/*V. litorea* chloroplast association.

### *A. Chloroplast Genetic Autonomy*

Originally, the possibility was considered that the chloroplast genome of *V. litorea* possessed an unusual coding capacity enabling it to code for proteins that are typically encoded by the nuclear genome in other photosynthetic organisms. To address this, the 115 kb chloroplast genome of *V. litorea* was sequenced and found to contain only 137 protein coding genes and all of the plastid rRNA genes (Table 1 and J. Lee, J.R. Manhart and M.E. Rumpho, unpublished). In terms of gene content, *V. litorea* cpDNA was found to be intermediate to *Porphyra*, a red alga (Reith and Munholland, 1993) and *Odontella*, a diatom (Kowallik *et al.*, 1995) (Table 1). Some of the photosynthetic genes present in the cpDNA of *V. litorea* but not in land plant chloroplast genomes include: Rubisco small subunit (*rbcS*), Rubisco expression protein (*cfxQ)*, several *psa* and *psb* genes, chaperonins DnaK (*dnaK)* and GroEL (*groEL*), ATP synthase CF1 subunits (*atpD* and *atpG*) and protein elongation factors Tu (*tufA*) and Ts (*tsf*) (Table 1). However, the *V. litorea* chloroplast genome is very much a "normal" non-green algal plastid genome and does not have any extensive or particularly unusual coding capacity to substitute for all or even a significant number of essential nuclear encoded chloroplast targeted proteins necessary to sustain the observed chloroplast activity.

*Table 1.* Partial listing of genes identified on the chloroplast genome of *Vaucheria litorea* and not found in other algal or land plant chloroplast genomes[1]

| Gene | Gene product | Vaucheria litorea | Cyanelle | Porphyra | Odontella | Nicotiana or Oryza sativa | Other Land Plants |
|---|---|---|---|---|---|---|---|
| *Photosynthesis* | | | | | | | |
| *rbcS* | ribulose bisphosphate carboxylase, small subunit | + | + | + | + | − | − |
| *cfxQ* | Rubisco expression protein | + | − | + | + | − | − |
| *psaD* | PSI, ferredoxin binding protein, subunit II | + | + | + | + | − | − |
| *psaE* | PSI, subunit IV, 18–20 kDa | + | + | + | + | − | − |
| *psaF* | PSI, plasticyanin-binding protein, subunit III | + | − | + | + | − | − |
| *psaK* | PSI, PS1-K polypeptide (P 37) | − | − | + | − | − | − |
| *psaL* | PSI, reaction centre subunit XI | + | − | + | + | − | − |
| *psaM* | PSI, reaction centre subunit M | + | + | + | + | − | + |
| *psbJ* | PSII, protein J | + | + | + | + | − | + |
| *psbV* | PSII, cytochrome C550 | + | + | + | + | − | − |
| *psbW* | PSII, PSII, protein W (13 kDa) | + | + | + | + | − | − |
| *psbX* | PSII, PSII, protein X (4.1 kDa) | + | + | + | + | − | − |
| *petF* | ferredoxin | + | + | + | + | − | + |
| *atpD* | ATP synthase CF1 subunit | + | + | + | + | − | − |
| *atpG* | ATP synthase CF1 subunit | + | + | + | + | − | − |
| *atpI* | ATP synthase CFo subunit IV | + | − | + | + | + | + |
| *Biosynthesis* | | | | | | | |
| *acpP* | acyl carrier protein | + | + | + | + | − | − |
| *chlB* | protochlorophyllide reductase chlB chain | + | + | + | − | − | + |
| *chlI* | magnesium chelatase subunit | + | + | + | + | − | − |
| *chlL* | protochlorophyllde reductase Fe-S ATP-binding protein | + | + | + | − | − | + |
| *chlN* | protochlorophyllde reductase chlN chain | + | + | + | − | − | + |
| *ilvB* | acetohydroxyacid synthase large subunit | + | − | + | − | − | − |
| *ilvH* | acetohydroxyacid synthase small subunit | + | − | + | − | − | − |
| *thiG* | thiG protein, thiamine biosynthesis | + | − | + | + | − | − |
| *Txn, Tln, Replication* | | | | | | | |
| *dnaB* | replication helicase subunit | + | − | + | + | − | − |
| *tsf* | elongation factor Ts | + | − | + | − | − | − |
| *tufA* | elongation factor Tu | + | + | + | + | − | − |
| *Miscellaneous* | | | | | | | |
| *clpC* | clp protease ATP-binding subunit | + | − | + | + | − | − |
| *dnaK* | hsp-70 type chaperone | + | + | + | + | − | − |
| *groEL* | 60 kDa chaperonin | + | + | + | + | − | − |
| *ftsH* | ATP-dependent Zn protease | + | − | + | + | − | − |
| *secA* | preprotein translocase subunit | + | − | + | + | − | − |
| *secY* | preprotein translocase subunit | + | + | + | + | − | − |

[1] Table modified from Kapoor and Sugiura (1998) to include *V. litorea.* +, present; − absent.

## *B. Algal Nuclei or Long-Lived RNA Species in the Sea Slug*

The retention of algal nuclear material by *E. chlorotica* has been ruled highly unlikely based on several different lines of evidence. Extensive observation of electron micrographs of adult animals has revealed no visual evidence of any foreign nuclei or nucleomorph structures (*V. litorea* itself also does not possess a nucleomorph) (Graves *et al.*, 1979; Mujer *et al.*, 1996; Rumpho *et al.*, 2000). Southern blot analysis employing a probe to the *V. litorea* nuclear internal transcribed spacer (ITS) region and DNA from *E. chlorotica, V. litorea* and *E. chlorotica* eggs yielded a positive signal for the algal DNA sample, but no signal for the animal or egg DNA samples (Green *et al.*, 2000). PCR analysis was also

carried out using primers designed against the *V. litorea* ITS and again negative results were obtained for animal and egg DNA supporting the absence of *V. litorea* nuclei in the sea slug.

The presence of long-lived algal nuclear transcribed RNA species was investigated in *E. chlorotica* by Northern blotting with a *V. litorea* phosphoribulokinase (*prk*) probe. As discussed in more detail below, this essential algal nuclear encoded enzyme is detectable at the protein and enzyme activity levels in sea slugs starved for several months, but no algal transcript has so far been detected in the animals by Northern blotting (M.E. Rumpho, unpublished).

### C. Unusual Stability of Isolated Chloroplasts and Macromolecules

R. Trench (1975) first proposed the idea that the chloroplasts themselves and the macromolecules in the kleptoplasts might be extremely stable or "robust." He referred to the latter as "macromolecule turnover in suspended animation," imparting an autonomous property to the rugged kleptoplasts outside their mother cell. To first successfully establish a kleptoplastic association, the algal chloroplasts must be able to not only withstand rapidly changing external conditions, but also the physical handling of being sucked from their own cytosol, passed through the gut of the animal and phagocytized into the digestive epithelium. In the case of *V. litorea*, this literally entails ripping the chloroplasts from their outer two membranes, the periplastid and ctER, remnants of the secondary endosymbiotic origin of *Vaucheria* plastids. As indicated earlier and shown in Fig. 3 and 4, these two additional membranes are not observed around the kleptoplasts in *E. chlorotica*. Rather, the kleptoplasts are typically found in direct contact with the sea slug cytosol, yet plastid structural integrity does not appear to be impaired. Electron micrographs reveal the chloroplasts take on a rounded appearance in the sea slugs (Fig. 3 and 4) compared to their more elongated shape in the alga (Rumpho *et al.*, 2000; Mondy and Pierce, 2003), but the tri-lamellar thylakoid structure and densely stained stroma is retained for months. Only after about eight months of starvation when the sea slugs have begun to lose chlorophyll and appear more like an autumn leaf, do the kleptoplasts begin to lose their fine structure (Mondy and Pierce, 2003).

The association of *C. fragile* chloroplasts with *E. viridis* was one of the first characterized longer-term (>7 d) kleptoplastic associations and led to further studies on the natural stability of these plastids. Trench and colleagues (R. Trench *et al.*, 1973a; R. Trench and Ohlhorst, 1976) observed that chloroplasts from the siphonales, especially *Codium*, exhibited an unusual robustness in isolation. Unlike isolated spinach chloroplasts which lose their ability to fix $CO_2$ within hours, isolated *C. fragile* chloroplasts were able to fix $CO_2$ for several days; 20% of control rates were maintained after 7 d isolation in continuous darkness (R. Trench *et al.*, 1973a; Gallop *et al.*, 1980). Direct exposure to seawater significantly decreased photosynthetic rates of the isolated chloroplasts, but they were fairly resistant to changes in osmotic pressure between 300 and 600 mM mannitol (Gallop *et al.*, 1980). Thus, it appears that *Codium* plastids are able to withstand the physical pressure of transfer from the algal filament to the sea slug and the plastids probably never come in direct contact with the seawater during the feeding/transfer process. Interestingly, even in the first attempts to introduce isolated chloroplasts into foreign host cells, chloroplasts of siphonaceous algae were chosen including *Caulerpa* (Giles and Sarafis, 1971) and *Vaucheria* (Bonnett, 1976). Giles and Sarafis (1974) later demonstrated that isolated *Caulerpa* plastids appear intact by phase contrast microscopy even after treatment with various detergents, enzymes, boiling in water, freezing, and sonication. It was necessary to use a French pressure cell to break them.

Chloroplasts isolated from *V. litorea* or *E. chlorotica* also display unusual "robustness" remaining structurally intact and functional ($O_2$ evolution, $CO_2$ fixation, and protein synthesis) for a minimum of 72 h after isolation (Green, 2001; Rumpho *et al.*, 2001; Green *et al.*, 2005). Longer-term structural studies have revealed that chloroplasts isolated from *V. litorea* remain greater than 25% intact for up to three weeks (as estimated by phase-contrast microscopy and oxygen evolution measurements) and are extremely difficult to rupture by osmotic stress (Green, 2001). While this unusual chloroplast stability surely aids in the uptake and assimilation process of the kleptoplasts in the animal's digestive system, it does not explain the feeding selectiveness of *E. chlorotica*, discussed earlier.

Do unusually stable plastids also contribute to unusual protein stability within these organelles? Evidence to date from Western blotting indicates that several plastid encoded proteins involved in photosynthetic electron transport (PSI and PSII complexes) and $CO_2$ fixation (Rubisco subunits) are present for several months in *E. chlorotica* (Green *et al.*, 2000). In addition, PRK, light harvesting proteins (Lhcp I and II), and a photosynthetic electron transport component (PetC), all nuclear encoded chloroplast proteins, are

also present and functional after several months starvation of the sea slugs (Pierce *et al.*, 1999, 2003; Rumpho *et al.*, 2001). If the genes for these essential chloroplast proteins that are unique to photosynthetic organisms are not present in the nuclear genome of the sea slug by way of horizontal gene transfer or substitution, then the possibility of unusual stability of proteins must be considered.

Chloroplast protein stability lasting for nine months as proposed here for sea slug kleptoplasts is unprecedented in large part due to the constant exposure of chloroplasts to light damage or photoinhibition. Although the sea slugs have some ability to shade themselves from excessive light in the sea by burrowing into the sediments, hiding under the algal mats, and reducing body exposure to the sunlight by closing their parapodia, they cannot fully escape the light energy in the illuminated aquaria. Protein turnover is essential for recovery from photoinhibition, in the removal of inactive or improperly assembled proteins, as well as for the maintenance of stoichiometry of multiple-subunit complexes (some of which may be a mix of nuclear and plastid encoded subunits) during times of limited availability of co-factors, acclimation to environmental stress, and recovery from heat denaturation (Adam, 1996; Scheurwater *et al.*, 2000). Both protein degradation and synthesis are indispensable to prevent the aggregation of damaged proteins and to allow for the resynthesis/reassembly of undamaged proteins. Turnover rates have been analyzed for only a few proteins, primarily those most sensitive to photodamage such as D1 (Aro *et al.*, 1993; Thomas *et al.*, 2001; Henmi *et al.*, 2004). Protein degradation or turnover rates have not been specifically examined in chromophytes, but sequencing of the *V. litorea* chloroplast genome has revealed the presence of one open reading frame with homology to the nuclear encoded protease FtsH and one ORF with homology to the Clp protease regulatory subunit ClpC (see list of chloroplast encoded genes in Table 1), both nuclear encoded in land plants (reviewed in Raghavendra, 1998). FtsH primarily degrades inactivated D1 which can be replaced by de novo synthesis in the kleptoplast. The Clp protease complex (requiring both ClpP and ClpC subunits) has been implicated in the degradation of abnormally folded, unassembled, and/or inactive proteins (Adam and Clarke, 2002). In land plants ClpC is nuclear encoded and ClpP is plastid encoded; opposite of what is observed for *V. litorea*. The absence of a gene source for the proteolytic subunit ClpP in the sea slugs may inhibit the formation of a functional enzyme complex in the kleptoplasts. No other proteases are present in the *V. litorea* cpDNA, suggesting that those proteases involved in acclimation-degradation (nuclear encoded DegP and an unidentified protease that degrades light harvesting proteins under high light conditions) in other organisms may not be functional in the kleptoplasts. It remains to be determined if a low level of protein turnover is typical of *V. litorea* chloroplasts in general or if it is an adaptation of the kleptoplasts to life in an animal cell, in part as a result of the loss of functional chloroplast protease complexes and better absorption of reactive oxygen species.

### *D. Lateral or Horizontal Gene Transfer from the Algal Nucleus to the Sea Slug Nuclear Genome*

Eukaryotic cells and genomes have evolved from more than one prokaryote and are thus chimeras (Margulis *et al.*, 2000). Increasing evidence indicates that lateral gene transfer (the exchange of genes between distantly related species) has played a key role in evolution. Gene transfers from organelle genomes to the host nucleus have contributed much to the present day eukaryotic genome. For example, in the genome of *Arabidopsis thaliana*, approximately 4500 genes (18% of the genome) are of cyanobacterial origin (Martin *et al.*, 2002). In organisms that acquired plastids by secondary endosymbiosis, genes have been transferred from both the nucleus and chloroplast of the secondary symbionts to the new host (Ishida and Green, 2002; Archibald *et al.*, 2003). Proposed pathways for gene transfer include the "bulk-transfer" of large fragments of organellar DNA or RNA intermediates that escaped membrane constraints and recombined with the nuclear genome (Thorsness and Weber, 1996; Martin, 2003; Timmis *et al.*, 2004). In plants, the cytochrome oxidase subunit II (COXII) gene was transferred from the mitochondrial genome to the nuclear genome via an RNA intermediate (Nugent and Palmer, 1991). In some instances, multiple independent transfers of the same gene have occurred, e.g., the information to encode the ribosomal protein RPS10 was transferred from the mitochondria to the nucleus numerous independent times via an RNA intermediate (Adams *et al.*, 2000). Examples of both bulk transfer of DNA and fragments of DNA have also been documented in various organisms including Arabidopsis in which the entire mitochondrial genome has been integrated into chromosome 2 of the nuclear genome (Lin *et al.*, 1999).

A similar process of bulk lateral gene transfer from the alga to the sea slug can be imagined in kleptoplastic associations. During the process of feeding on

the algal filaments, it is very likely that nuclei passed through the digestive system of the sea slug and were broken open. This would have presumably enhanced the transfer of algal nuclear genes or transcripts, including those encoding chloroplast-targeted proteins, to the nucleus of the sea slug. Short of sequencing the entire sea slug genome or thousands of sea slug ESTs (future projects), one is left to look for the transfer of algal nuclear genes to the sea slug nuclear genome on a one-by-one basis beginning with information from the presumed source of the plastids (*V. litorea* in this case). The first step is to identify those essential gene products that are not chloroplast encoded in *V. litorea*, not present at all in animal genomes, preferably not substitutable by another gene product in the animal, and essential for one of the processes observed in the kleptoplasts, e.g., photosynthetic carbon fixation and reduction. Based on this, researchers have focused on the light harvesting proteins (Lhcp) of photosystems I and II, the water-splitting protein (PsbO) and the reductive pentose phosphate (RPP) pathway enzyme, PRK.

The strongest support to date for lateral gene transfer in a sea slug is the transfer of a homologue of a fucoxanthin-chlorophyll a/c binding protein (Fcp) gene. Fcp belongs to the family of Lhcps that is unique to photosynthetic organisms, binds pigments, and is encoded in the nucleus (Durnford *et al.*, 1996; Lang and Kroth, 2001). An Lhcp homolog was detected by western blotting of proteins isolated from *E. chlorotica* throughout a nine month starvation period (Green *et al.*, 2000). De novo synthesis of Lhcp (or Vcp for vaucheriaxanthan-chlorophyll a/c binding protein in the case of *V. litorea*) is supported by radiolabeling studies of *E. chlorotica* (Hanten and Pierce, 2001; Pierce *et al.*, 1999, 2003). However, subsequent attempts to detect the corresponding gene in *E. chlorotica* by Southern blotting using cloned *V. litorea* LhcpI (GenBank #AF336985) or LhcpII (GenBank #AF336982) cDNA gene probes did not prove fruitful. PCR attempts employing homologous or heterologous degenerate primers also resulted in negative results for the sea slug, despite producing the expected gene products from the algal DNA. Pierce *et al.* (2003), have been more successful in obtaining positive Southern blot results supporting lateral gene transfer of an Fcp-like gene in kleptoplastic *E. crispata*. The origin of the kleptoplasts in this sea slug is unknown (Clark and Busacca, 1978; Jensen and Clark, 1983; Pierce *et al.*, 2003). Immunoprecipitation of radiolabeled products revealed that an Fcp-like homolog was de novo synthesized in *E. crispata*. Based on the known sequence of the Fcp-like protein purified from *E. chlorotica*, the authors designed primers to amplify *fcp* from *V. litorea*. This probe was then used in Southern blot experiments with *E. crispata* DNA. Sequencing of the Southern blot gene product and verification of sea slug flanking sequence are still necessary to validate a claim of lateral gene transfer.

A second protein targeted as a potential lateral gene transfer candidate is PRK, the enzyme essential for regenerating ribulose-1,5-bisphosphate in the plastid RPP pathway. Thirteen enzymes operate in the RPPP of which only two, Rubisco and PRK, are unique to the pathway (Hariharan *et al.*, 1998). All of the other RPP pathway enzymes have homologues in the cytosolic oxidative pentose phosphate pathway, present in both plant and animal cells (Debnam and Emes, 1999). Thus, the sea slugs could potentially provide all of the enzymes of the RPP pathway except Rubisco and PRK by retargeting (or randomly targeting) normally cytosolic proteins to the foreign plastids. Both subunits of Rubisco are chloroplast encoded in *V. litorea*, but PRK is invariably encoded by the nuclear genome in all known photosynthetic eukaryotic organisms. In agreement with this, no PRK gene was detected in the *V. litorea* chloroplast genome (Table 1). Western blotting with spinach or cotton anti-PRK revealed the presence of PRK protein throughout the lifetime of the sea slug and measurable enzyme activity in *E. chlorotica* (0.11 μmol $mg^{-1}$ protein $min^{-1}$ vs. 1.0 μmol $mg^{-1}$ protein $min^{-1}$ in the alga) even after several months of starvation. Unless PRK protein is incredibly stable, the only other possible source for the protein is the nuclear genome of the sea slug, having ruled out long-lived RNA species earlier. Preliminary Southern blot analyses of *E. chlorotica* DNA using a *V. litorea* PRK cDNA probe (GenBank # AF336986) have not yielded positive results. Additional experiments are in progress to determine if a PRK gene is present and expressed in the animal.

Generally, the genes for nuclear encoded plastid targeted proteins can be traced to a primary endosymbiont origin, but this is not always the case. In the chloroarachinophyte *Bigelowiella natans*, 21% of the genes for plastid targeted proteins are derived from a variety of organisms, including red algae, streptophyte algae, algae with red algal endosymbionts, and also bacteria (Proteobacteria and Pseudomonadaceae) (Archibald *et al.*, 2003). Phylogenetic analysis of *psb*O from the fucoxanthin-containing dinoflagellate *Karenia brevis* indicates that during tertiary symbiosis the original *psb*O gene in the dinoflagellate nucleus was replaced by a *psb*O gene from a haptophyte nuclear genome (Ishida and Green, 2002). Thus, the presence

of nuclear genes encoding plastid targeted proteins including PRK, LhcpI and II, and PsbO in *E. chlorotica* that have originated from sources other than *V. litorea* cannot be ruled out and may explain their elusive behavior.

## VI. Concluding Remarks

An overview has been presented here of the distribution and functional capacity of kleptoplasts in ciliates, foraminifera, and sacoglossans, especially the *Elysia* species. Readers are encouraged to explore the early literature related to this field and cited herein to learn more about the ecology of kleptoplastic associations. We have also presented various lines of evidence and proposed mechanisms which need further study in an attempt to explain the remarkable longevity, both physically and functionally, of the *Elysia chlorotica/Vaucheria litorea* kleptoplast association. Additional and more convincing evidence for lateral gene transfer between organisms of two different kingdoms is surely to be forthcoming in the near future.

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# Chapter 24

# The Apicoplast

Soledad Funes
*Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Butenandtstrasse 5, Munich 81377 Germany*

Xochitl Pérez-Martínez
*Departamento de Bioquímica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, 04510 Mexico*

Adrián Reyes-Prieto
*Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, 04510 Mexico*

Diego González-Halphen*
*Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, 04510 Mexico*

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*Author for correspondence, email: dhalphen@ifc.unam.mx

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## Summary

The apicoplast is an essential organelle characteristic of the apicomplexan parasites. It harbors its own genome and it is believed to be a chloroplast-derived organelle that originated by secondary endosymbiosis. Here, we address the more relevant properties of this organelle, an evolutionary relict of a once fully-functional algal chloroplast. We address how its highly-reduced plastid genome replicates and segregates, and how it gets transcribed and translated. We also describe the particular metabolism of the apicoplast, limited to certain pathways, including fatty acid and lipid biosynthesis, the non-mevalonate isoprenoid synthesis pathway, the biosynthesis of iron-sulfur clusters, and the *de novo* synthesis of heme groups. These metabolic pathways are of relevance as a preferred target for anti-parasitic drugs. The organelle also exhibits peculiar protein-import mechanisms. Numerous genes encoding apicoplast proteins are located in the nucleus, and the encoded proteins are targeted to the organelle in a process mediated by bipartite N-terminal extensions present in the protein precursors. The nature of the alga that was phagocytized and retained by the apicomplexan ancestor through a secondary endosymbiotic event remains controversial. We critically address the obscure evolutionary origin of the apicoplast and we review the evidence that has given rise to the so-called rhodophyte versus chlorophyte debate. The apicoplast is a fascinating organelle that has attracted many research groups in the last years. Our current knowledge about it its certainly limited, and its study is essential to understand the physiology and evolution of the apicomplexan parasites.

## I. Introduction

The Apicomplexa belong to the superphylum Alveolata (Gajadhar *et al.*, 1991; Wolters 1991), and are therefore relatives of dinoflagellates, ciliates, perkinsids, and two large groups of marine alveolates (Moreira and Lopez-Garcia, 2002). Alveolates exhibit two main morphological features: tubular mitochondrial cristae, and sacs (alveoli) under the plasma membrane (Patterson, 1999). In particular, Apicomplexans are immobile protists and obligate intracellular parasites of either invertebrate or vertebrate animals. The Apicomplexa is a large phylum of more than 4,600 known species (Ellis *et al.*, 1998), that are divided into four groups: coccidians (including members of the genera *Eimeria*, *Toxoplasma*, *Sarcocystis* and *Lankesterella*), haemosporidians (genera *Plasmodium*, *Leucocytozoon* and *Hepatozoon*), piroplasms (genera *Theileria* and *Babesia*) and gregarines (genera *Gregarina*, *Monocystis*, *Ophriocystis* and perhaps *Cryptosporidium*) (Vivier and Desportes, 1990; Leander *et al.*, 2003). Gregarines are found in invertebrates and prochordates (mainly worms and arthropods) (Vivier and Desportes, 1990). Coccidia occur throughout the animal kingdom and may be subdivided into Eimeriidae and Sarcocystidae subclades (Obornik *et al.*, 2002a). The *Plasmodium* lineage diverged from other apicomplexan lineages (piroplasmids and coccidians) several hundred million years ago, perhaps even before the Cambrian (Escalante and Ayala, 1995). This lineage comprises the malaria parasite *Plasmodium falciparum* (the causative agent of malaria) and its close relatives *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*, whose speciation occurred approximately 129 million years ago (Escalante and Ayala, 1995). Other representative apicomplexan species are the opportunistic pathogens of immunosupressed individuals like members of the genera *Toxoplasma* and *Cryptosporidium*. *Toxoplasma gondii* causes toxoplasmosis, a human disease characterized by lymphatic infections, mental retardation and blindness in adults, and severe neurological damage in the fetuses of infected pregnant women. In AIDS patients and in other patients with severe immunosupression, the disease causes encephalitis and dermatologic problems. The parasites of the genera *Eimeria* cause coccidiosis in rabbits, poultry, and cattle while *Sarcocystis* may contaminate sheep and pigs with sarcosporidiosis. Piroplasms like *Babesia* cause babesiosis in several groups of vertebrates including cattle, dogs, and sometime humans, while *Theileria* is the causative agent of East Coast fever in cattle. The most widely distributed apicomplexan may be *Toxoplasma*, followed by *Plasmodium,* piroplasms (*Babesia* and *Theileria*) and *Eimeria* (Vivier and Desportes, 1990). It remains to be ascertained if some marine free-living predators like the members of the genus *Colpodella*, are early-branching relatives of apicomplexans or of ciliates (Siddall *et al.*, 2001; Leander *et al.*, 2003). Therefore, free-living apicomplexans may exist.

Apicomplexans are ill-famed microorganisms because they are a scourge to humans and their livestock. These protists received their name after the apical complex, a typically structured complex of specialized organelles with cytoskeletal components (the conoid and associated structures) and secretory elements (dense granules, rhoptries and micronemes) that allow the attachment of the parasites to and penetration into their hosts (Black and Boothroyd, 2000). In contrast, microbodies like membrane-bound peroxisomes were thought to be missing in apicomplexan parasites (Ding *et al.*, 2000). Nevertheless, small vesicular structures with an estimated diameter of 100–300 nm, anterior to the *Toxoplasma gondii* nucleus, were identified as peroxisomes, by immunofluorescence studies using an anti-catalase antibody (Kaasch and Joiner, 2000). Therefore, the main parasite organelles seem to be the Golgi, mitochondria, micronemes, rhoptries, dense granules, peroxisomes, and the apicoplast.

The evolutionary origin of some cell organelles is relatively well established. Mitochondria are believed to have evolved from endosymbionts that derived originally from free-living $\alpha$-proteobacteria (Gray, 1999) probably related to extant members of the genus *Rickettsia* (Gray *et al.*, 2001). Chloroplasts are thought to have arisen from cyanobacteria (Martin *et al.*, 2002). It is widely accepted that the two independent processes of primary endosymbiosis that gave rise to these organelles occurred only once during eukaryote evolution. Other peculiar plastids, present in a limited set of eukaryotic lineages, suggest more complex organelle evolutionary origins (Archibald and Keeling, 2002). Such is the case of plastids surrounded by

*Abbreviations:* ACC – Acetyl-CoA carboxylase; ACP – Acyl carrier protein; ALAD – Delta-aminolevulinate dehydratase; DOXP – 1-deoxy-D-xylulose-5-phosphate; FabH – β-ketoacyl-ACP synthase III; FabI – Enoyl-ACP reductase; FabZ – β-hydroxyacyl-ACP dehydratase; FAS – Type I fatty acid synthase; FNR – Ferredoxin-NADP(+)-reductase; GAPDH – Glyceraldehyde-3-phosphate dehydrogenases; GFP – Green fluorescent protein; Hsp – Heat shock protein; PDF – Peptidyl deformylase; pfENR – *Plasmodium falciparum* enoyl acyl carrier protein reductase; plDNA – Plastid DNA; SPP – Stromal processing peptidase; V-$H^+$Ppase – Vacuolar proton-dependent pyrophosphatase.

more than two membranes like those in apicomplexans and other lineages like euglenoids, chlorarachniophytes, stramenopiles (heterokont algae), haptophytes, cryptophyta, and dinoflagellates. The presence of an intriguing and unique organelle in apicomplexan parasites, the "apicoplast," whose name derives from "apicomplexan-plastid" (McFadden *et al.*, 1996), is the subject of this chapter. The apicoplast (Fig. 1) is a recently discovered organelle of apicomplexan parasites. The apicoplast harbors its own genome and is believed to be a chloroplast-derived organelle. This organelle may have originated by secondary endosymbiosis, a process in which a eukaryotic cell engulfed and retained another eukaryote (a unicellular alga). The apicoplast, with its unique highly-reduced genome, its particular metabolism, its protein-import mechanisms, its obscure evolutionary origin, and its relevance as a target for antiparasitic drugs, is a fascinating subject of research. The relatively high number of reviews encompassing apicoplast structure and function that have appeared in recent years illustrates the impact of this organelle on the research interests of several groups (McFadden and Waller, 1997; McFadden and Roos, 1999; Roos *et al.*, 1999; Singh and Habib, 2000; Wilson, 2002; Foth and McFadden, 2003; Williams and Keeling, 2003; Ralph *et al.*, 2004a).

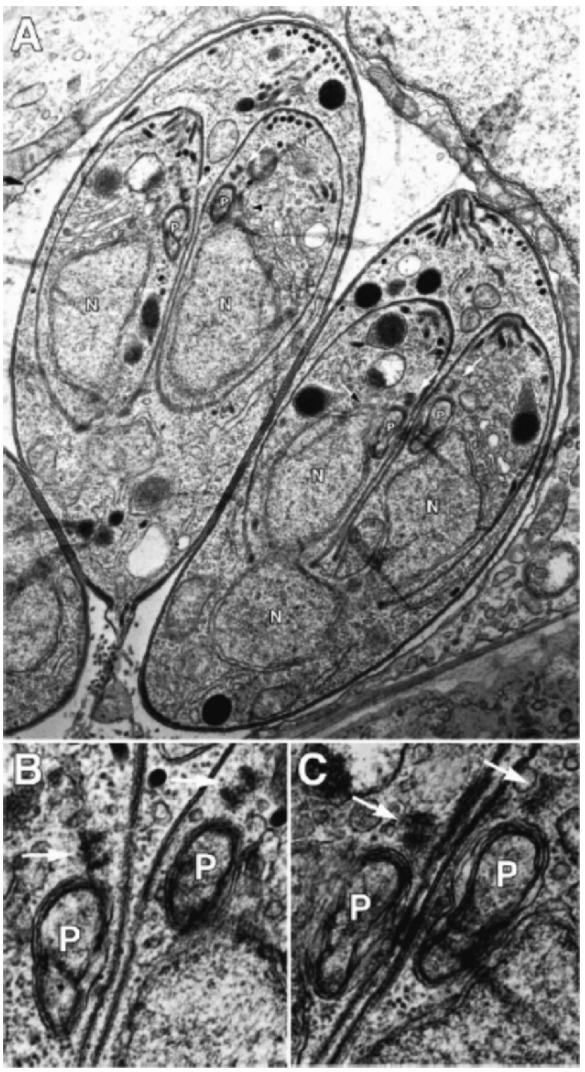


*Fig. 1.* Organization of the apicoplast during division of *T. gondii*. Panel A: two dividing parasites are present in a single parasitophorous vacuole, each containing two developing daughters. The apicoplast (P) exhibits four delimiting membranes. Panels B and C show the apicoplasts at higher magnification. The centrioles (white arrows) are present at the apical end of each plastid. Micrograph courtesy of Boris Striepen; reproduced from Striepen *et al.* (2000) by copyright permission of The Rockefeller University Press.

Some apicomplexan parasites lack an apicoplast; such is the case of *Cryptosporidium parvum*. Recent findings support the hypothesis that *Cryptosporidium* evolved from a plastid-containing lineage and subsequently lost its apicoplast during evolution, and that this organism may be the recipient of a large number of horizontally transferred genes, many of which are not present in other Apicomplexans (Huang *et al.*, 2004).

## II. A Brief History of the Studies on the Apicoplast

The early approaches to the nature of the apicoplast were circumscribed to studies of the cellular structure of apicomplexans (for a detailed historical account see Gleeson, 2000; for a compact scheme, see Ralph *et al.*, 2004a). The presence of organelle-like structures different from mitochondria was originally reported by microscopic studies in the late 1960's. The so-called "spherical bodies" were first identified in *Eimeria perforans* merozoites (Scholtyseck and Piekarski, 1965) and described as "große Vakuole mit kräftiger Wandung" (large vacuole with stout surrounds). This structure was also identified in avian malaria-like parasites described as "associated to" or a "contaminant of" isolated mitochondrial fractions (Kilejian, 1975). The spherical bodies were present in all *Plasmodium* species analyzed (Kilejian, 1991). Other names ascribed to these "spherical bodies" were "Dickwändige vakuole" (large vacuole with stout surrounds), "Hohlzylinder" (hollow cylinder), "Lamellärer Körper" (body with multiple walls) and "Golgi adjunct" (reviewed by Siddall, 1992).

Although morphologically identified as a distinct subcellular structure, the apicoplast function remained unknown.

In the early and mid-1970's extrachromosomal DNA molecules now known to be associated with the apicoplast were isolated using CsCl gradients from *Plasmodium knowlesi* (Gutteridge *et al.*, 1971), *Plasmodium berghei* (Chance *et al.*, 1972), *Plasmodium chabaudi* (Chance *et al.*, 1972) and *Plasmodium lophurae* (Kilejian, 1975). The isolated DNA from the avian malaria parasite *P. lophurae* was interpreted to be a "circular mitochondrial DNA" that appeared under the electron microscope to have a contour length of 10.3 μm and an estimated size of 27 kb (Kilejian, 1975). The presence of these circular molecules was subsequently confirmed in several other apicomplexan species: *P. berghei* (11.3 μm) (Dore *et al.*, 1983), *T. gondii* (13 μm) (Borst *et al.*, 1984), *P. knowlesi* (11.6 μm) (Williamson *et al.*, 1985), and *P. falciparum* (11.1 μm) (Gardner *et al.*, 1988). For several more years these extrachromosomal DNAs continued to be considered mitochondrial DNA, since the data available did not hinted towards a different interpretation.

A few years later the sequences of the legitimate mitochondrial genes *coxI* (cytochrome *c* oxidase subunit I) and *cytb* (cytochrome *b*) were obtained from tandem-arranged linear DNA molecules (6 kb length each unit) from the rodent parasite *P. yoelii* (Vaidya and Arasu, 1987; Vaidya *et al.*, 1989). Typically, apicomplexans contain extremely reduced mitochondrial DNAs (mtDNA), encoding two truncated ribosomal RNAs and only three components of the respiratory chain complexes: subunits I and III of cytochrome *c* oxidase (complex IV) and cytochrome *b* of ubiquinol: cytochrome *c* reductase (complex III) (Feagin, 2000). With the unambiguous characterization of a mitochondrial genome in apicomplexans, the nature of the other extrachromosomal element described in the previous section became an even more intriguing subject of research (Feagin, 1994). New evidence for the possible nature and origin of this genome came from the determination of the size of the molecule (35 kb) by restriction mapping (Gardner *et al.*, 1991a) and from the sequencing of the genes encoding portions the β and β' subunits of RNA polymerase (Gardner *et al.*, 1991b). With the recognition of two distinct extrachromosomal elements in apicomplexan parasites the location of the non-mitochondrial 35 kb DNA molecule became relevant. Kilejian (1991) suggested the possibility that the "spherical bodies" could be the site where this 35 kb DNA unit resided, while Wilson (1991) pointed out the lack of experimental evidence to ascertain a specific subcellular location. Indeed, as indicated below, it was another six years before the association of the 35 kb DNA with the "spherical bodies" or apicoplasts could be conclusively demonstrated (McFadden *et al.*, 1996; Köhler *et al.*, 1997).

With the sequence of the genes encoding the β and β' subunit of RNA polymerase in the 35 kb extrachromosomal element of apicomplexans (Gardner *et al.*, 1991b), similarities of the predicted proteins with several prokaryotic and chloroplast counterparts became evident. Phylogenetic analysis suggested that the *Plasmodium* RNA polymerase probably derived from an ancestor that harbored a typical chloroplast genome (Howe, 1992), and it was proposed for the first time that the "spherical bodies" of apicomplexan cells were vestigial secondary plastids (Wilson *et al.*, 1994). Another important clue to the identity of the 35 kb DNA was the identification of a plastid-like rRNA in *T. gondii*, similar to the previously reported one of *P. falciparum* that possessed a specific motif, absent from both cytosolic and mitochondrial rRNAs, that could explain the sensitivity of the parasite to macrolide-lincosamide antibiotics (Beckers *et al.*, 1995).

The complete mapping of the circular 35 kb element from *P. falciparum* (Wilson *et al.*, 1996) immediately suggested a similarity in organization to algal chloroplast genomes. The circular apicoplast DNA molecule resembled an extremely reduced plastid DNA (plDNA), where the photosynthetic genes have been specifically lost (Gardner *et al.*, 1994). Nevertheless, the apicoplast genomes contain a remnant set of genes encoding components that constitute the minimal set required for autonomous protein translation. The similarities in microstructure of apicoplasts with the chromist plastids, and the complete sequence of the apicoplast genome of *P. falciparum* confirmed the suggestion that apicomplexan plastid-like genomes were acquired by secondary symbiosis (Wilson *et al.*, 1996). At the same time, the sub-cellular location of the 35 kb DNA molecule was determined by a series of *in situ* hybridization experiments. Micrographs showed that the plastid-like genome was located inside a single oviform organelle, spatially associated with mitochondria in *T. gondii* (McFadden *et al.*, 1996). The plDNA was also identified by high-resolution *in situ* hybridization, and shown to localize in a distinct, separate organelle close to the nucleus of *T. gondii*: the apicoplast (Köhler *et al.*, 1997).

The presence of more than two surrounding membranes in the organelle was assumed early (Siddall, 1992) and discussed in the context of a possible secondary endosymbiotic origin of the oviform organelle

(Wilson *et al.*, 1996). New data have confirmed the existence of several membranes around the apicoplast (McFadden *et al.*, 1996; Köhler *et al.*, 1997), although the exact number (three or four) was not ascertained. In *T. gondii* (Köhler *et al.*, 1997) and other genera of the group, the presence of four membranes is well accepted, whereas in *P. falciparum* only three membranes are evident during the asexual stages of the life cycle (Hopkins *et al.*, 1999). Nevertheless, this seems to be a particular characteristic of *P. falciparum* that is probably related to its biological cycle. It is now thought that four membranes are present in coccidians, like *T. gondii* and *Goussia janae*, and in the haemosporidian *Garnia gonadatii.* Most probably, four membranes may represent the ancestral state of the apicoplast, and three membranes a derived character (Hopkins *et al.*, 1999; Foth and McFadden, 2003). Additionally, the targeting of cytosol-synthesized plastid proteins appears to be conserved both in *Toxoplasma* and *Plasmodium*, suggesting similar apicoplast membrane receptors in both organisms (Waller *et al.*, 2000; Foth and McFadden, 2003).

Independent of the actual number of surrounding membranes, the presence of more than two natural barriers suggests an origin by secondary endosymbiosis (Delwiche, 1999; Hopkins *et al.*, 1999; Maréchal, 1997). The presence of multiple membranes in other organisms like cryptomonads (Ludwig and Gibbs, 1985), euglenoids (Gibbs, 1978) and chlorarachniophytes (McFadden *et al.*, 1994) also implies an evolutionary origin by phagocytosis and enslavement of photosynthetic, unicellular algae. The two inner membranes may correspond to those derived directly from the cyanobacterial ancestor that gave rise to the inner and outer envelopes of the chloroplast (as in glaucophytes, rhodophytes and green plants). In contrast, the one or two extra membranes may have originated from the endomembrane system of the host: either from the phagosome and/or from the endoplasmic reticulum, or alternatively, from the plasma membrane of the primary host (reviewed in Archibald and Keeling, 2003). Several evidences suggest that the cryptomonad plastid is the result of a secondary endosymbiosis with a red alga, whereas for euglenoids and chlararachnyophytes the idea of a green algal origin has been favored (Gibbs, 1978). More recently, it has been proposed that the plastid of chromalveolates, a group that includes the superphylum alveolata, and therefore also the apicomplexans, evolved from a red algal ancestor (Harper and Keeling, 2003). Suggestions on the rhodophyte or chlorophyte origin of apicomplexans will be addressed below.

## III. What is the Physiological Role of the Apicoplast?

"What use is the plastid in Apicomplexa?" is a long-standing question (Gleeson, 2000), that is yet to be answered satisfactorily. Although the apicoplast genome is thought to be a relic of a much larger plastid precursor, the organelle is not a useless appendix, but an essential component of the apicomplexan protists.

The apicoplast is required for viability. This is confirmed by the observation that a plastid segregation mutant in *T. gondii* cannot survive after invasion of a second host cell (He *et al.*, 2001a). Expression and maintenance of the plastid genome also appears to be essential for the parasite survival, since it is the target for several antibiotics thought to have action in prokaryotes and other plastids. These inhibitor drugs affect expression of the plDNA at the level of replication, transcription or translation (McFadden and Roos, 1999). Treatment of apicomplexan parasites with some of these antibiotics, like macrolides, lincosamides, or ciprofloxacin, produce a "delayed death" phenotype in the parasite, where treatment inhibits survival only after the parasite invades a subsequent host cell (Fichera *et al.*, 1995; Fichera and Roos, 1997; Sullivan *et al.*, 2000).

In addition to photosynthesis, chloroplasts are the sites of starch storage, heme synthesis, nitrate and sulfate assimilation, fatty acid and lipid biosynthesis, the non-mevalonate isoprenoid biosynthesis pathway, the biosynthesis of iron-sulfur clusters, and the biosynthesis of essential amino acids. Even in those organisms that have lost the capability of carrying out photosynthesis, like the colorless alga *Prototheca wickeramii*, its plastid-targeted proteins participate in carbohydrate, amino acid, lipid, tetrapyrrole, and isoprenoid metabolism as well as *de novo* purine biosynthesis and oxidoreductive processes (Borza *et al.*, 2005). Thus, the metabolism of chloroplasts and of its evolutionary-related colorless plastids seems to be far more complex that the one taking place inside the apicoplast. Only some metabolic functions seem to have been retained in the apicoplast, and are thought to be the reason for the persistence of this organelle in apicomplexans. It is of course also possible that the apicoplast is harboring a so-far-unknown metabolic pathway exclusive to these parasites and that is the actual reason for the essential role of the apicoplast in the cell viability. Undoubtedly, the completion of the 23-megabase genome sequences of *P. falciparum* (Gardner *et al.*, 2002) and *P. yoelii* (Carlton *et al.*, 2002) represents a strong asset in the characterization of apicoplast function. In addition, the

*Plasmodium* Genome Database (http://PlasmoDB.org) represents a powerful tool that allows both researchers and malaria devotees to access and work with the current available sequence information (Roos *et al.*, 2002; Kissinger *et al.*, 2002; Bahl *et al.*, 2003). Also, the recent completion of the 9 megabase genome of *Cryptosporidium parvum* (Abrahamsen *et al.*, 2004) and the near approaching completion of *Toxoplasma* genome sequences will increase the wealth of information already available at the *Cryptosporidium* Genome Resources (http://cryptodb.org) (Puiu *et al.*, 2004) and the *Toxoplasma* Genome Resource (http://toxodb.org) (Kissinger *et al.*, 2003) respectively.

## IV. Structure and Expression of the Apicoplast Genome

### *A. A Plastid-like DNA Present in Apicoplasts*

Apicoplast genomes are compact molecules of around 35 kb, with gene content and arrangement highly conserved among different apicomplexan species (Lang-Unnasch *et al.*, 1998). A notable smaller molecule of 27 kb seems to be present only in *P. chabaudi* (Sato *et al.*, 2000), and a plDNA is absent in *C. parvum* (Zhu *et al.*, 2000a; Abrahamsen *et al.*, 2004). This genus could be an early branch of the apicomplexa. The absence of an apicoplast and its plastid genome can be due to a secondary loss during adaptation to intracellular life.

To date, the apicoplast genomes of *P. falciparum* (Wilson *et al.*, 1996), *T. gondii* (U87145, NC–001799) and *Eimeria tenella* (AY217738, NC–004823) (Cai *et al.*, 2003) have been completely sequenced. They encode approximately 68 genes, highly packed in a circular plDNA with almost no intergenic regions and with almost no introns. Most of the genes (60 out of 68 in *P. falciparum*) encode components which are involved in "housekeeping" functions, i.e., transcription and translation of the plastid genome (Fig. 2), the β, β′ and β″ subunits of the RNA polymerase (*rpoB, rpoC1* and *rpoC2* genes respectively), several ribosomal proteins from the large and small subunits (*rps* and *rpl* genes), a translation elongation factor (*tufA* gene), large and small rRNAs (*SSU* and *LSU rRNAs*), and a complete set of tRNAs that supports translation of the plDNA-encoded proteins (Preiser *et al.*, 1995). The β, β′ and β″ subunits of the RNA polymerase are similar to the corresponding subunits of cyanobacteria and chloroplasts, and differ from the mitochondrial proteins (Gardner *et al.*, 1991a). In the apicoplast genome

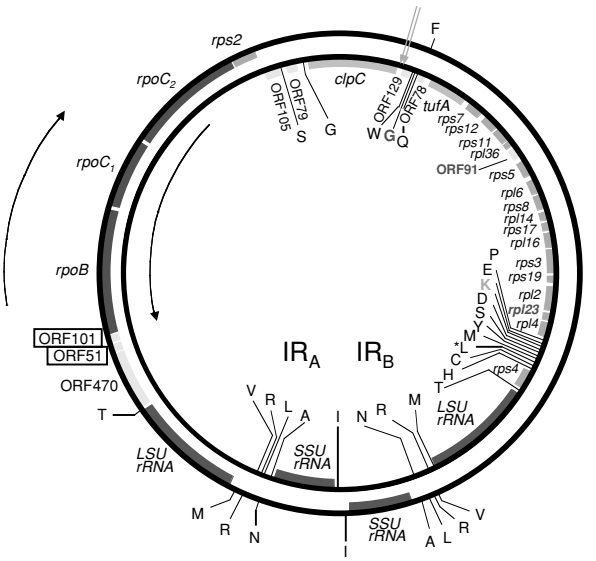


*Fig. 2.* (See also Color Plate 5, p. xxxviii.) Comparison of the gene content and arrangement of the plastid DNA among apicomplexa. The gene map of the circular 35 kb plDNA from *P. falciparum* is presented (modified from Wilson *et al.*, 1996). The two halves of the inverted repeat region are represented as $IR_A$ and $IR_B$. The black arrows indicate the direction of transcription of the genes in the outer and inner strands. The *P. falciparum* plDNA is compared with the *T. gondii* (Gene Bank accession number NC_001799) and *E. tenella* (Gene Bank accession number NC_004823) plastid genomes. Genes specified by red letters are absent in *T. gondii* and *E. tenella*. The $tRNA^L$ gene (asterisk) present in the tRNAs cluster is the only gene where an intron was found in *P. falciparum* and *T. gondii,* but it is absent in *E. tenella*. In the same cluster, the $tRNA^K$ (green character) is encoded in the opposite strand in *T. gondii* and *E. tenella*. ORF101 and ORF51 have a different location in *T. gondii* and *E. tenella*, indicated by the green arrows. ORF 129 is annotated as the *rpl11* gene in *T. gondii* and *E. tenella*.

there is also an ORF that specifies a ClpC-like molecular chaperone (*clpC* gene) and a highly conserved ORF (ORF 470 in *P. falciparum*) related to the chloroplast gene *ycf24*. There are also a small number of other putative ORFs whose significance is questionable (Wilson *et al.*, 1996), but may be involved in some still unknown function that is essential to the parasites. The plDNA contains an inverted repeat (IR) region of approximately 10.5 kb, which is present in chloroplast but not in mitochondrial genomes. The two halves of the inverted repeats are IR-A and IR-B, and encode duplicated large and small rRNA subunits and nine duplicated tRNA genes (Gardner *et al.*, 1991a; Wilson and Williamson, 1997). This region appears to be important for plDNA replication (see below), and electron microscopy studies sometimes show this region as a cruciform structure within the circular molecule (Borst

*et al.*, 1984; Wilson and Williamson, 1997), probably due to self-annealing of the inverted repeats.

In *P. falciparum* the genes of the plDNA follow the universal genetic code, whereas in *T. gondii* some coding regions contain UGA stop codons that are presumed to be read as tryptophans (Wilson *et al.*, 2003). In *Neospora caninum* the protein product of the *rpoB* gene has three in-frame UGA codons, which also appear to encode tryptophan residues (Lang-Unnasch and Aiello, 1999). There is a strong codon bias in plDNA due to its high A/T content (86.9% in *P. falciparum* and 78.4% in *T. gondii*). In *P. falciparum* only approximately 4.6% of the codons present contain G or C at the third position, 17% of all codons contain G or C at the second position and 15.3% at the first position (Preiser *et al.*, 1995; Wilson and Williamson, 1997).

## B. Segregation of the Plastid and its Genome

In *Plasmodium*, during sexual reproduction, the plDNA is transmitted by only one of the parental cells (Creasey *et al.*, 1994). Most *T. gondii* and *P. falciparum* cells have one plastid per cell (Köhler *et al.*, 1997; Striepen *et al.*, 2000; Waller *et al.*, 2000). The plDNA copy number has been estimated to be around six to 25 copies per cell in *T. gondii* (Fichera and Roos, 1997; Köhler *et al.*, 1997; Matsuzaki *et al.*, 2001), and from one to three (Wilson *et al.*, 1996 ; Köhler *et al.*, 1997; Williamson *et al.*, 2002) and up to 15 (Matsuzaki *et al.*, 2001) in *P. falciparum*. Faithful segregation of the plastid and the plDNA during cell division is essential for the parasite survival. Apicoplast segregation and plDNA replication and segregation are highly coordinated with cell division, probably to ensure the transmission of both the plastid and its genome to the daughter cells (Striepen *et al.*, 2000; Matsuzaki *et al.*, 2001). Plastids divide just before the separation of the two daughter cells (a process known as schizogony). The apicoplast shape changes during the parasite cell cycle, from ellipsoid in G1 or S stages to elongated dumbbell and U-shaped forms when the cells are close to division (Striepen *et al.*, 2000). The elongated plastid appears to be closely linked to the mitotic machinery, since the ends of the dividing organelle are consistently associated with the centrosomes (Fig. 1). This association is present even in non-dividing apicoplasts (Striepen *et al.*, 2000). The plDNA is present in the form of DNA-containing areas (nucleoids), and usually a single nucleoid is found in each organelle (Matsuzaki *et al.*, 2001). plDNA replication takes place early during plastid division, since the elongated plastid already contains two nucleoids that localize towards the ends of the organelle, and segregate equally into the daughter cells during the parasite mitotic reproduction (endodyogeny) (Striepen *et al.*, 2000; Matsuzaki *et al.*, 2001). Plastid division might be driven by a combination of the force generated by the mitotic spindle and the daughter pellicle growth (Striepen *et al.*, 2000). However, Matsuzaki *et al.* (2001) observed structures around the constriction site of the dividing plastid, and suggested that they could be similar to a plastid-dividing ring-related structure, similar to the one observed in chloroplasts (Miyagishima *et al.*, 2003).

## C. Replication of the Plastid Genome

Like mitochondrial DNA, plDNA replication takes place just before replication of the chromosomal DNA and the first nuclear division (Smeijsters *et al.*, 1994; Preiser *et al.*, 1996; Williamson *et al.*, 2002). Apicoplast genomes exhibit a highly conserved circular structure observed by electron microscopy and a highly conserved gene content and arrangement among different species (Kilejian, 1975; Dore *et al.*, 1983; Borst *et al.*, 1984; Wilson and Williamson, 1997). Nevertheless the *in vivo* topology of plDNA exhibits high variability. Pulse-field electrophoresis revealed that plDNA is mainly present as linear concatamer molecules in *E. tenella* (Dunn *et al.*, 1998) and *N. caninum* (Gleeson and Johnson, 1999). Similarly, less than 10% of the plDNA molecules of *T. gondii* are present *in vivo* as circular molecules, while the vast majority are linear molecules arranged in tandem repeats of 1 to 12 copies of 35 kb each (Williamson *et al.*, 2001). These linear molecules could be replicated by a rolling circle mechanism, starting from the middle of the inverted repeat, as observed in chloroplasts, bacterial plasmids and phages (Kolodner and Tewari, 1975; Novick, 1998; Khan, 2000). After the first round of replication, the linear molecule can be processed to produce a linear monomer. Otherwise, replication could continue and stop after the next round of replication to produce a dimer, and so on. In this manner, a mixed population of oligomers with different repeats of the basic 35 kb molecule can be obtained (Fig. 3). In contrast, in *P. falciparum* more than 97% of plDNA is present as covalently closed circular molecules of 35 kb, and only a small amount is present as linear monomeric molecules (Williamson *et al.*, 2002). In this case, plDNA follows a bidirectional replication from twin D-loops present at the inverted repeat segments where multiple replication origins are differentially activated (Williamson *et al.*, 2002; Singh *et al.*, 2003, 2005). An additional rolling-circle mechanism where replication involves

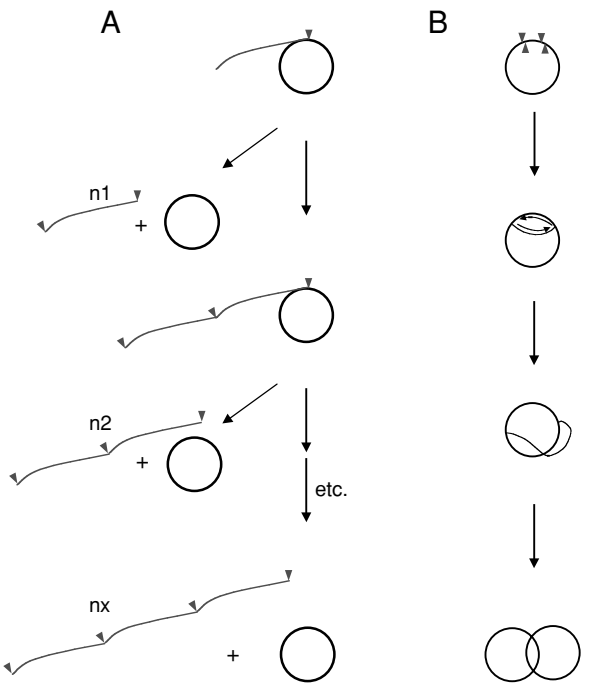


*Fig. 3.* (See also Color Plate 5, p. xxxviii.) Models for plDNA replication in *Plasmodium* and *Toxoplasma*. A) Rolling circle model for *T. gondii* plDNA. Replication initiates on a circular molecule at or near the center of the inverted repeat (∇). After one round of replication, the linear tail can be cut to produce a 35 kb linear monomer, and replication ceases. Processing is not always successful. If processing of the linear molecule fails, replication continues for a further complete round. If processing of the tail is successful, a linear 70 kb dimer is produced and replication ceases. If not, a third round of replication takes place, and so on. By this mechanism a linear molecule of up to 12 copies of the plDNA can be generated. Adapted from Williamson *et al.* (2001). B) D-loop replication model for the circular plDNA from *P. falciparum*. Replication starts from twin D-loop origins (τ) present in each half of the plDNA inverted repeat region. Each D loop is generated by unidirectional replication from an origin, resulting in displacement of a single-stranded region. The D loops might expand toward each other and fuse to form a Cairns structure, with replication proceeding bi-directionally around the plastid DNA. Modified from Wilson *et al.* (2003).

regions outside the inverted repeats has been proposed (Williamson *et al.*, 2002). In this case, the linear tails produced would be processed into circular molecules of 35 kb. Recently, several putative replication origins have been described within the inverted repeat region of *P. falciparum*, showing differential activation levels (Singh *et al*., 2005). However, it was recently proposed that more than two *ori* are present in each inverted repeat segment (Singh *et al.*, 2005). An additional rolling-circle mechanism where replication involves regions outside the inverted repeats has been proposed for a minor population of plDNA (Williamson *et al.*, 2002). In this case, the linear tails produced would be processed into circular molecules of 35 kb.

The enzymology of plDNA replication has only just begun. A bacterial-like topoisomerase type II activity has been associated with the plDNA of *P. falciparum* (Weissig *et al.*, 1997). The genes *gyrA* and *gyrB*, encoding two putative apicoplast-targeted subunits of DNA gyrase/TopoIV are present in the *P. falciparum* genome (Wilson, 2002; PlasmoDB database, accession numbers PFL1120c and PFL1915w), and homologs of the A and B subunits of the DNA gyrase/topoIV were recently cloned from *P. vivax* (Khor *et al.*, 2005). Furthermore, expression of the protein encoded in a recombinant fragment of the PvGyrB gene exhibited ATPase activity. The corresponding encoded protein region shares similarity with the ATP-binding domain of the B subunit from the bacterial DNA gyrase (Khor *et al.*, 2005).

## D. Transcription of the Plastid Genome

Transcription is an active process within the plastid of *P. falciparum*. Plastid transcript accumulation levels vary during the cell cycle, and are more abundant in the later stages of the erythrocytic cycle, as demonstrated for the *rpoB*/C rRNA genes (Feagin and Drew, 1995) and for the *tufA* gene (Clough *et al.*, 1999). Transcription has also been demonstrated for the *rpoB/C* genes (Feagin and Drew, 1995), for the large and small ribosomal subunits (Gardner *et al.*, 1991b, 1993, 1994), for all the tRNAs (Preiser *et al.*, 1995), as well as for *ClpC*, *ORF470*, *tufA*, and some ribosomal proteins (Wilson *et al.*, 1996). Detection of possible polycistronic and precursor mRNAs for some of the plastid genes has been hampered due to the difficulties of northern blot analysis that in some cases produce smearing signals (Wilson *et al.*, 1996). However, the map of the plastid genome suggests that at least four polycistronic transcripts could be present (Wilson *et al.*, 1996). The *rpoB/C* genes appear to be part of a polycistronic unit (Gardner *et al.*, 1991b; Fichera and Roos, 1997) as well as some genes encoding tRNAs (Preiser, *et al.* 1995). In addition, some ribosomal protein encoding genes like *rpl2-rpl23* and *rps3-rps19* are cotranscribed (Wilson *et al.*, 1996).

Plastids usually contain two types of RNA polymerases. One is nucleus-encoded and resembles the T3 and T7 phage RNA polymerase. The other is a prokaryote-like enzyme composed of subunits α, β, β′ and β″ (encoded by the plastid genes *rpoA*, *rpoB* and $rpoC_1$ *and* $rpoC_2$ respectively). Apicomplexan parasites lack the α subunit in their plDNA, and the β′ subunit is encoded by two separate genes *rpoC1* and *rpoC2*, as previously observed for plastids and

cyanobacterial genomes (Wilson *et al.*, 1996). There is no direct evidence that the *rpoB/C* genes present in the plDNA encode for a RNA polymerase active in plastid transcription or if there is an alternative nucleus-encoded phage-like RNA polymerase. However, the antibiotic rifampicin, which is an inhibitor of both the prokaryotic and chloroplast β subunits of RNA polymerases (Surzycki, 1969; Campbell *et al.*, 2001) also inhibits the *in vitro* and *in vivo* transcription of the apicoplast in *Plasmodium* species (Strath *et al.*, 1993). This strongly suggests that the plastid-encoded RNA polymerase subunits are active in the malaria organelle.

### *E. Translation of the Plastid Genome*

To date, no direct detection of plastid-synthesized proteins with specific antibodies is available. However, the apicomplexan plDNA encodes a complete set of tRNAs (25 in *P. falciparum*) (Preiser *et al.*, 1995), and as judged by electron micrographs, the apicoplast contains ribosome-like particles (McFadden *et al.*, 1996). Detection of polysomes (Wilson *et al.*, 1996) that contain rRNAs and mRNAs in *P. falciparum* plastids supports the idea that protein synthesis in the plastid does take place (Wilson *et al.*, 1996; Roy *et al.*, 1999). Furthermore, the antibiotic anisomycin binds to the apicoplast ribosomes, and not to their cytosolic counterparts (Roy *et al.*, 1999). Some of the ribosomal proteins are encoded in the nuclear genome of *T. gondii*. That is the case of the S9 and L28 ribosomal proteins, whose precursors exhibit a plastid targeting sequence (Waller *et al.*, 1998). The import of nucleus-encoded ribosomal proteins is strong evidence that ribosome assembly must occur inside the apicoplast. Plastid translation also seems to occur inside the apicoplast since a putative plastid-like peptidyl deformylase (PDF) is present in the genome of *P. falciparum* (Giglione *et al.*, 2000) and was found to be active when over-expressed in *Escherichia coli* (Bracchi-Ricard *et al.*, 2001). PDF removes the N-formyl groups from bacterial and organellar proteins. Compared to the bacterial PDF, the *Plasmodium* gene predicts an N-terminal extension that could target the protein to the apicoplast (Meinnel, 2000).

The only direct genetic evidence for active translation of plDNA is the finding of mutations within the *LSU* rRNA that confer resistance to the antibiotic clindamycin in *T. gondii* (Camps *et al.*, 2002). In addition, several antibiotics that inhibit prokaryote translation have been found to target the translation machinery of the apicoplast (reviewed in McFadden and Roos, 1999; see below).

### *F. Why has the Plastid Genome Been Retained?*

The reduced plDNA from the malarial and related parasites is probably the smallest known plastid genome. Therefore, the majority of metabolic pathways that take place in the apicoplast must be specified by nuclear genes whose products are imported into the organelle (Waller *et al.*, 1998). The question,"why has the plastid genome been retained?" inevitably arises again and again. Plastid DNA expression is an active process, essential for the cell survival. In particular, it seems to be critical the for synthesis of two plastid-encoded putative "housekeeping" proteins encoded by the genes *ClpC* and ORF470/*ycf24.* ClpC has similarity to the ClpC/ClpA molecular chaperones present in plastids and *E. coli* respectively (Wilson *et al.*, 1996). The chloroplast ClpC is a regulatory subunit of the Clp protease complex. This complex seems to be involved in the normal turnover of some stromal enzymes and regulatory proteins, as well as in removing damaged polypeptides (Adam and Clarke, 2002). ClpC has two ATP-binding domains characteristic of group 1 Clp/Hsp100 proteins, but only one is conserved in the *Plasmodium* gene (Wilson *et al.*, 1996). The other "housekeeping" protein encoded in the plastid genome is the ORF470/*ycf24* also present in the plastids of the red algae *Porphyra purpurea* and *Cyanidium caldarum* and in the plDNA of the diatom *Odontella sinensis* (Williamson *et al.*, 1994). This gene corresponds to a bacterial homologue known as *sufB*, which forms part of the bacterial *suf* operon (comprising six genes, *sufA, -B, -C, -D, -S,* and -E), involved in iron homeostasis, iron-sulfur cluster formation, and oxidative stress response (Ellis *et al.*, 2001; Wilson *et al.*, 2003). It has been proposed that ORF470/*ycf24* is essential, and one of the reasons why the apicoplast has protein synthesis capabilities (Law *et al.*, 2000). Also, ORF470/*ycf24* could be involved in the iron metabolism of malaria parasites, like the assembly and/or maturation of iron-sulfur clusters, which would serve to convert apoferredoxin into ferredoxin (Ellis *et al.*, 2001; Wilson *et al.*, 2003). The predicted functions of ClpC and ORF470/*ycf24* remain to be confirmed experimentally.

## V. Protein Targeting to Apicoplasts

Although plDNA is essential for the survival of the apicomplexan parasites, most of the proteins that are necessary for the apicoplast function are nucleus-encoded.

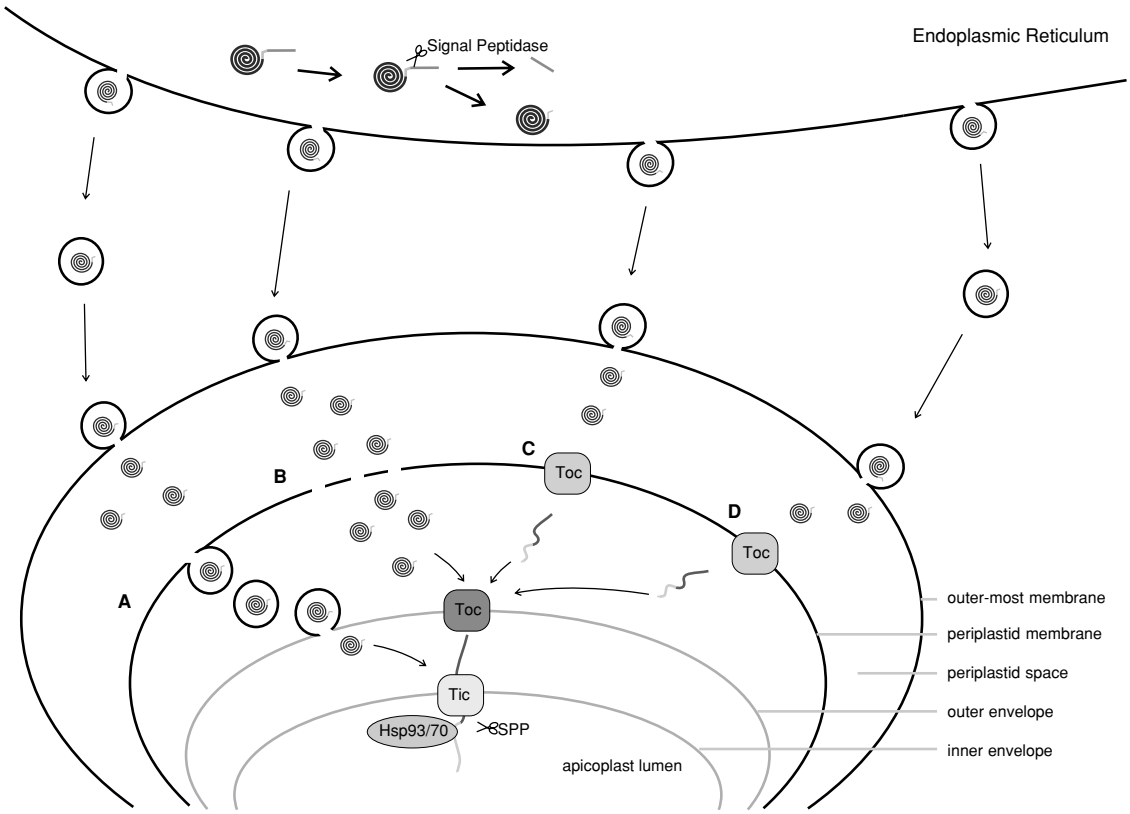


*Fig. 4.* (See also Color Plate 6, p. xxxix.) Targeting of apicoplast proteins. Apicoplast proteins are synthesised as preproteins with N-terminal bipartite presequences. The first segment directs the protein to the endoplasmic reticulum where is cleaved by a signal peptidase. Later, it is delivered to the outer-most membrane of the apicoplast via vesicles. The periplasmic membrane is crossed via vesicles (A), pores (B), or secondary Toc complexes (C and D). After the precursor protein contacts the outer envelope, conserved Toc and Tic machineries mediate the final transport to the apicoplast lumen where a Stromal Peptidase (SPP) digest the transit peptide. See text for details.

According to *in silico* predictions, more than 500 proteins must be synthesized in the cytosol and imported into the organelle in order to ensure its proper function (see section on apicoplast metabolism below). The first clue about the targeting pathway to the apicoplast was suggested on the basis of the apicoplast structure and the presence of four membranes. Apicoplast proteins are first targeted to the endoplasmic reticulum, guided by the presence of a signal peptide, and further on directed to the plastid (Bodył, 1999; Waller *et al.*, 1998; Fig. 4).

As compared to other organelles like mitochondria and chloroplasts, where targeting signals, transit peptides, and import machineries have been extensively dissected (and still new details on those processes are discovered every day) (Neupert and Brunner, 2002; Soll and Schleiff, 2004), the studies of protein targeting to the apicoplast have flourished only recently, and the full comprehension on the mechanisms involved seems to be still remote.

Two main machineries have been described so far for protein translocation across the outer and inner envelopes of a chloroplast: the TOC and the TIC translocases (for Translocase of the Outer and Inner envelope of the Chloroplast respectively) (see Chapter 3). It is not so hard to imagine that during the evolution of the apicoplast, the translocases of the original chloroplast have been conserved, especially because the presence of a plant transit peptide located after the signal peptide of a secreted protein can successfully direct the green fluorescent protein (GFP) to the apicoplast of *T. gondii* (Foth and McFadden, 2003).

From the protein targeting point-of-view, the apicoplast as a chloroplast-derived organelle may present some advantages and some disadvantages in protein import and sorting when compared to protein import in the original chloroplast. On one hand, during the evolution of the apicoplast the thylakoid membranes have been lost and therefore the problem of protein

sorting within the "apicoplast-stroma" has been automatically solved. On the other hand, the one or two additional membranes surrounding the original endosymbiotic plastid represent extra physical barriers for protein import.

### A. Apicoplast Targeting Signals

Numerous genes encoding apicoplast proteins are located in the nucleus, and the encoded proteins are targeted to the organelle in a process mediated by a bipartite N-terminal extension. The first part of this apicoplast leader sequence is a signal peptide that targets proteins to the secretory pathway (Waller *et al*., 1998, 2000). The second is a chloroplast-like transit peptide region required to direct proteins from the secretory pathway into the apicoplast (DeRocher *et al.*, 2000; Waller *et al.*, 2000; van Dooren *et al.*, 2002).

The essential role of each of the apicoplast targeting sequence segments, and some of their structural characteristics, have been shown both in *P. falciparum* and in *T. gondii* in a series of experiments that exploit the formation of protein chimeras of targeting signals to GFP. Signal peptides target proteins to the secretory pathway in all eukaryotic cells. They are characterized by the presence of three structural elements: a short positively charged amino-terminal segment, a central hydrophobic segment, and a more polar carboxy-terminal segment that is recognized by the signal peptidase (reviewed in Emanuelsson and von Heijne, 2001). The signal peptide segment of apicoplast-targeted proteins share the same structural characteristics that can be identified *in silico* by prediction programs like SignalP (http://www.cbs.dtu.dk/services/SignalP/; Nielsen *et al.*, 1997; Waller *et al.*, 1998, 2000; Foth *et al.*, 2003; Bendtsen *et al.*, 2004). The second part of the apicoplast targeting signal shows some similarities to the chloroplast transit peptides. For the *in silico* prediction of *P. falciparum* apicoplast targeting signals, two algorithms have been developed: PATS (http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php) (Zuegge *et al.*, 2001; Waller *et al.*, 1998, 2000) and PlasmoAP (http://www.plasmodb.org/restricted/PlasmoAPcgi.shtml) (Foth *et al.*, 2003). The predicted features of apicoplast targeting signals are supported by the fact that a chloroplast transit peptide fused to the N-terminus of GFP can direct this reporter protein to the apicoplast in *T. gondii* (reviewed in Foth and McFadden, 2003). *T. gondii* transit peptides can also direct import *in vitro* of the reporter protein GFP into pea chloroplasts (DeRocher *et al.*, 2000).

Several features of the original chloroplast transit peptides seem to have adapted to the apicoplast conditions. The evolutionary pressures that act on the transit peptides may have led to the maintenance of their structural features independently of their extremely weak amino acid sequence conservation (Yung and Lang-Unnasch, 1999; Harb *et al.*, 2004; Ralph *et al.*, 2004b).

In the organisms studied so far, the primary sequence of the transit peptide depends largely on the codon usage and A/T content of each genome: the *P. falciparum* transit peptides are richer in asparagine, glycine, proline and arginine, the same observation is true for the chloroplast transit peptides of *Arabidopsis thaliana* and *Glycine max* that also present genomes enriched in A/T; in plants like rice and barley residues such as alanine, glycine, proline and arginine are favored (Ralph *et al.*, 2004b). It seems that despite the differences in amino acid composition, as long as the transit peptide is composed by a high number of basic residues—avoiding acidic ones—the function of the peptide is not altered (Foth *et al.*, 2003).

In the case of plant transit peptides, it has been observed that phosphorylation of serines and threonines can occur and could be one of the mechanisms used to distinguish between chloroplast pre-proteins and other organellar pre-proteins (mitochondrial and peroxysomal precursors) (Waegemann and Soll, 1996). This process has not been observed in apicoplast transit peptides, probably because in contrast to the plant situation, the apicoplast presents an extra membrane barrier with different characteristics, avoiding the need for these protective mechanisms (Ralph *et al.*, 2004b).

For chloroplast transit peptides, it has been proposed that the recognition of the outer envelope by the transit peptide relies on the plastid-specific lipids, and that upon lipid interaction the transit peptide can adopt specific secondary structures that facilitate further activity of the translocation machinery (Bruce, 2000). Despite the presence of similar galactolipids to those found in chloroplasts, the presence of a periplastid membrane in the apicoplast suggested that the influence of these galactolipids on the apicoplast transit peptides is unlikely (reviewed in Ralph *et al.*, 2004b).

An interesting case of fine regulation of the traffic routes in *T. gondii* was reported for a mitochondrial iron-containing superoxide dismutase (TgSODB2) which possess a presequence consisting of a hydrophobic N-terminal signal peptide followed by an amphipatic mitochondrial presequence. In the

*Table 1.* Comparison of chloroplast transit peptides to apicoplast transit peptides (TP)

| TP characteristic | Chloroplast | Apicoplast |
|---|---|---|
| Length | One segment of around 20–150 aa. | Bipartite. First segment consists of a signal peptide of maximum 16 to 34 aa followed by a transit peptide of maximum 80 aa. |
| Amino-acid enrichment | In serines and threonines. But it also depends on the codon usage of each organism. | Serines and threonines in *T. gondii* and lysines and asparagines in *P. falciparum*. High bias to basic residues and lack of acidic residues. |
| Hsp70 binding domain? | Yes. | Yes. Not absolutely required but may be important for increased fidelity during the import process. |
| Conserved primary or secondary structure | No. In aquous solution forms a random-coil structure; but in contact with outer envelope galactolipids can form specific secondary structures. | Unknown. |
| Peptidase processing | Yes. Inside of the chloroplast stroma by the stromal processing peptidase. | Yes, two. First, cleavage of the signal peptide after the protein enters the endomembrane system. Second, cleavage of the transit peptide in the apicoplast lumen. |
| Interaction with galactolipids | Yes. | Unknown, but unlikely. |
| Prediction program | ChloroP: http://www.cbs.dtu.dk/services/ChloroP/ | SignalP (only for the signal peptide) http://www.cbs.dtu.dk/services/SignalP-2.0/ PATS (designed only for *P. falciparum*) http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php PlasmoAP (designed only for *P. falciparum*) http://www.plasmodb.org/restricted/ PlasmoAPcgi.shtml |
| Phosphorylation | Yes, on serines and threonines residues. However this is not always observed. | Unknown, but unlikely. |

absence of the signal peptide, the protein remains in the cytoplasm, and deletion of the presequence leads to secretion of the protein. Strikingly, the change of a single charged residue within the hydrophobic sequence directs the protein to the apicoplast (Brydges and Carruthers, 2003). A possible role of TgSODB2 was discussed, but this observation could also be an indirect hint that possibly the secondary structure adopted by the targeting signals can play a decisive role during the correct localization of proteins in the apicomplexan parasites. A general comparison of the chloroplast transit peptides and the apicoplast transit peptides is shown in Table 1.

The processing of the transit peptide of nuclear-encoded apicoplast proteins requires plastid-associated activity, as shown by studies carried out with *T. gondii* apicoplast segregation mutants (He *et al.*, 2001b). Furthermore, protein import into the apicoplast is stage regulated. Newly-synthesized GFP containing a plastid targeting sequence was recruited into *Plasmodium* secretory pathway early (0 to 12 h) in its development in red cells. The protein chimera was released into the parasitophorous vacuole. However, import into the apicoplast and processing to mature GFP did not occurred until 18 to 36 h in development (Cheresh *et al.*, 2002).

The presence of a signal peptide followed by a transit peptide is necessary and sufficient to deliver a protein into the apicoplast, however, the precise mechanism of protein translocation through the four membranes in order to reach the apicoplast stroma is still unknown.

### *B. The Role of the Secretory Pathway in Targeting Apicoplast Proteins*

If apicoplasts have three or four membranes, the main problem facing protein targeting is how a protein can cross two (or at least one) additional membranes before their initial contact with the plastid outer envelope.

Targeting of nuclear encoded apicoplast proteins commences via the secretory pathway in the endoplasmic reticulum. The set of proteins linked to this secretory pathway includes several factors that are active in

the invasive stages of the parasite (like all those directed to the rhoptries, micronemes, parasitophorous vacuole, and the parasitophorous vacuole membrane) as well as apicoplast-specific proteins (van Dooren *et al.*, 2000).

The nucleus-encoded apicoplast ribosomal protein S9 of *T. gondii* was fused with GFP to study protein domains required for apicoplast targeting (DeRocher *et al.*, 2000). The N-terminal 42 amino acids of the S9 ribosomal protein directs secretion of GFP, indicating that targeting to the apicoplast proceeds through the secretory system. Large sections of the S9 predicted transit sequence could be deleted with no apparent effects on GFP import into the apicoplast. The predicted transit peptide domain of the S9 targeting sequence is able to direct proteins to the mitochondrion *in vivo* and to chloroplasts *in vitro*. Consistently, in *P. falciparum*, the apicoplast targeting signal (the signal peptide followed by the transit peptide) of the nucleus-encoded acyl carrier protein directs the delivery of the reporter GFP into the apicoplast; but when the signal peptide is deleted, the GFP accumulates in the parasite's cytoplasm (Waller *et al.*, 2000; Foth *et al.*, 2003).

The above data indicate that the signal peptide is necessary and sufficient to allow a protein to enter the endomembrane system of the secretory pathway. If this signal peptide is removed, the protein accumulates in the cytosol, and the contact of the precursor protein with the apicoplast seems to be lost. The signal peptide is cleaved by a signal peptidase after its entrance into the endoplasmic reticulum (ER).

In *T. gondii* the addition of Brefeldin A (an inhibitor of early steps of the traffic between the ER and the Golgi apparatus) does not seem to influence the localization of apicoplast proteins, supporting the idea that apicoplast targeting is independent of the Golgi apparatus. However, this compound also blocks the removal of the transit peptide (DeRocher *et al.*, 2005). Thus, the precise influence of a disruption of the Golgi targeting remains to be elucidated, and the possibility remains that proteins like Sec7 GTP/GDP exchange factors without canonical apicoplast targeting signals could be targeted to intermediate compartments that may regulate the targeting/maturation process.

## C. Crossing the Middle Boundary

The following step is less clear: why and how a protein can go through the second membrane (the periplastid membrane)? So far there are several hypothesis proposed, all of them still lacking strong experimental support (reviewed in Foth and McFadden, 2003). Three different possibilities are addressed below (see also Fig. 4).

### 1. Pore Crossing

The periplastid membrane may contain large pores through which proteins can freely pass, without requiring any special targeting mechanism or signal (Cavalier-Smith, 1999; Kroth and Strotmann, 1999). This is a very simple explanation that involves the absence of a special translocation mechanism, and that presumably would require only the "availability" of the transported protein in an unfolded state. Free crossing through the membrane pores, could be favored in the direction to the apicoplast by the contact of the transit peptide with the TOC machinery of the apicoplast outer envelope, and/or by chaperone proteins present in the periplastid space.

### 2. Vesicle Transport

Another simple explanation, could involve the formation of small transport shuttles (that contain the transported proteins) by invagination of one membrane. As soon as this vesicles contact the next membrane they fuse together delivering the proteins into the next compartment. This hypothesis was first proposed on the basis of the presence of a "periplastid reticulum" (a complex of vacuoles and tubules) in the periplasmic space of some algae (Gibbs, 1981). However, it has been noticed that if this would be the transport mechanism, the vesicle-shuttle would deliver the proteins directly into the inner envelope space (the space between the most inner envelopes) bypassing the contact area with the TOC complex, therefore making its presence unnecessary (discussed in Foth and McFadden, 2003). One direct observation that could favor this mechanism, is the involvement of the parasitophorous vacuole during the trafficking of apicoplast proteins (Cheresh *et al.*, 2002).

### 3. Presence of a Second TOC Complex in the Periplastid Membrane

A different proposal involves the presence of a second TOC machinery in the periplastid membrane. It was proposed that the TOC complex subunits can be directed to both the outer envelope and the periplasmid membrane, by a dual targeting mechanism. The presence of this "external" TOC complex could interact

with the transit peptides and translocate them through the periplastid membrane in the same way that occurs during the crossing of the outer envelope (van Dooren *et al.,* 2001). Alternatively, the components of each TOC complex (the periplasmic and the outer envelope ones) could be different. Those subunits involved in the receptor function of the complex are necessary only in the periplasmic TOC, whereas the actual channel has to be present in both. If the two channels are connected in any way, then the translocation through both membranes could be simultaneous.

### *D. Crossing the Chloroplast-derived Outer and Inner Envelope Membranes*

Once a pre-protein has reached the outer envelope, most likely it will follow the same pathway used by the chloroplast proteins that carry a cleavable presequence: the preproteins are recognized by receptors of the TOC complex in a GTP-dependant manner. These preproteins cross the outer envelope through an aqueous pore and are then transferred to the TIC complex. As soon as the transit peptide enters the apicoplast lumen, chaperones such as Hsp93 and/or Hsp70 could bind to the protein preventing its retrograde movement. Finally, the transit peptide could be cleaved by a stromal processing peptidase homologue (SPP) and the mature protein folded with the assistance of Cpn60 (a GroEL homologue) (van Dooren *et al.*, 2002; Foth and McFadden, 2003; Foth *et al.*, 2003; the chloroplast pathway is reviewed in Soll and Schleiff, 2004).

Protein import into mitochondria and chloroplasts is energy-dependent and requires the presence of an electrochemical gradient across their inner membranes. Since the apicoplast lacks a photosynthetic machinery, no electrochemical gradient is expected to be built. Therefore, relative large amounts of GTP may be consumed in the translocation process.

Although we already know the basic principles of protein targeting into the apicoplast, this knowledge is limited to those proteins that contain a "typical cleavable apicoplast targeting sequence." It is still unclear if there are other targeting pathways to this organelle (for example recognition of internal-non-cleavable signals). It will be also of major interest to find out if the composition and "molecular environment" of each apicoplast compartment involves different targeting mechanisms, or if these factors can modify in some way the transport pathway. Another topic that remains unclear is the variety of sorting mechanisms within the different compartments.

## VI. Metabolism and Inhibitor Drug Targeting

### *A. Apicoplast Metabolism*

Wilson (2002) categorized the known and potential apicoplast components into two main classes: *i*) those involved in plastid "housekeeping" functions, including replication of DNA, components involved in the form and division of the organelle, protein synthesis and heme biosynthesis and *ii*) those that participate in the apicoplast metabolic pathways, mainly fatty acid and lipid biosynthesis and isoprenoid biosynthesis. The majority of these predicted components must be nucleus-encoded. PlasmoDB, the Malaria Database, allows one to retrieve all the sequences of proteins that are predicted to be targeted to the apicoplast (http://plasmodb.org/restricted/Apicoplast.shtml). From a search (carried out on February 2004), 551 sequences could be retrieved. Only 165 (30%) of these putative apicoplast-targeted proteins could be ascribed to distinct metabolic routes: 25 were related to nucleic acid metabolism, 25 were tRNA-ligases, 16 were ribosomal proteins, 21 were enzymes related to lipid metabolism, eight to carbohydrate metabolism, three to amino acid metabolism, seven were chaperones and heat shock proteins, 13 were proteases, eight were transporters and other membrane proteins, two were related to iron-sulfur cluster biogenesis, and 37 were related to various other metabolic pathways. The vast majority (70%) were annotated as hypothetical proteins. This immediately indicates that only a partial segment of the overall metabolic pathways that take place in the apicoplast is known. Furthermore, few of the participating components in these pathways have been characterized to date. These known enzymes are limited to fatty acid and lipid biosynthesis, the non-mevalonate isoprenoid biosynthesis pathway, the iron-sulfur cluster biosynthesis, and the *de novo* synthesis of heme groups. The Shikimate pathway, essential for the synthesis of aromatic compounds, was once thought to be localized in the apicoplast (Roberts *et al.*, 1998), but is now believed to be cytosol-localized (Fitzpatrick *et al.*, 2001; Roberts *et al.*, 2002).

More recently, using a bioinformatic approach, 545 proteins (other than the 23 proteins that are apicoplast-encoded) were predicted to be functional in the *P. falciparum* apicoplast (Ralph *et al.*, 2004a). This worked confirmed the presence of proteins involved in the maintenance of the organelle, as well as proteins involved in four biosynthetic pathways in the

apicoplast, related to the metabolism of fatty acids, isoprenoids, iron-sulfur clusters and heme groups. Also, a large proportion of genes (69%) were found to encode apicoplast-targeted proteins with unknown function.

## 1. Fatty Acid and Lipid Biosynthesis

Fatty acid and lipid biosynthesis seems to be a major function of the apicoplast (Seeber 2003). Fatty acids are essential for the synthesis of membrane lipids in Apicomplexa and other compounds like phosphoglycerides (Dieckmann-Schuppert *et al.*, 1992; Elabbadi *et al.*, 1997), sphingolipids and glycosphingolipids (Gerold and Schwarz, 2001), and glycosylphosphatidylinositol (Gowda *et al.*, 1997). For some time, it was thought that *Plasmodium* was incapable of *de novo* fatty acid synthesis. Nevertheless, *P. falciparum* is able to incorporate radioactively-labeled acetate and malonyl-CoA into fatty acids with chains from 10 to 14 carbons (Surolia and Surolia, 2001). This fatty acid synthesis pathway represents the plant chloroplast- and bacteria-like type II pathway distinct from the type I pathway of animals (including humans) (Waller *et al.*, 2003a). The enzymes of the type II pathway do not associate in a complex, in contrast with those of type I (Coombs and Müller, 2002). Several enzymes that participate in fatty acid biosynthesis like the acyl carrier protein (ACP), the β-ketoacyl-ACP synthase III (FabH) and an acetyl-CoA carboxylase (ACC) are localized in the apicoplast (Waller *et al.*, 1998, 2000; Jelenska *et al.*, 2001; Coombs and Müller, 2002). These type II fatty acid biosynthetic enzymes are present also in other chromists, including cryptomonads, heterokonts, and haptophytes (Ryall *et al.*, 2003). In addition, other fatty acid biosynthesis-related enzymes have been identified as nucleus-encoded proteins that may be targeted to the apicoplast, such as the β-hydroxyacyl-ACP dehydratase (FabZ) and an enoyl-ACP reductase (FabI) (Waller *et al.*, 1998; McLeod *et al.*, 2001; Surolia and Surolia, 2001). In *T. gondii* and *P. falciparum* lysates, radiolabeled uridine diphosphate galactose is incorporated into monogalactosylcerebrosides, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol, which are major lipids of algae and land plants synthesized within the chloroplast (Maréchal *et al.*, 2002). *C. parvum*, which lacks an apicoplast, contains a type I fatty acid synthase (FAS) that is located in the cytosol (Zhu *et al.*, 2000b). This enzyme is clearly different from the thiolactomycin-sensitive, apicoplast-targeted type II FAS enzymes identified in *T. gondii* and *P. falciparum*.

The presence of the pyruvate dehydrogenase complex (PDH) in the apicoplast of *P. falciparum* has also been demonstrated. This complex provides the acetyl-CoA for fatty acid synthesis inside the plastid. Since this PDH is the only one encoded in the *P. falciparum* genome; apicomplexans may be lacking the mitochondrial PDH version (Ralph, 2005; Foth *et al.*, 2005).

Two enzymes involved in lipoic acid synthesis, an essential cofactor of alpha-keto acid dehydrogenase complexes, the lipoic acid synthase and the lipoyl-ACP:protein N-lipoyl transferase, are also localized in the *P. falciparum* apicoplast (Wrenger and Muller, 2004).

## 2. The Non-Mevalonate Isoprenoid Biosynthesis Pathway

Isopentenyl diphosphate is the universal precursor of isoprenoids. In mammals and fungi, isoprenoid biosynthesis is achieved via the acetate/mevalonate pathway. Classical inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase do not work on *Plasmodium*, indicating that the mevalonate pathway is absent in this parasite. However, two enzymes that are central to a mevalonate-independent pathway have been described in *P. falciparum*: a 1-deoxy-D-xylulose-5-phosphate (DOXP) reductoisomerase (AF111813) (Jomaa *et al.*, 1999; Ridley, 1999; Vial, 2000) and the DOXP synthase (Soldati, 1999). Both enzymes are necessary for the synthesis of isopentenyl diphosphate in bacteria and plants. The DOXP reductoisomerase precursor of *Plasmodium* carries a putative bipartite apicoplast targeting sequence, and fusions of this presequence to GFP are successfully imported into apicoplasts (Jomaa *et al.*, 1999).

## 3. Iron-Sulfur Cluster Biogenesis

Iron-sulfur cluster biogenesis takes place both in the mitochondrion and in the apicoplast (Seeber, 2002). Since apicomplexan parasites like *Plasmodium* exhibit intimate contact between the apicoplast and mitochondrion (Hopkins *et al.*, 1999; Bannister *et al.*, 2000), an exchange of iron-sulfur clusters between these organelles might be possible. In addition, some nucleus-encoded proteins that can be directly involved in the apicoplast iron sulfur cluster biogenesis have been identified. Such is the case of the two plant-like redox enzymes ferredoxin-NADP(+)-reductase (FNR) and its redox partner ferredoxin, a small [2Fe-2S] protein. FNR has been identified in *T. gondii* and ferredoxin

in *P. falciparum* and *T. gondii* (Striepen *et al.*, 2000; Vollmer *et al.*, 2001). Homologous sequences have also been identified in other apicomplexan genome databases (Seeber, 2002). Furthermore, it was shown that FNR and ferredoxin of *T. gondii* interact productively favoring electron transfer from NADPH to yield reduced ferredoxin both *in vitro* and *in vivo* (Pandini *et al.*, 2002). *In vivo*, ferredoxin would then serve as a reductant for some specific metabolic reactions, including iron-sulfur cluster formation (Neuhaus and Emes, 2000). Other possible participants of iron-sulfur cluster formation, such as NFU (a putative scaffold for iron-sulfur cluster assembly) and NFS (a putative cysteine desulfurase) have also been identified as possible apicoplast-targeted proteins in the apicomplexan databases (Zuegge *et al.*, 2001). In addition, the lipoic acid synthase, an enzyme that requires an iron-sulfur cluster, is functional in *T. gondii* and is targeted to the apicoplast (Thomsen-Zieger *et al.*, 2003).

### *4. de novo Synthesis of Heme Groups*

In early studies on the apicoplast, it was thought that *de novo* synthesis of heme groups did not took place in this organelle, since it was considered a pathway active only in the mitochondrion (Surolia and Padmanaban, 1992). However, the delta-aminolevulinate dehydratase (ALAD), the second enzyme of the heme synthesis pathway of *P. falciparum*, has been overexpressed and characterized. The enzyme has been localized to the apicoplast of the malaria parasite as shown by immunoelectron microscopy (Dhanasekaran *et al.*, 2004). Additional immunofluorescence studies have shown that ferrochelatase, the terminal enzyme of the heme-biosynthetic pathway (the parasite encoded isoform), also localizes to the apicoplast in *P. falciparum* (Varadharajan *et al.*, 2004)

## *B. Inhibitor Drug Targeting and Control of Parasitic Diseases*

Malaria is undoubtedly one of the most severe of all parasitic diseases and one of the world's greatest health problems. Up to 500 million clinical cases worldwide have been estimated, and malaria represents the third greatest cause of death in children under age five. Chloroquine (Aralen) used to be the pharmacological drug of choice for all forms of malaria in the acute stage. Nevertheless, some strains of *P. falciparum* have become resistant to this inhibitor drug, representing a serious health problem. Current antimalarial strategies also involve prolonged treatments of combination therapies such as atovaquone/proguanil (Malarone) or arthemeter/lumefantrine (Riamet, Coartem) (Touze *et al.*, 2002; Dupouy-Camet, 2004). The development of new inhibitor drugs is (and must be) a continuing process. The three general strategies for development of antiparasitic drugs are: (i) to target enzymes that are unique to the parasite, (ii) to target enzymes present both in the parasite and the host, but which are indispensable only for the parasite, and (iii) to target enzymes with the same biochemical functions both in the parasite and in the host that exhibit differential pharmacological sensitivities. Since the apicoplast is an organelle unique to the parasitic apicomplexans, it is automatically identified as an ideal target for chemotherapeutic drugs (Roos, 1999; Soldati, 1999; Ralph *et al.*, 2001; Maréchal and Cesbron-Delauw, 2001). It is of both research and medical interest to study this specific inhibitor drug targeting; therefore, efforts in this area are currently being made in many laboratories.

Among the possible apicoplast metabolic pathways that may be target for inhibitor drug development are the apicoplast replication, transcription, and translation machinery, the plant-like fatty acid synthesis machinery, and the non-mevalonate isoprenoid biosynthesis pathway (Seeber, 2003).

### *1. Inhibitor Drugs that Target the Apicoplast Replication, Transcription and Translation Machineries*

The replication of the apicoplast genome in *Plasmodium* and *Toxoplasma* could be specifically inhibited using ciprofloxacin, a fluoroquinolone drug that targets a type II topoisomerase DNA gyrase (Fichera and Roos, 1997; Weissig *et al.*, 1997; Williamson *et al.*, 2002) and cleaves the *Plasmodium* plDNA (Weissig *et al.*, 1997). Also, clindamycin was shown to inhibit plastid replication (Fichera and Roos, 1997). These results allowed one to link apicoplast function with parasite survival, validating this organelle as an effective inhibitor drug target.

Several antibiotics that inhibit prokaryote translation have been found to target the translation machinery of the apicoplast (reviewed in McFadden and Roos, 1999). The large rRNA subunit is the putative target of thiazole-containing peptide antibiotics like thiostrepton and micrococcin. These antibiotics bind to a conserved GTPase domain of the eubacterial ribosomal large subunit (Ryan *et al.*, 1991; Lu and Draper, 1995). Thiostrepton, which appears to bind to the plastid 23S rRNA of *Plasmodium* but not to the one of

*Toxoplasma* (Clough *et al.*, 1997), can inhibit growth of *Plasmodium* both *in vitro* (Clough *et al.*, 1997; McConkey *et al.*, 1997) and *in vivo* (Sullivan *et al.*, 2000). Also, micrococcin was found to be a more potent inhibitor than thiostrepton of *Plasmodium* protein synthesis and growth (Rogers *et al.*, 1998). In addition, the translation elongation factor EF-Tu of *Plasmodium* (encoded by the plastid *tufA* gene) was found to be a target of unrelated antibiotics like kirromycin, enacyloxin IIa, and GE2270. These inhibitor drugs can also bind *in vitro* to recombinant over-expressed plastid EF-Tu of *P. falciparum* (Clough *et al.*, 1999).

*T. gondii* is sensitive to several inhibitors of prokaryotic translation (clindamycin, azithromycin, macrolides and tetracyclines) (Beckers *et al.*, 1995; Derouin, 2001). Direct genetic evidence was obtained supporting the idea that apicoplast translation is the target for clindamycin in *Toxoplasma*. Two independent *Toxoplasma* clones with strong and stable clindamycin resistance were selected. These mutants exhibited a G → U point mutation at position 1857 of the apicoplast large-subunit rRNA (Camps *et al.*, 2002).

Other antibiotics (macrolides, chloramphenicol and doxycycline) may also target the apicoplast ribosome. Several of them, including azithromycin, chloramphenicol, clindamycin and macrolide antibiotics exhibit a peculiar mode of action upon *T. gondii* tachyzoites, in which the inhibitor drug effects are observed only in the second infectious cycle. Intracellular tachyzoites treated with clindamycin replicate as untreated controls. Also, the inhibitor drug-treated parasites emerge from infected cells and invade new host cells as efficiently as untreated controls. Nevertheless, replication within the second host is dramatically slowed (as a function of the concentration and duration of the original antibiotic treatment) (Fichera *et al.*, 1995).

## 2. Inhibitor Drugs That Target the Apicoplast Fatty Acid Synthesis Machinery

Inhibitor drugs that target the fatty acid biosynthesis of the apicoplast may be promising for future therapeutic approaches. Thiolactomycin and triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, an antibacterial agent) have been reported to target the type II fatty acid biosynthesis pathway in *Plasmodium*. Triclosan (an inhibitor of enoyl-acyl-carrier-protein reductase, FabI) inhibits both *Plasmodium* ($IC_{50}$ of 0.7 μM) (Surolia and Surolia, 2001) and *Toxoplasma* growth (Surolia and Surolia, 2001; Beeson *et al.*, 2001; McLeod *et al.*, 2001). Also thiolactomycin (an inhibitor of α-ketoacyl-ACP synthase III) inhibits the growth of *P. falciparum in vitro*, with an $IC_{50}$ around 50 μM (Waller *et al.*, 1998). Humans, in contrast, utilize a type I fatty acid synthesis pathway, which is resistant to triclosan. Several analogues of thiolactomycin have been recently synthesized, some of which exhibit up to six fold-greater efficacy than the original inhibitor drug (Waller *et al.*, 2003a). These discoveries thus pave the way for novel approaches to the treatment of malaria (Surolia *et al.*, 2002). Two additional enzymatic activities, acetyl-CoA carboxylase (ACC) and enoyl-ACP reductase, respond to inhibitors previously identified for bacteria and plants, and show potential as targets for small-molecule inhibitors in a stand-alone or combination chemotherapy (Gornicki, 2003). Also, growth inhibition of *T. gondii* by aryloxyphenoxypropionate herbicides was correlated with the inhibition of the apicoplast ACC by these compounds (Zuther *et al.*, 1999; Jelenska *et al.*, 2002). In particular, the carboxyltransferase domain of ACC seems to be the target for these inhibitors (Zagnitko *et al.*, 2001). In contrast, the cytosolic *T. gondii* ACC is resistant to these herbicides. Both *T. gondii* apicoplast and cytosolic ACCs are resistant to cyclohexanediones, another class of drugs that inhibit chloroplast ACC (Jelenska *et al.*, 2002).

The structural basis of triclosan binding has been sorted out (Perozzo *et al.*, 2002). The *P. falciparum* enoyl acyl carrier protein reductase gene, encoding a putative apicoplast-targeted 50-kDa PfENR protein, was cloned and expressed. Recombinant PfENR was crystallized in three forms, as a complex with NADH, as a complex with triclosan and NAD(+), and as a complex with NADH and two triclosan analogs.

Since several enzymes participate in Type II FAS, there are also several alternative routes that may work in its metabolic pathway, allowing a rapid adaptation of the parasites to inhibitor drug pressure (Coombs and Müller, 2002). Chemotherapeutic drugs effectiveness could be rapidly surpassed by the high metabolic plasticity of apicomplexan parasites.

## 3. Inhibitor Drugs That Target the Non-Mevalonate Isoprenoid Biosynthesis Pathway

The non-mevalonate isoprenoid biosynthesis pathway is essential for *P. falciparum* survival, since DOXP-inhibitors like the antibiotic fosmidomycin and FR-900098 strongly inhibit cultures of this parasite (Jomaa *et al.*, 1999).

Other chemotherapeutic drugs like toltrazuril and ponazuril, of wide veterinary use as anticoccidial agents, seem to cause their initial damage within the

apicoplast and the tubular mitochondrion of *Neospora caninum* tachyzoites, thus destroying two of the most important apicomplexan cell organelles (Darius *et al.*, 2004).

### *4. Inhibitor Drugs That Target the Apicoplast but Whose Precise Site of Action Remains to be Ascertained*

The apicoplast is also thought to be a target for quinolones. The *in vitro* activity of 24 quinolones against *T. gondii* was assessed and their $IC_{50}$ determined in tissue culture (Gozalbes *et al.*, 2000). The quinolones most potent against *T. gondii* were trovafloxacin, grepafloxacin, gatifloxacin, and moxifloxacin.

One may ask how many of the above-mentioned inhibitor drugs will actually prove to be clinically useful. Many of the apicoplast enzyme inhibitors tested *in vitro* or in cell cultures remain to be proven pharmacologically safe and effective.

## VII. Evolutionary Origin of the Apicoplast

For certain protists, the nature of the eukaryote that was engulfed in the secondary endosymbiotic process seems to be pretty well established: a chlorophyte alga in the case of Chlorarachniophyta and Euglenophyta, and a rhodophyte alga in the case of Criptophyta, Heterokontophyta and Haptophyta (Fig. 5). In contrast, the nature of the unicellular alga that originated the apicoplast remains controversial. Apicoplast genes exhibit very high evolutionary rates, so they are difficult to localize precisely in phylogenetic analyses (Moreira and Philippe, 2001). Phylogenetic reconstruction analyses have placed the apicoplast gene sequences close to either chlorophyte or rhodophyte sequences, giving rise to the so-called "green *versus* red" debate in this field of literature (Fig. 5).

### *A. Evidence for a Chlorophyte Origin of the Apicoplast*

#### *1. Similarity of tufA Genes*

Based on phylogenies constructed with the *tufA* gene (which encodes the protein synthesis elongation factor Tu) a green algal ancestor was proposed because the apicomplexan plastids consistently affiliated with green algae (Köhler *et al.*, 1997). Nevertheless, single gene analyses are not free of long branch attraction

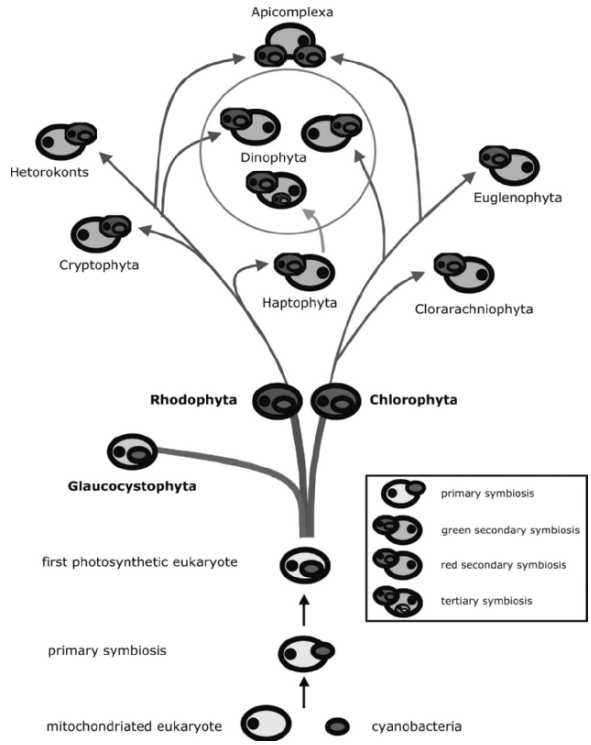


*Fig. 5.* (See also Color Plate 7, p. xl.) Phylogenetic origins of plastids with a possible origin of apicoplasts. See text for details.

artifacts, so analysis based on a larger set of a series of genes (concatenated genes) is usually preferred.

#### *2. The Presence of Acidocalcisomes*

Acidocalcisomes are subcellular organelles containing pyrophosphate and calcium that are present in Trypanosomatidae and Apicomplexa (Marchesini *et al.*, 2000; Diniz *et al.*, 2000; Docampo *et al.*, 2005). Acidocalcisomes contain a typical vacuolar proton-dependent pyrophosphatase (V-$H^+$PPase). *P. falciparum* contains two classes of plant-like vacuolar-type H(+)-pyrophosphatases (McIntosh *et al.*, 2001), while the green alga *Chlamydomonas reinhardtii* contains a V-$H^+$PPase similar to that of acidocalcisomes (Ruiz *et al.*, 2001). In phylogenetic analysis carried out with V-$H^+$PPase sequences, the apicomplexans, trypanosomatids, land plants, and *C. reinhardtii* clearly affiliate in a monophyletic clade (Hannaert *et al.*, 2003). It remains to be explored if acidocalcisome-like structures are widely spread and present in other protists and algae. In particular, it would be of interest to know if rhodophyte algae also contain a vacuolar pyrophosphatase, and if so, how does it affiliate in phylogenetic

analyses. At this moment, the presence of homologous genes encoding these enzymes in apicomplexans and chlorophyte algae indirectly supports a green ancestry for the parasites.

### 3. The Presence of Fragmented cox2a and cox2b Genes of Probable Chlorophyte Origin in Apicomplexan Parasites

In some chlorophyte alga like *C. reinhardtii, Polytomella* sp., *Scenedesmus obliquus* and *Volvox carterii,* subunit II of cytochrome *c* oxidase (COXII) is encoded by two separate nuclear genes (*cox2a* and *cox2b*). This is in contrast with what happens in the vast majority of eukaryotes, where COXII is encoded by a single mitochondrial gene. The algal *cox2a* gene encodes the COXIIA subunit, which comprises the N-terminal., membrane-embedded half of a regular COXII protein. The COXIIB polypeptide, encoded by the nucleus-localized *cox2b* gene, corresponds to the soluble C-terminal half of a conventional COXII protein (Pérez-Martínez *et al.*, 2001). Chlorophyte COXIIA and COXIIB subunits are imported into the mitochondrion and incorporated into cytochrome *c* oxidase. These fragmented algal subunits contain unique extensions (a C-terminal extension in COXIIA and a N-terminal extension in COXIIB) not present in orthodox COXII subunits. These extensions may participate in the assembly of the COXIIA and COXIIB polypeptide fragments, giving rise to a heterodimeric COXII subunit functionally equivalent to classical COXII subunits (Pérez-Martínez *et al.*, 2001). A complex sequence of events were required for the appearance of nucleus-encoded functional fragmented *cox2* genes in an ancestor of chlorophyte alga: i) an insertion in an ancestral mitochondrial *cox2* gene which did not disrupted COXII function, ii) cleavage of the mitochondrial *cox2* gene carrying the insertion into two functional fragments (*cox2a* and *cox2b*), iii) independent migration of the fragmented genes to the nucleus, iv) insertion and functional activation of the relocalized genes, and v) inactivation and elimination of the corresponding *cox2a* and *cox2b* mitochondrial copies. Fragmented *cox2a* and *cox2b* genes are also present in Apicomplexa, including *Plasmodium* (Funes *et al.*, 2002; Gardner *et al.*, 2002) and *Toxoplasma* (Funes *et al.*, 2002). The corresponding COXIIB subunits also contain N-terminal extensions that exhibit similarity to its chlorophycean counterparts. In particular, the motif PxxxPxxY, absent in all canonical COXII subunits, is conserved in the N-terminal extensions of COXIIB subunits of chlorophytes and apicomplexans. Therefore, it was proposed that a proto-apicomplexan host may have engulfed a green alga and retained its fragmented *cox2* genes, which eventually replaced the function of the host mitochondrial *cox2* gene. Since other green algae like *Ulvophyceae* and *Trebouxiophyceae* exhibit orthodox, mitochondrial, unfragmented *cox2* genes, the green algal endosymbiont of the proto-apicomplexan must have been a member of the class *Chlorophyceae.* An independent phylogenetic analysis of *cox2* sequences that included mitochondrial sequences from ciliates, suggested that the splitting, migration, and functional relocalization of the *cox2* gene might have happened independently in chlorophytes and apicomplexans (Waller *et al.*, 2003b). Nevertheless, two other independent analyses, based on different databases, failed to show affiliation of ciliate *cox2* sequences with apicomplexans and chlorophyte alga, and are clearly in discordance with the possibility of independent splitting of *cox2* genes in different lineages (Funes *et al.*, 2003; Hackett *et al.*, 2004). Out of the more than 1382 sequences of COXII subunit sequences found in the databases, only three of them (0.21%) present insertions, and only the chlorophyte and apicomplexan ones appear to be fragmented. Most likely, the singular series of events that led to functional *cox2* gene splitting occurred only once in evolution, and the apicomplexan *cox2a* and *cox2b* genes may be considered of chlorophyte origin (Funes *et al.*, 2003).

### 4. Phylogenetic Analyses Have Related the Apicoplast Genome With Euglenoids

Several workers have proposed a relationship of apicomplexan parasites with euglenoids, and therefore, indirectly with a green algal origin. Apicomplexans and euglenoids appeared as sister groups in phylogenetic reconstructions based on plastid small subunit ribosomal RNAs (Egea and Lang-Unnasch, 1995, 1996), *rpo*B genes (Gardner *et al.*, 1994), and *rpo*C genes (Howe, 1992). All these works seemed to be limited by the extreme AT bias of the apicoplast sequences and by the limited number of sequences included. More recently however, the complete sequence of the apicoplast genome of the coccidian *Eimeria tenella* (Cai *et al.*, 2003) has allowed the reexamination of apicoplast evolution by phylogenetic reconstructions using a larger database. Maximum likelihood (ML) and Bayesian inference (BI) methods have been used to analyze a concatenated data set of sequences of the apicoplast-encoded rpo proteins (rpoB, rpoC1 and rpoC2 proteins). These proteins are large in size, and provide

a large number (1214) of amino acid alignable positions. Phylogenetic analyses using both ML and BI approaches yielded similar tree topologies, all of which placed apicomplexans close to *Euglena* (Cai *et al.*, 2003). These results led the authors to conclude that if the apicoplasts did originated from an endosymbiotic event with a red alga (see below), then the proto-apicomplexan host might have already contained some primary green plastid genes.

## B. Evidence for a Rhodophyte Origin of the Apicoplast

### 1. Phylogenetic Analyses of Ribosomal RNA Genes Have Suggested Support for a Red Algal Affinity

A rhodophyte origin for Apicomplexa was proposed based on a phylogenetic reconstruction made with the genes encoding the plastid 16S and 23S rRNAs (Zhang *et al.*, 2000). As stated above, reconstructions based on a single gene are taken with care, and analysis of a larger set of genes or of gene clusters is preferred.

### 2. The Phylogenetic Relationship Between Dinoflagellates and Apicomplexans

Dinoflagellates are a very diverse group of organisms, several of which are photosynthetic. Their plastids are thought to have originated by the secondary endosymbiosis of a red alga. Some species may have acquired their plastids by a tertiary endosymbiotic event from a haptophyte-like ancestor (Zhang *et al.*, 2000) (Fig. 5). Due to the relatively close phylogenetic relationship between dinoflagellates and apicomplexans, a common rhodophyte origin of all alveolates was proposed (Cavalier-Smith and Beaton, 1999). The more recent chromalveolate hypothesis proposes a rhodophyte ancestry for the plastids of all chromists (heterokont algae, cryptophytes and haptophytes) and alveolates, and assumes that a unique endosymbiotic event gave rise to this supergroup (Cavalier-Smith, 2003). Apicomplexan and dinoflagellate plastid gene sequences are divergent and difficult to compare. In addition, the dinoflagellate genes reside on single gene mini-circles that seem to mainly encode photosynthetic components (Cavalier-Smith and Beaton, 1999; Zhang *et al.*, 1999), and therefore its ribosomal components have not been subjected to phylogenetic analysis. The phylogenies of dinoflagellates based on nucleus-encoded SSU-rRNA genes suggest multiple plastid losses and replacements in this lineage (Saldarriaga *et al.*, 2001). Therefore, Dynophyta may have originated both from red and green lineages (Morden and Sherwood, 2002). It is therefore not improbable that dinoflagellate and apicomplexan lineages may have also originated by independent secondary endosymbiotic events.

### 3. Gene-Cluster Analysis of the Apicoplast Genome Suggests a Close Relationship With Red Algal Plastid Genomes

The cluster-organization of plastid genes encoding ribosomal proteins - the so-called plastid super-operon, a concept first suggested by (Ohta *et al.*, 1997) - has been extensively analyzed (Williamson *et al.*, 1994; McFadden and Waller, 1997; Stoebe and Kowallik, 1999). In particular, the *S*12/*S*10 operon of cyanobacterial origin, containing the genes *rps12*, *rps7*, *fus*, *tufA* and *rps10*, is usually located in the 5′ end of the super-operon in the green algal lineage, while in red algae, this cluster is appended to the 3′ end. The highly reduced apicoplast genomes of several species also exhibit the rearrangement of the *S*12/*S*10 operon in the 3′ end. This has lead to the suggestion that, irrespective of the massive gene reduction observed in the apicomplexan plastid genome, the cluster-organization of genes indicates its rhodophytic ancestry. Blanchard and Hicks (1999) made a comprehensive study of this plastid super-operon involving overall genomic structure, gene content, primary sequence analysis, and also considering the presence or absence of introns. These phylogenetic analyses ambivalently placed the genus *Plasmodium* either close to *Euglena* (a protist of reliable green algal ancestry) or to *Odontella* (a species of well-grounded red algal ancestry). Nonetheless, the authors concluded that apicoplasts do not belong to the green plastid lineage. Other factors that led to this conclusion were the presence of the *clpC* and *ycf24* genes in the plDNA, which are only present in the red lineage and consistently absent in the green one. Also, the presence in the apicomplexan plastid of the ribosomal protein genes *rpl3*, *rpl6*, *rps5*, and *rps17*—a set of genes absent in all green-lineage chloroplast genomes—reinforced their interpretation. Nevertheless, even in phylogenetically related species, important changes of gene order in the ribosomal protein encoding super-operon happen with high frequency. Large rearrangements of individual genes and of gene clusters can be observed when comparing plastid genome sequences of closely related species. For example, the plastid sequences of three relatively close-related green algal species, *Chlorella vulgaris*

[NC_001865], *Nephroselmis olivacea* [NC_000927], and *C. reinhardtii* (Maul *et al.*, 2002), show numerous events of inversion of short dispersed repeats, gene loss, and changes in gene order (Maul *et al.*, 2002).

Gene cluster analysis presupposes that reduction of the original photosynthetic plastid genome involved the elimination of the genes encoding the genes for photophosphorylation, keeping the organization of the remaining genes (mainly those encoding ribosomal components) relatively intact. For example, the non-photosynthetic parasitic plant *Epifagus virginiana* (GenBank NC–001568) has a 70 kb plastid genome that maintains a gene cluster organization—except for the lost photosynthetic genes—almost identical to the 156 kb tobacco chloroplast genome (GenBank NC_001879) (Wolfe *et al.*, 1992). Nevertheless, analysis of other reduced plastid genomes show that a specific deletion of photosynthetic genes does not necessarily leave behind the same arrangement of the remaining genes. Comparison of the 73 kb plastid sequence of the colorless flagellate *Astasia longa* (GenBank NC_002652) to the one of its photosynthetic counterpart *E. gracilis* (143 kb, GenBank NC_001603) shows that numerous inversion events have accompanied the loss of photosynthetic genes (Gockel and Hachtel, 2000). Also, comparison of the reduced plastid genome of the colorless unicellular alga *Prototheca wickerhamii* (54 kb), closely related to the one of the green alga *C. vulgaris* (150.6 kb, accession NC_001865) shows that a complicated series of gene rearrangements have occurred (Knauf and Hachtel, 2002). Thus, the present day plastid DNAs of several colorless protists are not only the result of highly specific deletions (loss of photosynthetic genes), but also of multiple rearrangements of the remaining individual genes. One should consider the possibility that similar rearrangements (deletions and inversions) may have occurred during the reduction of the original endosymbiont plastid genome that eventually gave rise to the apicoplast genome. These massive rearrangements may have constructed a new gene-cluster organization that by chance, resembles that of a red algal plastid. Also, the genome reduction process during the symbiogenesis of the enslaved alga that gave rise to the apicoplast may have been unique to the Apicomplexan lineage. The apicoplast genome gene clustering may have been further rearranged by acquisition of mitochondrial genes by lateral gene transfer (Obornik *et al.*, 2002b).

The organization of the ribosomal protein superoperon is the strongest evidence supporting the rhodophyte origin of apicoplasts. The similar organization of genes in the apicoplast genomes of several apicomplexans is in accordance with a process in which an ancient rhodophyte plastid genome was strongly reduced, losing all its photosynthetic-related genes but keeping its few remaining genes in their original organization. Nevertheless, as stated above, it is possible that gene rearrangements have masked the evolutionary story of the apicoplast genome compaction.

## *4. Comparison of Apicomplexan and Dinoflagellate Plastid-Targeted Proteins Like GAPDH*

Plants, green algae, red algae, and euglenoids contain two glyceraldehyde-3-phosphate dehydrogenases (GAPDH) enzymes, a cytosol-localized, NADH-dependent GAPDH (catabolic) enzyme of eukaryotic origin, and a plastid NADH- or NADPH-dependent GAPDH enzyme (anabolic) of probable cyanobacterial origin (Henze *et al.*, 1995). In contrast, in apicomplexa and dinoflagellates, both GAPDH enzymes are closely related and appear to be of eukaryotic origin. It was therefore proposed that the nucleus-localized gene encoding the cytosolic GAPDH duplicated in a common ancestor of apicomplexans and dinoflagellates (Fast *et al.*, 2001). This duplication event was followed by the acquisition of a nucleotide sequence encoding a targeting sequence in one of the copies that allowed import of its corresponding GAPDH into the apicoplast. Eventually, the nucleus-localized gene took over the function of the existing cyanobacterium-derived plastid gene. This was taken as evidence that plastids found in these two lineages originated by a single secondary endosymbiosis in a common ancestor. Recently, new GAPDH sequences from chromalveolates were obtained and included in a phylogenetic analysis. Intriguingly, the plastid-targeted GAPDH of *T. gondii* appeared more strongly related with the one from haptophytes (marine algae), than the one from dinoflagellates. Haptophytes and *T. gondii* constitute a monophyletic clade sister of all other chromalveolates (Harper and Keeling, 2003). These data and the absence of putative plastid-targeted GAPDH in *Plasmodium* raise the possibility of independent acquisition of plastid-targeted GAPDH in some apicomplexans like *T. gondii,* and losses in some other lineages like *Plasmodium* (Harper and Keeling, 2003). Therefore, the possibility of independent origins remains open, since analysis of GAPDH sequences in other dinoflagellates suggests that the acquisition of its corresponding genes may have occurred more than once and by different mechanisms (Fagan and Hastings, 2002).

### C. Evidence for a Combined Chlorophyte and Rhodophyte Origin of the Apicoplast

A data set of 6,480 unique expressed sequence tags were generated from the dinoflagellate *Alexandrium tamarense*, a taxon that is sister to apicomplexans. Forty-eight nucleus-localized genes of *A. tamarense* were identified as photosynthetic genes whose protein products functionally pertain to the plastid. Phylogenetic analysis indicated that these genes have both red and green algal origins (Hackett *et al.*, 2004). The presence of genes of green algal origin was interpreted to have originated by multiple gene transfers from different green algae, or by at least two processes of secondary endosymbiosis: that of a green alga followed by a red one. Although it remains to distinguish between these two possibilities, it is evident that a mosaic of genes from red and green algal origins is present in this dinoflagellate.

### D. The Red vs. Green Debate on the Origin of Apicoplasts is Alive, but May be Resolvable

Phylogenetic trees reconstructed with different molecular markers have related apicomplexans with green algae (Köhler *et al.*, 1997), red algae (Williamson *et al.*, 1994; McFadden and Waller, 1997; Stoebe and Kowallik, 1999), land plants (Dzierszinski *et al.*, 1999; Huang *et al.*, 2004), euglenoids (Blanchard and Hicks, 1999; Cai *et al.*, 2003), cyanobacteria (Huang *et al.*, 2004) and even eubacteria (Huang *et al.*, 2004). Both the highly diverged sequences of apicomplexan protists and the still limited size of the sequence database may contribute to this large variety of phylogenetic affiliations. Still, the available evidence points towards a unicellular alga as the precursor of apicoplasts. If apicoplasts are indeed of rhodophyte origin, it means that phylogenetic reconstructions based on a single gene, such as the analysis of *tufA* genes is misleading. Also, it may suggest that functional fragmentation and migration of mitochondrial *cox2* genes occurred independently at least twice in two different and unrelated eukaryotic lineages (chlorophytes and apicomplexans). Alternatively, chlorophyte-like split *cox2* genes may have been acquired by a lateral gene transfers event. A rhodophyte origin of apicomplexan parasites also predicts that red algae will contain acidocalcisome-related structures and a V-H+PPase homolog. If on the contrary, apicoplasts are indeed of chlorophyte origin, the so-called plastid super-operon may not be as conserved as previously thought. A green alga containing a plastid genome with multiple rearrangements may have been the original endosymbiont. Its plastid genome, modified after a process of multiple inversions and strong reduction, may nowadays resemble that of a red algal plastid genome (deceptively so). The presence of red and green genes in dinoflagellates may also explain why apicomplexans exhibit both rhodophyte and chlorophyte characteristics. Although the establishment of mitochondria and chloroplasts by endosymbiosis followed by symbiogenesis perhaps occurred once in evolution, secondary endosymbiosis may have more opportunities for success, and it may have been a more common phenomenon. In the case of apicomplexans a double endosymbiotic event, the engulfment of a green alga followed by the engulfment of a red alga by a highly phagocytic ancestor, may have occurred. The highly organized endocytic compartments of extant apicomplexans like *T. gondii* and *Plasmodium* spp. suggests a past and present avid phagotrophic nature for these organisms (Robibaro *et al.*, 2001). This wide-spread protist may have devoured and retained the genes of other algae (both chlorophytes and rhodophytes): simply following the "you are what you eat hypothesis" (Doolittle, 1998). It is not hard to imagine an insatiable predator (a protist version of a *Tyrannosaurius rex*) that may have engulfed (and retained) sequentially both green and red algal species.

Although claims have been made that the "red-versus-green" debate was settled (Fast *et al.*, 2001), clearly there is still conflicting evidence for the apicoplast ancestry (Gleeson, 2000; Palmer, 2003; Waller *et al.*, 2003b; Funes *et al.*, 2003). We conclude that the red-green debate is actual and legitimate: more phylogenetic analyses, more experimentation, and a wider exploration of extant protist plastid and nuclear genomes (specially of Rhodophytes) will be required to settle the issue. In the meanwhile, neither a red nor a green origin should keep permeating the apicoplast literature as a dogma.

## VIII. Future Studies and Prospects for Disease Control

Although identified on the basis of morphology many years ago, the apicoplast is a relative recently described organelle, with much of what we know learned during the 1990's. Therefore, its discovery and characterization lags several decades behind as compared to work done in other better-characterized organelles like mitochondria and chloroplasts. As in any relatively

young field, the work done on apicoplasts has been fragmentary, and only some aspects have been fully developed. Glimpses of some of the main metabolic routes have been obtained, and a partial characterization of targeting and protein import has been developed. The fact that the plastid seems to be indispensable for parasite survival, identifies it as an ideal target for future therapeutic purposes. Two major approaches will surely bring further development to the field: first, the exhaustive analysis of the recently completed genomes of *P. falciparum* (Gardner *et al.*, 2002) and *P. yoelii* (Carlton *et al.*, 2002), next, the development of biochemical techniques that may circumvent current technical problems like the low yield of parasite cultures and the isolation of pure apicoplast fractions. Preliminary analysis of the *P. falciparum* genome has already provided remarkable peculiarities of the apicomplexan biology: the presence of numerous inserts within the globular domains of proteins, a unique transcription apparatus, a relative high-degree of mutability in the DNA repair system, and the presence of "animal-like" adhesion modules in its cell-surface molecules (Aravind *et al.*, 2003). Phylogenetic analyses of the 500 to 600 nuclear genes whose protein products are predicted to localize to the apicoplast suggest a relationship of *Plasmodium* to plants (Gardner *et al.*, 2002) and to cyanobacteria (Aravind *et al.*, 2003). The characterization of an apicoplast proteome, and the confirmation that all predicted apicoplast-targeted proteins (Ralph *et al.*, 2004a) are indeed apicoplast components, will allow the completion of our currently fragmented image of the structure and function of this organelle. There is no doubt that more biochemical studies of the apicoplast are required, therefore, efficient techniques to allow the isolation of the organelle in reasonable quantities are needed for the direct study of its proteome and its subsequent metabolic characterization. Of special interest for future studies, are the 381 proteins that contain apicoplast-targeting sequences, and are therefore predicted to be apicoplast-localized, whose function remains a mystery.

It is likely that some of the inhibitor drugs that target the apicoplast will be useful in clinical applications. However, extensive clinical use will prompt the development of resistance in parasites (Coombs and Müller, 2002). Medicine and Veterinary will keep considering apicomplexan parasites as a scourge to humanity and the apicoplast as a chemotherapeutic drug target par excellence. So it should be. Nevertheless, from a broader biological point of view, apicomplexans may be visualized as organisms that contain a remarkable evolutionary gene mosaic and that are exquisitely adapted to their hosts. The apicoplast is the extant witness of still obscure endosymbiotic processes that occurred long ago. A full confession from this witness yet remains to be obtained.

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# Chapter 25

# The Role of Plastids in Gravitropism

Maria Palmieri and John Z. Kiss*
*Botany Department, Miami University, Oxford, OH 45056, U.S.A.*

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## Summary

In contrast to most animals, plants are largely sessile, so these organisms have had to develop survival strategies that differ from those of most animals. Whereas many animals migrate in search of favorable environmental conditions (i.e. water, food supply, climate, low predation), plants can adjust to their environment through directed growth (i.e. tropisms). For instance, plant roots develop in soil to optimize anchorage and absorption of water and nutrients. Shoots develop above the ground, and their morphology is tailored to suit lighting needs and optimize placement in wind currents. Thus, plants direct their growth in response to local environmental conditions.

*Author for correspondence, email: kissjz@muohio.edu

*Robert R. Wise and J. Kenneth Hoober (eds.), The Structure and Function of Plastids,* 507–525.

Before plants can exhibit growth relative to an environmental cue, they must first sense such a stimulus. For instance, gravity represents a constant and ubiquitous acceleration vector that plants can sense in order to direct the growth of their various organs. Gravity sensing occurs in specialized cells termed statocytes, which include the columella cells of the root cap and the endodermis (or starch sheath) of stem-like organs (e.g. coleoptiles, pulvini, rhizomes, shoots, stems and reproductive stalks). These statocytes possess sedimentable organelles (amyloplasts) that contain dense starch grains. Changes in the direction of the gravity vector are sensed when these amyloplasts settle towards gravity, and the plant then directs the growth of its organs accordingly. This process is known as gravitropism.

This chapter describes the evidence that points to amyloplasts as the gravity-sensing organelles in plants. We present the various models and corresponding evidence regarding how the mechanical stimulus of amyloplast sedimentation leads to plant gravity perception. In addition, an overview is provided of some of the types of research tools that are used and what contributions they have made to the field of gravitropism. Lastly, future research directions are discussed.

Briefly, amyloplasts are putative gravisensing organelles in higher plants because their presence in statocytes is required for the gravity response in all graviresponding plant organs. This assertion is confirmed by experiments utilizing (1) optical tweezers, (2) laser ablation of statocytes, (3) mutants which lack amyloplast starch and/or amyloplasts and/or statocytes, (4) surgical removal of statocytes and (5) amyloplast displacement using high gradient magnetic fields. These studies demonstrate that not only must amyloplasts be present within statocytes, but also they must settle in response to gravity in order for the corresponding plant organ to possess a full gravitropic response.

Several models have been developed to explain how the mechanical stimulus of amyloplast sedimentation leads to plant gravity perception. Studies utilizing pharmacological depolymerization of the F-actin cytoskeleton demonstrate enhanced gravitropic curvature of plant roots and shoots in the absence of an intact F-actin cytoskeleton. This suggests that the actin cytoskeleton plays an important role in gravity perception and/or gravity transduction. One model—the tensegrity model—suggests that the cytoskeleton forms a mesh through which the amyloplasts passively fall, thereby creating forces of slack and tension in the actin filaments. It is hypothesized that the actin filaments are connected to stretch receptors in the plasma membrane, and that the collective forces of slack and tension induced by the amyloplasts are transferred to these stretch receptors via the actin filaments.

Another model, the actin-tether model, proposes that amyloplast-induced forces of slack and tension are transferred to stretch-receptors in a fashion similar to that described above, except that discrete connections are proposed to exist between the actin filaments and the amyloplasts. Although these are the two dominant hypotheses proposed to explain the role of the actin cytoskeleton in signal perception/transduction models of root gravitropism, to date, no discrete connections have been found, either between F-actin filaments and amyloplasts or between F-actin filaments and membrane-bound stretch receptors. Finally, a new model emerges highlighting the vacuole as a key player in stem gravitropism.

Many recent technological innovations promise exciting new possibilities for the study of gravitropism. Through the use of clinostats, centrifuges and space flight, it has become possible to alter the gravitational field to which plants are exposed. Experimentation under these conditions provides valuable insight into mechanisms of gravity perception. Computer-based feedback systems have allowed us to study gravitropic curvature while maintaining constant gravity stimulation at a given locus on a plant organ. Finally, microarrays and gene profiling reveal gravity-induced alterations in gene expression at the genome level.

*Abbreviations:* AtsPLA$_2$β – *Arabidopsis thaliana* secretory low molecular weight phospholipase A$_2$ enzyme; Adp – ADP-glucose pyrophosphorylase; ARG1, AGR2 – altered response to gravity; ARL2 – ARG1-LIKE2; GFP – green fluorescent protein; grv2 –gravitropism defective 2; GSA – gravitropic set-point angle; GUS – β – glucuronidase; HGMF – high gradient magnetic field; HSP70 – heat shock protein 70; Lat-B – latrunculin; MF –microfilament; PA phospholipase – A$_1$ phosphatidic acid preferring phospholipase A$_1$ enzyme; Pgm – phospoglucomutase; RPM – random positioning machine; SCR – ScaRecrow; Sgr – shoot gravitropism; SNARE – soluble *N*-ethyl-maleimide-sensitive-factor attachment-factor receptor; t – SNARE – target membrane – SNARE; v – SNARE – donor vesicle – SNARE.

## I. Introduction

This chapter considers the role of starch-containing plastids, known as amyloplasts, in plant gravitropism. We first present a broad overview of gravitropism, followed by an in-depth review of the role of amyloplasts in gravitropism. The review is primarily limited to a discussion of starch-containing plastids, although there are other types of structures that may function in gravity sensing. For instance, vesicles containing barium sulfate function as statoliths in some lower

plant taxa in which all three phases of gravitropism occur within the same cell (for review, see Sievers *et al.*, 1996). *Chara* provides an example of this phenomenon, as the rhizoids and protonemata of this alga possess vesicles that contain barium sulfate and function as statoliths (Wang-Cahill and Kiss, 1995; Kiss, 1997).

Next, the chapter provides a brief historical perspective of the field, followed by a presentation of the evidence in favor of amyloplasts as gravisensors, a discussion of current research on the role of plastids in gravitropism, a consideration of the types of tools used to study gravitropism, and, lastly, a look to future research that needs to be performed in order to solve unanswered questions. We intend that this chapter will convey to the reader an accurate sense of gravitropism as an exciting research field with a history that stretches back for centuries, and we hope that it showcases the recent breakthroughs in the field, as well as exciting possibilities for the future.

### *A. Sensing of the Environment by Plants*

All living organisms must sense and respond to their environment. This includes abiotic factors such as light, gravity and temperature, as well as biotic factors such as predation and competition. Different organisms have developed various means of sensing and responding to environmental stimuli. In contrast to most animals, plants are largely sessile. As such, plants have had to develop survival strategies different from those of animals. In general, plants respond to their environment by exercising control over their physiology, morphology and growth. The particular response employed by the plant depends upon the stimulus and needed outcome. For example, plant roots can reach deep into the earth for anchorage, water and nutrients, while the morphology of the above ground portions of the plant can be tailored to suit lighting needs and optimize placement in wind currents. Thus, there are many ways in which plants exhibit directed growth in response to their environment.

### *B. Plant Tropisms*

The directed growth in response to an external stimulus is known as a tropism. Numerous tropisms have been identified in plants, including: hydrotropism (water; see Takahashi, 1997), phototropism (light; see Hangarter, 1997; Correll and Kiss, 2002), gravitropism (gravity; see Kiss, 2000), thigmotropism (touch; see Fasano *et al.*, 2002; Jaffe *et al.*, 2002) and electrotropism (electricity; see Wolverton *et al.*, 2000). Unlike animals, which can move to avoid certain stimuli or to find more favorable environmental conditions, most plants remain rooted in place for the duration of their lifespan. They grow towards favorable conditions and away from unfavorable conditions by actively determining the direction of their growth in response to factors such as those listed above. The remainder of this chapter will focus on plant gravitropism, and specifically upon how plants perceive, maintain and change the orientation of their organs with respect to gravity. The sequence of events that takes place when and after a change in gravity vector is sensed also is considered.

## II. Gravitropism

Gravitropism is the directed growth of plant organs in response to gravity. In general, stems grow upwards and roots grow downwards. However, plant organs often maintain a specific orientation in the gravity field that is not directly parallel to the gravity vector. Thus, the *gravitropic set-point angle* (GSA) is used to define the angle at which a plant organ is oriented with respect to gravity (Digby and Firn, 1995; Mullen and Hangarter, 2003). The GSA varies from species to species, with respect to organ, and can also be modified by the plant in response to environmental cues such as light. Because gravity affects plants in such complex ways, it is not difficult to imagine that plants have developed sophisticated mechanisms to sense and respond to this ubiquitous stimulus.

Although gravity acts equally upon every plant organ, not every cell necessarily contains gravisensing capabilities. Plants in the higher taxonomic divisions perceive gravity within specialized cells termed *statocytes* (Sack, 1991; Kiss, 2000). Statocytes vary in location and ultrastructure, depending upon the specific organ. Gravity perception in roots takes place in the columella cells, which are located in the central region of the root cap (Fig. 1a), whereas shoots sense gravity in the endodermis, which is a single layer of cells surrounding the vascular tissues (Fig. 1b).

The available evidence overwhelmingly supports the hypothesis that gravity sensing occurs within plant statocytes by the sedimentation of dense, starch-filled plastids (amyloplasts) contained within statocytes (or in some lower taxa, through the sedimentation of vesicles filled with barium sulfate). Thus, according to Sack (1997): “In short, there is no wild-type gravitropic

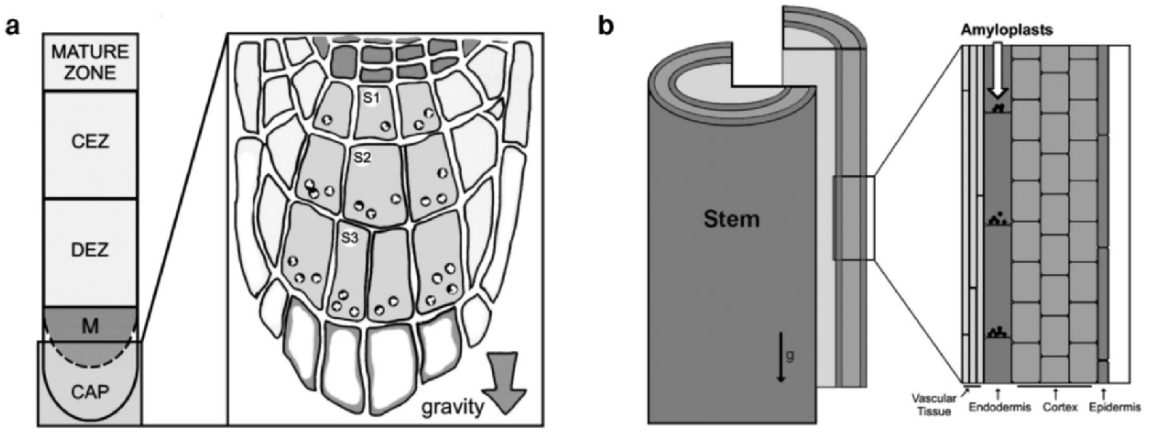


*Fig. 1.* (See also Color Plate 7, p. xl.) Diagram of a generalized segment of a plant root (a) and stem (b) with an enlarged view of a cross-section from each. (a) A root consists of several growth zones. The meristematic region (M) produces undifferentiated cells that first elongate and then become part of the distal elongation zone (DEZ). As more cells arise from the meristematic region the elongating cells in the DEZ become progressively farther from the meristematic region, and enter the central elongation zone (CEZ). Subsequently, the cells continue to elongate and progress away from the meristem until they enter the maturation zone (mature zone), where they differentiate. Within the root cap, some cells develop into columella cells, and these are the gravity-sensing cells of the root. In *Arabidopsis*, the columella cells comprise three layers termed story one, story two and story three (S1, S2 and S3, respectively). The columella cells contain amyloplasts (white on black circles) which sediment in the direction of the gravity vector (arrow). (b) A stem consists of (from the outside in) an epidermal layer (epidermis), a cortical zone (cortex), an endodermal layer or layers (endodermis) and vascular tissue. The endodermal cells contain membrane-bound organelles filled with starch grains (amyloplasts, open arrow) that sediment in response to gravity (g, black arrow). Fig. 1a is redrawn from Swatzell and Kiss (2000).

[plant] cell or [plant] organ known which lacks amyloplast (or barium sulfate vesicle) sedimentation". Nonetheless, other gravity sensors may exist in addition to amyloplasts, and there are several candidates for such intracellular gravity-sensing structures—including the nucleus (Kordyum and Guikema, 2001) and the entire protoplast itself (Wayne *et al.*, 1990). The hypotheses regarding these structures and plant gravity perception have evolved over time, and a brief overview of the hypotheses along with the evidence for each is discussed below, followed by an in-depth discussion of amyloplasts and their role in gravitropism.

## A. Historical Perspective

Two centuries ago, Thomas Andrew Knight (1806) reported that plants orient their organs in response to gravity. Knight constructed a rotating wheel with which germinating seeds could be exposed to different gravitational vectors and forces. By controlling both the orientation and the rotational velocity of the device, he was able to produce centrifugal forces of varying strengths that were capable of unidirectionally counteracting and/or opposing the force due to gravity. He then affixed seeds to the wheel and analyzed the direction of growth of the radicles and hypocotyls once germination occurred. Knight discovered that the seedlings responded to the centrifugal force in the same manner that they responded to the force produced by gravitational acceleration. Thus, he concluded that roots and shoots direct their growth with respect to gravity.

Later in the century, Charles and Francis Darwin (Darwin and Darwin, 1881) were among the first to propose that gravity sensing in plant roots takes place in the root cap. They performed studies on three legumes (*Vicia faba*, *Pisum sativum* and *Phaseolus multiflorus*), as well as cotton (*Gossypium herbaceum*), corn (*Zea mays*) and a gourd (*Cucurbita ovifera*). The Darwins found that gravitropic curvature was eliminated by excision of the tips of the radicles, but curvature was restored once the tips regenerated. Furthermore, curvature was not abolished if excision was delayed until after the plants had been gravistimulated for an hour or two (Darwin and Darwin, 1881). Thus, they proposed that gravitropic sensing occurs specifically in the root tip and that the information is subsequently transmitted to the region of the root where gravitropic curvature occurs.

About the same time that the root tip was determined to contribute to gravitropism, amyloplasts

were implicated as part of the gravity-sensing apparatus. Gottlieb Haberlandt (1900) and Bohumil Němec (1900) supported the statolith (dense, movable object) theory. The statolith theory suggested that plant gravity sensing occurs when starch grains trigger graviperception by settling in response to gravity; thus, the starch grains function as statoliths (i.e. dense, moveable particles). This theory was supported by observations that all gravitropic plant organs studied seemed always to contain cells possessing sedimentable starch grains. For example, these statolith-containing cells, termed statocytes, were observed in the columella cells of root tips as well as in the endodermis (starch sheath) of all stem-like organs and aerial roots. Thus, these pioneers in the field of gravitropism recognized a correlation between statolith sedimentation and gravitropic competence; however, the exact mechanism of statolith action and the subsequent sequence of events leading to gravitropic curvature remained unknown.

## B. Phases of Gravitropism

### 1. Perception

Gravitropism can be divided into three stages: perception, transduction and response (Sack, 1991; Salisbury, 1993). The perception phase occurs in statocytes. However, there is evidence that root gravity perception can also occur *outside* of the putative root statocytes (columella cells), but the mechanism for this type of perception is unknown (Wolverton *et al.*, 2002a). For statocytes, the perception phase is characterized by gravity exerting an accelerational force on a susceptor present within the statocytes. This effects a change in the potential energy of the susceptor such that the new orientation of the plant organ with respect to gravity is perceived. There are several candidates for the gravity susceptor, including organelles such as the nucleus and/or amyloplasts, and also the entire protoplast itself.

A long-standing debate surrounds two competing hypotheses regarding the perception phase of gravitropism. The gravitational pressure model, or, more commonly, the protoplast pressure model (Wayne *et al.*, 1990) asserts that gravity is sensed when the mass of the entire protoplast is acted upon by gravity, causing forces of compression along the lower portion of the cell and tension along the upper portion of the cell between the plasma membrane and the cell wall. According to this model, these forces of slack and tension relate the gravity signal via stretch receptors that are proposed to be located in the plasma membrane. In contrast, the starch-statolith model (Sack, 1997) postulates that the plant gravisensing apparatus consists of dense organelles which sediment inside of the statocytes upon reorientation of the plant (or organ or cell) with respect to gravity (Sack, 1991). The data that support the protoplast pressure model are not necessarily inconsistent with the starch statolith model, and both mechanisms might act in the perception of gravity (MacCleery and Kiss, 1999; Kiss, 2000).

### 2. Signal Transduction

Signal transduction is the second phase of gravitropism. It occurs when dissipation of the potential energy of gravistimulated amyloplasts results in the production of chemical signals that ultimately trigger a growth response. Many intracellular species have been implicated in gravity signal transduction including the vacuole, endoplasmic reticulum, cytoskeleton and various ions. Exactly how signal transduction occurs is unknown because the pathway has not been completely characterized. However, it seems that the perception and signal transduction phases overlap temporally, and that these two phases may also possess some common intracellular components (e.g. the cytoskeleton). There are two models concerning the role of the cytoskeleton in mechanisms of gravitropism: the actin-tether model (Baluška and Hasenstein, 1997) and the tensegrity model (Yoder *et al.*, 2001). Each of these models envisions interactions between amyloplasts and the F-actin cytoskeleton; however, the former model imagines discrete connections between the amyloplasts and the microfilaments, whereas the latter conceives the F-actin cytoskeleton as a restraining mesh through which the amyloplasts must fall. There also is evidence suggesting a role for the vacuole in gravity signal transduction, which gives rise to a third model that is applicable to stem-like organs (Morita *et al.*, 2002; Kato *et al.*, 2002b).

### 3. Response

The response phase of gravitropism is characterized by directed growth in response to gravity. The growth response is elicited by auxin concentration gradients that form across gravistimulated organs such that more of this hormone is present in the lower portion, as compared to the upper portion of the organs. This theory of auxin-mediated differential growth is known as the Cholodny—Went theory (reviewed in Trewavas,

1992). The auxin redistribution is preceded by $Ca^{2+}$ and $H^+$ fluxes, with enhanced $Ca^{2+}$ concentrations present along the lower portions of gravistimulated roots and stems. Auxin redistribution is accomplished when auxin efflux carriers are relocated from the former *bottom* to the new *lower side* of the statocytes, and auxin is therefore transported laterally to the new lower side of the organ (Ottenschläger *et al.*, 2003). In roots, the increased auxin concentration enhances growth on the upper side of the organ, so that the root grows in a downward direction. In stem-like organs, the enhanced auxin concentration stimulates growth on the lower side, resulting in upward growth of the organ. Why increases in auxin concentration on the lower side of both roots and stems cause such different responses in these organs is unknown.

### C. Gravity Perception in Plants

There are a myriad of intracellular structures that may act as gravity susceptors in protists, fungi and plants. These include, but are not limited to: paracrystalline protein bodies (Fries *et al.*, 2002), lipid globules (Grolig *et al.*, 2004), barium sulfate-filled vesicles (Schröter *et al.*, 1975), starch-filled amyloplasts (Sack, 1991, the nucleus (Kordyum and Guikema, 2001), and the entire protoplasm (Wayne *et al.*, 1990). However, for higher plants, there are two main models for gravity perception. One suggests that perception occurs when gravitational forces act upon the protoplast, and the other proposes that starch-filled amyloplasts function as sedimentable statoliths that perceive gravity. Each of these models will now be discussed.

#### 1. Protoplast Pressure Model

The protoplast pressure hypothesis (Wayne *et al.*, 1990; Wayne and Staves, 1996) suggests that the mass of the entire protoplasm causes tension and compression at the top and bottom of the plasma membrane, respectively. According to this model, these forces may relate the gravity signal to membrane-bound stretch receptors located in the plasma membrane. Support for the gravitational pressure hypothesis (Wayne *et al.*, 1990) is primarily based upon studies of gravity-dependent cytoplasmic streaming with the Characean algae *Nitellopsis* and *Chara*, which have no sedimenting amyloplasts, yet still sense and respond to gravity. For instance, vertically oriented internodal cells of Characean algae exhibit a gravity-dependent polarity of the cytoplasmic streaming (Wayne *et al.*, 1990; Staves *et al.*, 1995). The direction of this streaming is reversed when the cells are placed in a comparatively viscous medium (Staves *et al.*, 1997) and abolished when the cells are placed horizontally (Wayne *et al.*, 1990). The data that support this model are not necessarily inconsistent with the other prevalent model (Wayne and Staves, 1996; Kiss, 2000), and it has been proposed that multiple mechanisms may act in the perception of gravity (Barlow, 1995; Sack, 1997; MacCleery and Kiss, 1999).

#### 2. Starch Statolith Model

The starch statolith hypothesis (Sack, 1997; Kiss, 2000) proposes that the force due to gravity is perceived by the sedimentation of dense, starch-filled amyloplasts that function as statoliths within the statocytes. There are many different formulations of the hypothesis, but in general the following characteristics are common to all plastid-based gravity perception models: (1) change in the position of the plant/organ with respect to gravity increases the potential energy of the amyloplasts; (2) energy is dissipated by the settling of the plastids towards and/or onto the new cell bottom; (3) changes in the direction of the gravity vector are perceived by the plant due to the action of the amyloplasts; and (4) perception leads to a transient decrease in the cytosolic proton concentration (with concomitant proton extrusion into the apoplast), and possibly also an increase in cytosolic calcium ion concentration, lateral redistribution of auxin efflux carriers, and auxin redistribution to the lower side of the organ. Lastly, it is this auxin redistribution that leads to the differential growth known as gravitropic curvature. In order to thoroughly explore the role of plastids in gravitropism, it is now necessary to address the following questions in detail. (1) Are amyloplasts gravisensors? (2) If amyloplasts are gravity-perceiving organelles, how do they work? The first question is answered below and the second is the focus of the next section.

Several lines of evidence indicate that root columella cells and shoot endodermal cells function as statocytes, and that the amyloplasts within these cells function as statoliths. For reviews on this topic see Volkmann and Sievers (1979), Sack (1991), Salisbury (1993), Sack (1997), Fukaki and Tasaka (1999) and Kiss (2000). Mutants lacking an endodermal layer (Fukaki *et al.*, 1998), amyloplasts within the endodermal layer (Fujihira *et al.*, 2000), or starch in the endodermal amyloplasts (Weise and Kiss, 1999) are defective in gravitropism. Also, complete removal of the columella cell layer, either by decapitation or laser

ablation, results in loss of root gravitropic curvature (but not root growth), suggesting that gravity sensing takes place in the columella region of the root cap. Blancaflor *et al.* (1998) selectively ablated *Arabidopsis* columella cells using a laser, and then analyzed the effects of such treatment on gravicurvature. In this way, the group constructed a map that correlates each columella cell in the root cap to its relative contribution to root gravisensing. Results indicated that the central cells of story two contributed the most to gravisensing, followed by the central cells of story one (Fig. 1). It was also determined that the amyloplast settling occurred most quickly in the central story two (S2) cells, followed by the central story one (S1) cells. In fact, this study provided strong support for the starch-statolith hypothesis because analysis of amyloplast sedimentation rates amongst the various columella cells demonstrated that the statocyte cells with the fastest sedimentation rates made the greatest contribution to gravisensing.

Studies of starch-deficient mutants have been instrumental in highlighting the role of amyloplasts in graviperception. There are several mutants available that are starchless (e.g. *pgm*, *adg*) or are starch-deficient (e.g. ACG20, ACG27). Studies with these mutants demonstrate that amyloplasts possessing a full complement of starch are necessary for full gravitropic competence in roots and hypocotyls of *Arabidopsis thaliana* and *Nicotiana sylvestris* (Kiss and Sack, 1989, 1990; Kiss *et al.*, 1989, 1996, 1997; Vitha *et al.*, 1998). Starch-deficient mutants display a reduction in gravitropic sensitivity, but mild hypergravity conditions (of 2 to 10 *g*) brought about by centrifugation resulted in increased amyloplast sedimentation and restoration of wild-type curvature response to the starch-deficient mutants (Fitzelle and Kiss, 2001). In addition, gravitropic sensitivity was correlated to amyloplast sedimentation in *Arabidopsis* roots (MacCleery and Kiss, 1999), further emphasizing the role of amyloplasts as sedimenting statoliths.

Additional evidence supporting the role of amyloplasts as gravisensors comes from the use of magnetophoresis, which has been used to move amyloplasts within the statocytes while the plant organ is held at its natural gravitropic set-point angle (Fig. 2). The dense concentration of starch within amyloplasts makes these organelles significantly more diamagnetic than the rest of the statocyte; consequently, amyloplasts are repelled by high gradient magnetic fields (HGMF). This repulsion mimics the force due to gravity on the amyloplasts but retains the rest of the plant in a "non-gravistimulated" state. Results of these HGMF studies

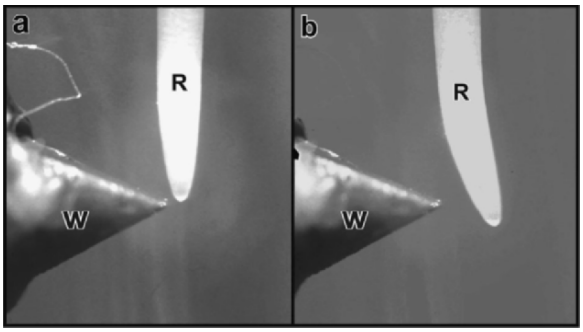


*Fig. 2.* Photograph of a flax (*Linum usitatissimum*) root exposed to a high gradient magnetic field. The ferromagnetic wedge (W) produces a high gradient magnetic field that generates a force similar to that produced by gravity. Once the root tip is exposed to this force, the amyloplasts inside the root cap are repelled from the wedge (see Kuznetsov and Hasenstein, 1996). This mimics the force caused by gravity, causing the root tip to grow away from the wedge. (a) Root tip at the beginning of the experiment. (b) Root tip after 1 hour of exposure to the high gradient magnetic field with the root tip growing away from the wedge. Photographs courtesy of Dr. Karl Hasenstein, University of Louisiana at Lafayette, USA.

indicate that amyloplast movement induces gravitropic curvature in roots (Kuznetsov and Hasenstein, 1996), coleoptiles and hypocotyls (Kuznetsov and Hasenstein, 1997) and inflorescence stems (Weise *et al.*, 2000). Further support for the starch statolith hypothesis comes from the fact that the starchless plastids of *pgm* mutant *Arabidopsis* roots and shoots were not repelled by the HGMF, and gravitropic curvature did not occur (Kuznetsov and Hasenstein, 1996; Weise *et al.*, 2000). Taken together, data from these different types of experiments underscore the importance of amyloplasts to gravity sensing.

Interestingly, the latter study also demonstrated that hypocotyls (as well as roots) have spatially separated sites of perception and response, whereas inflorescence stems do not. That is, a continuous stretch of inflorescence stems exhibit both graviperception and gravicurvature. The gravity signal originates in the endodermis and is transmitted radially to the cortical tissue (Fig. 1b). In contrast, graviperception occurs in the root tip, while gravicurvature happens in the root elongation zone (Fig. 1a). Similarly, gravity sensing in hypocotyls occurs more towards the hook, while curvature occurs farther down the length of the hypocotyl. This suggests a difference in the gravitropic mechanisms of hypocotyls versus inflorescence stems—an idea that is supported by experimental evidence, as will be discussed later. However, a commonality amongst these organs is that gravity sensing occurs intracellularly, while gravicurvature occurs at the level of tissues

and organs. How the gravity signal gets transmitted from the sites of perception to the responding loci is the subject of signal transduction.

## D. Gravity Signal Transduction in Plants

### 1. Calcium and Signal Transduction

Now that we have delved into the necessity of amyloplast sedimentation for full gravitropic sensitivity, the question of how amyloplasts propagate the gravity signal comes to the forefront. Exactly how the mechanical motion of the amyloplasts is transduced into a physiologically relevant signal remains a matter of considerable debate. However, studies have shown that, following gravistimulation, calcium ion ($Ca^{2+}$) concentrations increase in the cytosol (Plieth and Trewavas, 2002) and cell wall, particularly in cells along the slower-growing flank of the gravistimulated organs (Björkman and Cleland, 1991). Therefore, $Ca^{2+}$ seem to play a role in gravitropic signal transduction. Since stretch-activated $Ca^{2+}$ channels have been discovered in animal signal transduction systems, it is hypothesized that similar structures may exist in plants. It is envisioned that these proposed channels are connected to microfilaments, and that when these microfilaments are impacted by settling amyloplasts, they stimulate the $Ca^{2+}$ channels to release $Ca^{2+}$ into the cytosol (Sievers and Braun, 1996; Baluška and Hasenstein, 1997; Yoder *et al.*, 2001).

Alternatively, intracellular stores of $Ca^{2+}$ may also be released into the cytosol by other structures besides calcium channels. For instance, $Ca^{2+}$ may be sequestered by ligands in the cytosol (Yoder *et al.*, 2001) or stored inside the vacuole (Morita *et al.*, 2002), and may contribute to transduction when they are released as a result of plastid interaction with these or other intracellular structures, such as a specialized form of endoplasmic reticulum (termed nodal ER), the tonoplast, or F-actin microfilaments. Since no specific evidence demonstrates the source of the increase in intracellular $Ca^{2+}$, all of these ideas merit further investigation.

### 2. Cytoskeleton and Signal Transduction

#### a. Actin–Tether Model

Plant cells contain two cytoskeletal elements that have been studied in regards to gravitropism: microtubules composed of α- and β-tubulin and F-actin microfilaments composed of G-actin monomers. The presence of microtubules (Nick *et al.*, 1990; Blancaflor and Hasenstein, 1993; Himmelspach *et al.*, 1999) and F-actin microfilaments (MF; Collings *et al.*, 2001; Yoder *et al.*, 2001; Yamamoto and Kiss, 2002) in root and stem statocytes has been demonstrated using fluorescence staining and electron microscopy. Experiments indicate that microtubules may play a role in gravitropism of certain stem-like organs, such as coleoptiles (Blancaflor, 2002). However, F-actin microfilaments participate in shoot and root gravitropism, since depolymerization of the F-actin cytoskeleton causes an overshoot in the gravitropic response of these organs (Yamamoto *et al.*, 2002; Hou *et al.*, 2003).

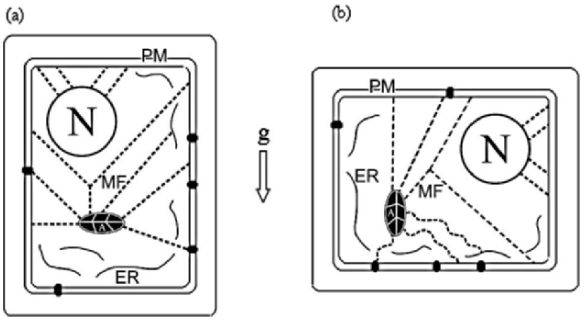


*Fig. 3.* The actin-tether model of gravity perception. (a) The amyloplasts (A) are physically connected to actin microfilaments (MF). (b) Reorientation with respect to the gravity vector (g) causes amyloplast displacement, which in turn produces tension or slack in the attached microfilaments. The cumulative forces of slack and tension on the MF relate the gravity signal to cortical ER and/or ion channels (filled ovals) in the plasma membrane (PM). N = nucleus. ER = endoplasmic reticulum. MT = microtubule. Redrawn from Baluška and Hasenstein (1997).

There are two main hypotheses regarding the interaction between amyloplasts and the F-actin cytoskeleton during signal transduction. The actin-tether model (Baluška and Hasenstein, 1997) hypothesizes that the amyloplasts are physically connected to the actin filaments (Fig. 3). Reorientation with respect to the gravity vector causes amyloplast displacement, which, in turn, produces tension or slack in the attached microfilaments. This combination of tension and slack may be related to membrane-bound stretch receptors that in turn transduce the signal.

#### b. Tensegrity Model

The tensegrity model (Yoder *et al.*, 2001) proposes that the amyloplasts are not physically bound to the actin filaments (Fig. 4). Rather, they are enmeshed in an MF network, and the amyloplasts disrupt the MF arrays as

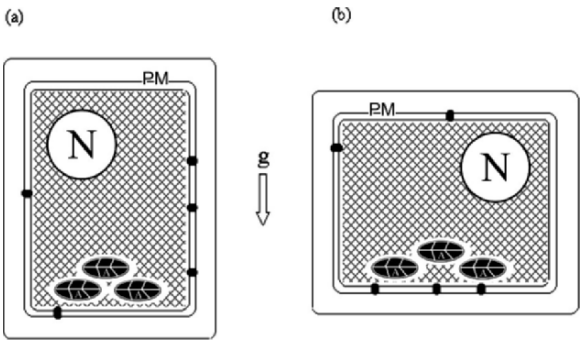


*Fig. 4.* The tensegrity model for gravity perception/transduction. (a) Amyloplasts (A) are enmeshed in an actin microfilament matrix (hatched pattern). (b) Gravistimulated amyloplasts disrupt the microfilament arrays as they traverse the cell, and the collective pressures transduce the signal to stretch receptors or ion channels (filled ovals) in the membrane. N = nucleus. g = gravity vector. PM = plasma membrane. Redrawn from Yoder *et al.* (2001).

they traverse the cell (due to gravistimulation). It is proposed that these collective pressures placed on the MF network may transduce the signal to stretch receptors or ion channels in the membrane. An alternative hypothesis is that the microfilaments restrain the plastids in such a way as to moderate their impact onto some touch-sensitive surface that may be located at the periphery of the (root) statocyte (Yoder *et al.*, 2001). This may result in a release of cytosolic $Ca^{2+}$ stores previously sequestered by ER-associated protein ligands. A specialized form of endoplasmic reticulum (nodal ER) was identified in the periphery of maize columella cells (H.-Q. Zheng and Staehelin, 2001). It was proposed that this nodal ER might shield the plasma membrane from sedimenting amyloplasts, and impart directionality to the gravity signal after impaction from amyloplasts, either by release of $Ca^{2+}$, or through physical connections to plasma membrane receptors (H.-Q. Zheng and Staehelin, 2001).

Although these are the two dominant hypotheses regarding the interaction of amyloplasts and F-actin in signal transduction, studies also suggest that F-actin can influence auxin transport (Cox and Muday, 1994; Butler *et al.*, 1998; Muday, 2000; Sun *et al.*, 2004). Furthermore, the possibility still exists that it is the movement of the plastids *away* from their original positions of rest that educes the graviresponse (Volkmann and Sievers, 1979), as opposed to their movement towards or arrival at a new destination. Lastly, plastid interaction with the tonoplast of the stem statocyte may mobilize $Ca^{2+}$ and/or $H^+$ stores from the vacuole, causing $Ca^{2+}$ and/or $H^+$ translocation into the cytosol (Morita *et al.*, 2002; Kato *et al.*, 2002b). The hypothesis that amyloplast interaction with the vacuole may be essential for gravitropism of stem-like organs will now be discussed, and the proposed transduction models will be presented.

## 3. Vacuole and Signal Transduction

It has been proposed that the central vacuole plays a role in gravisensing in stem-like organs (Clifford *et al.*, 1989; Morita *et al.*, 2002; Haswell, 2003). To understand why this hypothesis has been proposed, it is necessary to examine the ultrastructural features of both root and shoot statocytes (Fig. 5a–c). Stem endodermal cells are much larger, more elongated and possess large central vacuoles (Fig. 5a, b) as compared to root statocytes (Fig. 5c). Thus, amyloplasts in endodermal cells must interact much more intimately with the vacuole as they sediment.

This conclusion is supported by studies which have shown that the vacuoles in endodermal cells are deformed by displacement of amyloplasts (Volkmann *et al.*, 1993). Also, amyloplasts in wild-type plants have been shown to traverse the central vacuole during the course of sedimentation (Clifford *et al.*, 1989; Morita *et al.*, 2002), while amyloplasts of the agravitropic *zig/sgr*4 mutants do not sediment with gravity and are excluded from the vacuole (Saito *et al.*, 2005). Thus, it appears that amyloplast sedimentation through the vacuole is necessary for normal shoot gravitropism. In contrast, the vacuoles of columella cells are relatively small; they do not appear to represent significant obstacles to amyloplast movement in the root statocyte (Sack, 1991; compare Fig. 5b and 5c) and there is no evidence that gravistimulated amyloplasts pass through their interior. Also unlike endodermal cells, the nucleus is often located exclusively in the proximal portion of columella cells (Fig. 5c). As a result, there is potentially much less hindrance to amyloplast movement in root statocytes as compared to stem statocytes.

Another major ultrastructural difference between endodermal cells and columella cells is that, in addition to smaller vacuoles, columella cells possess nodal endoplasmic reticulum in the cortical region of the cell. This nodal ER, which may be a sink for calcium ions in the cell, potentially shields the plasma membrane from the amyloplasts, and also it may provide directionality to the gravity signal when it is impacted by the sedimenting amyloplasts (Yoder *et al.*, 2001; H.-Q. Zheng and Staehelin, 2001). To date, no such structures have been documented in stems. These large differences in ultrastructure between stem and root statocytes may

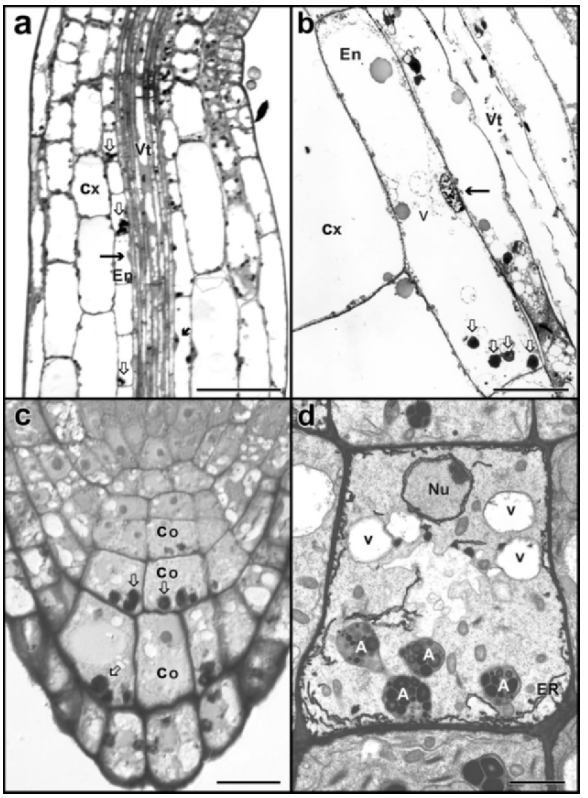


*Fig. 5.* Micrographs of hypocotyls and root tip of young seedlings demonstrating ultrastructural differences between the statocytes of stems and roots. (a) Brightfield micrograph of a hypocotyl. Amyloplasts (open arrows) are settled along the distal portion of the endodermal cells (En). Nuclei (solid arrows) are appressed to the plasma membrane. The endodermal cells have a large central vacuole. V = vascular tissue. Cx = cortical cell. Bar = 50 μm. (b) Electron micrograph of an endodermal cell (En) from a hypocotyl. Amyloplasts (open arrows) are visible, as is the central vacuole (V). Because the vacuole occupies such a large proportion of the endodermal cell volume, amyloplasts in the endodermis necessarily interact more with the vacuole as they traverse the cell (as compared to columella cell amyloplasts). Cx = cortical cell. Vt = vascular tissue. Solid arrow = nucleus. Bar = 10 μm. (c) Brightfield micrograph of a root cap. The columella cells (Co) are located centrally within the root cap. Amyloplasts (open arrows) are visible within the columella cells. Bar = 10 μm. (d) Electron micrograph of a columella cell of a root cap. Each columella cell has a proximally located nucleus (Nu) that under normal circumstances does not sediment with gravity. Several small vacuoles are visible (V). Note the relative size difference between the columella cell vacuoles and endodermal cell vacuoles. Columella cells also contain a well-developed endoplasmic reticulum (ER) network along their periphery. Bar = 2 μm.

reflect differing gravitropic mechanisms amongst the respective plant organs.

In addition to differences in ultrastructure suggesting different gravitropic mechanisms between roots and stems, various mutants have been identified that support this same conclusion, and also point to the vacuole as a potential participant in gravitropism. Examples include *grv*2, which functions in endocytosis and is required for normal amyloplast interaction with the vacuole (Silady *et al.*, 2004). Also, shoot gravitropism (*sgr*) mutants have been identified that possess impaired gravitropic responses in the aerial organs, yet the roots show normal gravitropic responses. *sgr*2, *sgr*3 and *sgr*4 (*sgr*4 was renamed *zig* because of the zig-zag phenotype of the stems). These strains possess mutations in genes whose expression products function vacuolar transport from the Golgi apparatus to the vacuole (H. Zheng *et al.*, 1999; Kato *et al.*, 2002b; Yano *et al.*, 2003) and vacuolar formation (Morita *et al.*, 2002). The mutants also exhibit abnormal amyloplast sedimentation (Fukaki *et al.*, 1996; H. Zheng *et al.*, 1999) and aberrant vacuole-like structures (Kato *et al.*, 2002a). When each of these mutants was transformed with an endodermis-specific promoter (*SCR*; SCaRecrow) attached to the respective wild-type gene, endodermal cell morphology, amyloplast sedimentation and gravitropic response were restored.

ZIG was found to be identical to AtVI11 (H. Zheng *et al.*, 1999), which is a v-SNARE (donor vesicle—Soluble *N*-ethyl-maleimide-sensitive-factor Attachment-factor REceptor) that is involved with vesicle transport processes—including transport to the vacuole. On the other hand, *SGR3* encodes a target membrane SNARE (t-SNARE; AtVAM3p) that localizes to the prevacuolar compartment and the vacuole, and has been shown to associate with AtVI11 in endodermal cells (Yano *et al.*, 2003). *SGR2* encodes a PA phospholipase $A_1$ enzyme (Kato *et al.*, 2002a). As such, it may play a role in gravitropic signal transduction by affecting vesicular trafficking, amyloplast distribution through the vacuolar membrane or by producing fatty acids and/or lysophospholipids to act as signaling molecules. Taken together, these results suggest that the vacuole may play an important role in amyloplast sedimentation and graviperception in stem-like organs.

Interestingly, another phospholipase enzyme has been implicated in shoot (but not root) gravitropism. Both silencing and overexpression studies suggest that *Ats*PLA$_2\beta$, a secretory low molecular weight phospholipase $A_2$ enzyme is involved in shoot gravitropism. Although *Ats*PLA$_2\beta$ is expressed exclusively in hypocotyls and shoots, this protein does not localize to amyloplast membranes or to the vacuole; rather, it is secreted into the cell wall/extracellular space, and it seems to mediate shoot gravitropism ultimately by affecting intracellular and apoplastic pH, and by promoting auxin-induced cell elongation (Lee *et al.*,

2003; Ryu, 2004; Wang, 2004). Other proteins besides *Ats*PLA$_2\beta$ have been identified that influence gravitropism yet do not localize to the vacuole. These proteins will now be discussed.

### *4. Proteins and Signal Transduction*

Although a preponderance of evidence suggests that sedimenting amyloplasts play a vital role in gravity perception, the mechanism by which this perception is transformed into a response has yet to be determined. Plastid interaction with the cytoskeleton and/or the vacuole are two hypothetical means by which this transduction can occur. Although no direct plastid/cytoskeleton interactions have been observed, myosin-like proteins have been localized to statolith membranes in *Chara* rhizoids (Braun, 1996). This is important because myosin and myosin-like proteins associate with the F-actin cytoskeleton. In addition, ARG1 (Altered Response to Gravity; Sedbrook *et al.*, 1999; Boonsirichai *et al.*, 2003) and ARL2 (ARG1-LIKE2; Guan *et al.*, 2003) have been implicated in mechanisms of the early phases of gravitropism. ARG1 is a protein that is required for normal gravitropic response, and is purported to interact with the cytoskeleton and participate in vacuole-associated pathways. This protein is a type II DNAJ-like protein with a DNA-J domain at the N terminus, a coiled-coil region at the C terminus, and a hydrophobic region in between. DNA-J proteins possess a myriad of functions, including protein folding and partitioning into organelles, modulation of HSP70 ATPase activity and signal transduction. Since the ARG1 coiled-coil region bears significant sequence homology to the coiled-coils of cytoskeleton-interacting proteins, it is proposed that ARG1 may interact with the cytoskeleton (Sedbrook *et al.*, 1999).

ARG1 has been implicated in other gravitropic processes. It has been demonstrated that alkalinization of columella cytosol occurs within seconds after gravistimulation of wild-type roots and is necessary for normal gravitropic response (Scott and Allen, 1999; Fasano *et al.*, 2001). *Arg1-2* null mutants lack both this alkalinization and a normal gravitropic response (Boonsirichai *et al.*, 2003). Interestingly, *arg1-2* null mutants also do not exhibit a lateral auxin gradient following gravistimulation, as determined by GFP studies and by studies utilizing GUS fusion to an auxin-responsive promoter. As was discussed earlier, establishment of a lateral auxin gradient following gravistimulation is necessary for the gravitropic curvature response. Expression of wild-type ARG1 in the null mutants restores the cytosolic rise in pH, the lateral auxin gradient necessary for gravitropic curvature (Ottenschläger *et al.*, 2003), and a normal gravitropic response. Recent studies demonstrate that ARG1 is a peripheral membrane protein that may participate in the same vesicle transport pathways as auxin efflux carriers that promote basipetal auxin transport in the root. Taken together, these data suggest that ARG1 may modulate gravity-induced cytosolic pH changes, vesicle trafficking of auxin transport machinery and gravity signal transduction (Boonsirichai *et al.*, 2003).

## III. Methods to Study the Role of Plastids in Gravitropism

### *A. Clinostats and Centrifuges*

One approach to studying plant gravitropism is to alter the gravitational field to which the plant is exposed. Beginning with Knight in 1806, various devices have been constructed to alter and/or "cancel" the effects of gravity upon statocytes. One example is the centrifuge, which increases gravitational acceleration. As was discussed previously, studies have shown that centrifugation can restore gravitropic sensitivity to starch-deficient mutants by causing plastid sedimentation (Fitzelle and Kiss, 2001). In another experiment, *Arabidopsis thaliana* hypocotyls and *Vigna angularis* epicotyls were centrifuged after their mechanoreceptors were pharmacologically blocked with lanthanum and gadolinium (Soga *et al.*, 2004). Centrifugation did not cause the typical hypergravity-induced growth suppression when the mechanoreceptors were blocked; however, gravitropism was unaffected by the pharmacological treatment (Soga *et al.*, 2004). This suggest that hypocotyls and epicotyls have different mechanisms for sensing gravity versus hypergravity.

Another instrument used to alter the effects of gravity on plant tissues is the clinostat. Clinostats rotate specimens around either one or two axes (Kraft *et al.*, 2000). In this way, the direction of the gravity vector relative to the plants is kept in a constant state of flux. Plastids are kept mobile and never settle in any one direction in order to cancel out the unilateral effects of gravity. There are several caveats regarding one-axis clinostats (also called two-dimensional clinostats). If the rotation is either too fast or not fast enough, then unwanted side effects can occur. For instance, if the rotation is too fast (or if the plant is too far from the rotation axis), then a centrifugal force develops that can be perceived by the plant (Hoson *et al.*, 1997). Two-axis clinostats were

built in an attempt to overcome these and other related problems.

In general, the two-axis clinostat, otherwise known as the random positioning machine (RPM), shows more promise for plant gravitropism research than other designs (Kiss, 2000; Kraft *et al.*, 2000). RPMs do not affect a significant change in the germination rate of many species, and they also seem to have a negligible effect on physiological parameters such as cell wall extensibility and sugar composition, sap osmotic potential, sugar translocation and polar auxin transport (Hoson *et al.*, 1997). However, results from three-dimensional clinorotation studies are conflicting. Several results indicate that plastid positions within RPM specimens closely approximate plastid positions in microgravity (e.g. Kraft *et al.*, 2000) while results from other studies indicate otherwise (e.g. Smith *et al.*, 1997).

In addition, some evidence suggests that the RPM might have adverse physiological effects (Smith *et al.*, 1997). For instance, columella cells of *Trifolium repens* specimens that were continuously clinorotated on an RPM for three days displayed increased vacuolation, cell wall degradation and an apparent reduction in amyloplast number. Also, the RPM increased the senescence rate of *Avena sativa* leaves, and it has been shown to affect peg formation in cucumber (Hoson *et al.*, 1997). Thus, more studies would be beneficial to further determine the adverse physiological effects this device may have on specimens. As with any method, there are advantages and caveats to using clinorotation. Nonetheless, the advantages of using two-axis clinostats make them valuable tools in gravitropism research.

## B. Feedback Systems

A confounding variable to the study of gravitropism is that the gravity stimulus changes as gravitropic curvature proceeds and the organ reorients to its gravitropic set-point angle. Therefore, a new tool has been developed to overcome the effects of diminishing stimulus on the gravitropic response. Known as the "ROTATO", this device is composed of a rotating stage coupled to a video digitizer via a feedback system (Mullen *et al.*, 2000). The ROTATO has the ability to maintain a particular locus of an organ in a fixed position with respect to gravity (Fig. 6), and because of this, gravity exerts a continuous and unidirectional force upon that position.

In a relatively short time, this tool has been shown to be valuable in the study of gravitropism, phototropism and interactions between these two tropisms. For instance, Mullen *et al.* (2000) observed only a 10-minute delay in the onset of gravicurvature following

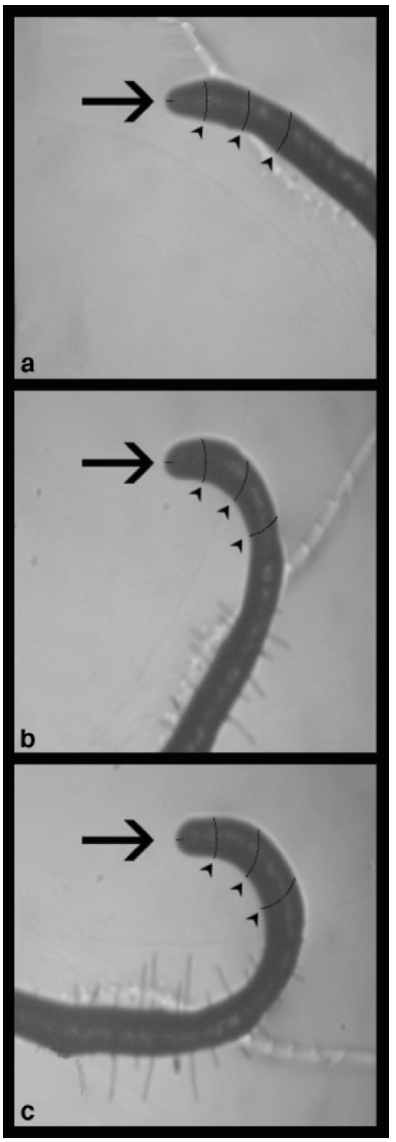


*Fig. 6.* Time-lapse photographs of an *Arabidopsis* root tip mounted on a rotating stage (ROTATO) and positioned such that the root tip remains constant during gravistimulation by reorientation. (a) Root tip after it has been mounted to the stage for 30 minutes. The longitudinal axis of the root tip (arrow) is maintained perpendicular to gravity. The three black lines (arrowheads) represent 330 μm, and are generated by the computer program as reference points for maintaining the position of the root tip (see Mullen *et al.*, 2000). (b) The same root tip once it has been on the ROTATO for 5 hours. Note that although the longitudinal axis of the root tip (arrow) has remained constant (compare arrows in Fig. 6a and b), gravitropic curvature has occurred, as is evidenced by the increasing angular distance of the rest of the root with respect to the horizontal. (c) After 9.5 hours on the ROTATO, the position of the root tip remains unchanged. However, the rest of the root has curved nearly 180° and is now pointing in the same direction as the root tip, and it is possible to obtain continuous gravitropic root curvature using the ROTATO (see Mullen *et al.*, 2000). Photographs courtesy of Melanie J. Correll (Miami University, USA).

reorientation of *Arabidopsis* roots via ROTATO. This is in contrast to 30-minute lag times observed previously, possibly because of increased plastid perturbation from more crude methods of reorientation. In addition, using ROTATO, Wolverton *et al.* (2002a, b) found that maintaining a region 4 to 5 mm from the tip in the vertical position causes gravicurvature even after the root tip reaches vertical. This corroborates previous findings that although root gravity sensing occurs primarily in the tip, gravity sensing also seems to occur outside of this region (Haberlandt, 1900; Wolverton *et al.*, 2002a). Further study is necessary to determine the nature and mechanisms of this second sensory phenomenon.

The ability of the ROTATO to maintain a constant gravistimulus has also been useful for studying root phototropism. Root phototropism is a weak response in comparison to gravitropism (Ruppel *et al.*, 2001), and by standardization of the gravistimulus with ROTATO, Kiss *et al.* (2003) observed 30° to 40° phototropic curvatures in wild-type *Arabidopsis* roots, as opposed to the 5° to 10° curvatures previously obtained without this instrument. The increased sensitivity of this technique also allows enhanced resolution of subtle differences in root phototropic responses, such as with analysis of phytochrome mutants (Correll and Kiss, 2002, 2005; Correll *et al.*, 2003).

## C. Space Flight Studies

Space flight studies have been particularly useful in the study of gravitropism (see Perbal *et al.*, 1997) because it can be advantageous when studying gravitropism to observe plant responses to a stimulus (e.g. centrifugal acceleration) in the absence or near absence of gravity, and the microgravity conditions encountered in low earth orbit provide an environment that is effectively devoid of unilateral gravity. This is in contrast to earth-based experimentation, where it is nearly impossible to completely negate the effects of gravity. The utility of microgravity-based gravitropism studies is exemplified in analyses of amyloplast positions in microgravity. It has been shown that during space flight, amyloplasts do not achieve a random positioning (Perbal *et al.*, 1987; Smith *et al.*, 1997). This phenomenon has been attributed to connections between the plastids and the actin cytoskeleton (Driss-Ecole *et al.*, 2000).

Microgravity conditions have also been utilized to study gravitropic sensitivity in starch-deficient mutants of *Arabidopsis* (e.g. Kiss *et al.*, 1998a, b, 1999). For instance, wild-type *Arabidopsis*, two starch-deficient lines and one starchless mutant were grown in microgravity. A 1*g* stimulus was applied in microgravity with an on-board centrifuge, and a clinostat was employed along with the ground control. This design promoted accurate detection of gravitropic sensitivity because the gravistimulus was administered and then effectively removed (due to the microgravity environment) subsequent to various controlled periods of stimulation via the centrifuge. Results demonstrated a correlation between increased starch content and gravitropic sensitivity, and lent further support to the starch-statolith model of gravity perception.

Different methods have been used in the course of gravitropic studies to characterize the dose response curve of the gravitropic reaction as well as the amount and duration of gravistimulation necessary to produce a detectable response (the threshold level). Here again, space flight studies have shown their utility by dramatically altering our concept of presentation time (Perbal *et al.*, 2002). Presentation time ($t_p$) is the minimal duration of gravistimulation necessary to induce a curvature response (Sack, 1991). A formerly popular model for estimating the presentation time is the L-model, which assumes that curvature has a linear relationship to the log of the dose of gravity stimulation (in $g \cdot s$). According to this model, the presentation time can be determined by plotting the dose response curve, fitting the points to a regression and then extrapolating back to zero (Perbal *et al.*, 2002).

However, studies have shown that this model does not accurately represent the data for the lower (Hejnowicz *et al.*, 1998) and higher (Perbal *et al.*, 1997) doses of gravistimulation. Therefore, another model—the hyperbolic model—was proposed by Perbal *et al.* (2002) after examination of space flight data. Unlike the L-model, the hyperbolic model does not extrapolate backwards to determine the threshold level of gravistimulation; rather, this model purports that theoretically there may not be a presentation time, and so the dose response curve is placed through the origin. This notion of a vanishingly short presentation time is in line with an actomyosin concept of gravity signal transduction, since minute disturbances in amyloplast position can be translated quickly to the F-actin cytoskeleton according to this model.

## D. Microarrays and Gene Profiling

Microarrays are a relatively recent and powerful tool for studying mechanisms of gravitropism, although, to date, few studies of this nature have been performed. Moseyko *et al.* (2002) probed approximately one-third of the *Arabidopsis* genome in an attempt to analyze gravity-induced changes at the transcriptional level.

The experimental design included extracting RNA from dark-adapted plants that were (1) vertically oriented, (2) rotated 90° for 15 minutes, (3) rotated 90° for 30 minutes and (4) rotated 360° within a 10 second interval. Results indicated expression changes in many genes whose products are already suspected players in gravitropism, including calcium-binding and calmodulin-like proteins, proton exchanging proteins, expansins and auxin-induced proteins. Surprisingly, changes in expression levels of the auxin carriers AUX1 and PIN2 were not evident, and it was suggested that these might be regulated post-translationally. However, some new genes were implicated in gravitropism, including ethylene-responsive elements, oxidative burst proteins and heat shock proteins.

Results from Moseyko *et al.* (2002) also demonstrated that many genes that are regulated in response to gravitropic reorientation are also regulated in response to mechanical stimulation. Another potential caveat to this pioneering work is that the RNA was extracted from entire seedlings, and therefore does not discern between roots and shoots. Because the gravitropic response and presumptive gravitropic mechanisms may vary between roots and shoots, it would be valuable to analyze these tissues separately.

Kimbrough and coworkers (2004) did exactly that. *Arabidopsis* root tips were analyzed by microarrays following gravistimulation and mechanical stimulation. Results identified several genes that were differentially expressed specifically in response to gravity and not to mechanical stimulation. Also, because the experiment was performed as a time course study, temporal resolution of gene expression was possible. Five genes were identified that are specifically and transiently upregulated within 2 minutes following gravistimulation: a pentacyclic triterpene synthase, expressed protein of unknown function At2g16005, a cys-protease, S-adenosyl-L-Met:carboxymethyltransferase and a major latex related protein. The specific roles these proteins play in gravitropism are yet to be determined.

Most of the gravity-specific changes in transcript abundance occurred between 5 and 15 minutes following gravistimulation (Kimbrough *et al.*, 2004). Several transcription factors of known function were identified, including: HFR1 (phytochrome A activator), *At*HB-12 (homeobox transcription factor) and KNAT1 (fate determination of shoot meristem cells). At 30 minutes following gravistimulation, no apical meristem transcription factors were upregulated. Although these transcription factors affect different stages of shoot and floral development, until now they had not been implicated in physiological processes of the root. Thus, this study points to new avenues of research that may further our understanding of the cellular mechanism of gravitropism.

## IV. Future Studies

Although exciting progress has been made in the field of gravitropism in recent years, much is yet to be discovered. We have yet to isolate and characterize proteins from membranes of the amyloplast outer envelope that interact with the actin cytoskeleton and/or other structures related to gravitropic sensing and/or signal transduction. Evidence suggests that ARG1 and/or ARL2 might interact with the cytoskeleton, but there is no evidence to indicate that they associate with amyloplast membranes. And while two SNAREs, AtVI11 (a v-SNARE) and AtVAM3p (a t-SNARE), are membrane proteins that have been implicated in shoot gravitropism (Yano *et al.*, 2003), neither has been shown to be targeted to plastids. Even so, these proteins seem to be involved in the early events of signal transduction. Another protein, SGR2, encodes a phospholipase A1 enzyme (Kato *et al.*, 2002a), the products of which may participate in gravitropic signal transduction because defects in this gene disrupt normal shoot gravitropism. This protein may also interact with the two SNAREs previously mentioned, but its precise role in gravitropism is unclear. Thus, much is still unknown about the proteins involved in the early stages of gravitropic sensing and gravity signal transduction.

In addition, the precise nature of the interactions between sedimenting amyloplasts and the actin cytoskeleton remains unknown, as well as whether amyloplast sedimentation is an active or passive process. It has been established that the normal gravitropic response is disrupted upon depolymerization of the cytoskeleton with the F-actin depleting drug latrunculin B (Lat-B; Yamamoto *et al.*, 2002). Lat-B has an advantage over other actin-disrupting drugs such as cytochalasins because it binds with a high specificity to the G-actin monomers, which F-actin comprises, as well as the barbed end of the actual F-actin filaments (Spector *et al.*, 1999). Because of this, introduction of Lat-B into cells can cause complete cellular depletion of the actin cytoskeleton and an "overshooting" of gravicurvature in *Arabidopsis* shoots (Yamamoto and Kiss, 2002; Yamamoto *et al.*, 2002) and roots (Hou *et al.*, 2003). However, it is still unclear why this response occurs.

Interestingly, amyloplast sedimentation studies demonstrate that Lat-B abolishes amyloplast saltations in all organs studied. Also, Lat-B has a dramatically different effect on amyloplast sedimentation in roots

versus shoots. That is, it enhances gravity-oriented amyloplast movement in root columella cells (Hou *et al.,* 2004), while in general it arrests this movement in hypocotyls (Palmieri and Kiss, 2005) and inflorescence stems (Saito *et al.*, 2005)—although one or more "rogue" amyloplasts still seem to retain mobility. Why a small group of amyloplasts retains mobility when F-actin is disrupted remains unknown. Also, why amyloplast mobility is inhibited upon F-actin disruption is unclear. One possible scenario is that increased cellular concentrations of F-actin fragments and G-actin monomers may result in a more viscous cytosol and prevent the amyloplasts from sedimenting or even from undergoing saltations.

However, a recent study using *Chara* rhizoids suggests that Lat-B *decreases* the viscosity of the cytosol (K. Hasenstein, personal communication). It would be interesting to see if Lat-B produces the same effect in *Arabidopsis* endodermal cells. If so, this would support the notion of an F-actin dependent system of active transport for amyloplasts in endodermal cells. Also, it seems plausible that introduction of pharmacological agents which stabilize actin and/or arrest the actomyosin machinery could impede plastid movement without significantly affecting cytosolic viscosity.

Several other proteins have been identified that may be involved in the early phases of gravitropism, and each may also associate with the actin cytoskeleton. First, myosin-like proteins have been targeted to statoliths of *Chara* rhizoids (Braun, 1996), but their presence and role in higher plants can only be speculated at this time. Second, ARG1 encodes another protein that potentially interacts with the F-actin cytoskeleton and is essential for normal gravitropism. Also, a $\beta_1$-integrin-like protein was localized to the plasma membrane of *Arabidopsis* (Swatzell *et al.*, 1999), but the role of this protein in gravitropism has not yet been elucidated. Emerging technologies in molecular biology will help to solve some of these questions.

Not only do we not know precisely how the cytoskeleton interacts with sedimenting amyloplasts, but also we are unsure how it mediates pH fluxes in gravireponding roots, and the role it plays in evoking the $Ca^{2+}$ flux which occurs following gravistimulation. Studies have shown that reorientation of a plant root with respect to the gravity vector results in a transient cytosolic pH increase (Scott and Allen, 1999; Fasano *et al.*, 2001), and a subsequent $Ca^{2+}$ increase in the cytosol (Plieth and Trewavas, 2002). How these gradients are formed is not completely clear, but ARG1 may play a key role, since evidence suggests that (1) it mediates proton extrusion into the apoplast, (2) it presumably associates with the cytoskeleton and (3) it also may be involved in auxin efflux carrier redistribution following gravistimulation (Boonsirichai *et al.*, 2003).

Lastly, much more study remains on the interactions between gravitropism and other tropisms. Although gravity has a strong effect on all biological systems, plants exhibit directed growth responses to a variety of other stimuli such as light, touch and water. The corresponding tropisms: gravitropism, phototropism, thigmotropism and hydrotropism interact to determine a plant's final physiological responses (Correll and Kiss, 2002; Steed *et al.*, 2004). For example, while primary roots of *Arabidopsis* are positively gravitropic, they also exhibit a negatively phototropic response to blue light (Correll *et al.*, 2003) as well as a positively phototropic response to red light (Ruppel *et al.*, 2001). Lateral roots also exhibit a positive phototropism to red light, and it has been proposed that this may help to optimize the orientation of the roots within the soil (Kiss *et al.*, 2002). Although roots potentially respond to light, gravity and touch, the relative strengths of these tropistic responses are not equal, and are as follows: gravitropism > blue light negative phototropism > red light positive phototropism.

Often, mutations affecting one tropism will also affect other tropisms. Examples include the *WAVY GROWTH* mutant *wav1*, which is defective in the blue-light receptor phototropin 1 (Okada and Shimura, 1990) and exhibits abnormal thigmotropism and gravitropism. Also, *wav2* (Mochizuki *et al.*, 2005) mutants have altered root responses to gravity, light and touch. The complexity of the interactions between each of the tropisms can be more fully comprehended when considering that plants possess many different light receptors, some of which have overlapping absorption capabilities and redundancies in their functional pathways (Whippo and Hangarter, 2003). And because phototropism is relatively weak in roots as compared to gravitropism, there can be severe functional limitations to ground-based studies of phototropism in roots. Engineering has enabled some of these limitations to be overcome, as is the case with using the feedback system ROTATO (Kiss *et al.*, 2003). However, microgravity provides the optimal environment for studying the interactions between phototropisms without the imposition of gravitropism on the growth responses. For this reason, plans are under way to conduct phototropism experiments as well as other plant development experiments on the International Space Station.

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# Chapter 26

# Chloroplast Movements in Response to Environmental Signals

Yoshikatsu Sato
*Division of Evolutionary Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444-8585, Japan*

Akeo Kadota*
**Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Minami-Osawa 1-1, Hachioji, Tokyo 192-0397, Japan*

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## Summary

Plants use light not only as an energy source for photosynthesis but also as an environmental signal for developmental regulation. In most plants, the photosynthetic organelle, the chloroplast, does not rest in the same position of the cell but instead relocates in response to external stimuli in order to maximize photosynthetic activity. Genetic and reverse genetic studies using *Arabidopsis thaliana* have allowed rapid progress in our knowledge of this field. Extensive advances in the last five years have identified and characterized new components controlling chloroplast movement,

*Author for correspondence, email: kadota-akeo@c.metro-u.ac.jp

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including blue-light receptors, phototropins and an actin-binding protein associated with chloroplast movements called "CHUP1" (chloroplast unusual positioning 1). This chapter gives integrated current information about the mechanisms of chloroplast movement. In the first part of this review, we summarize most recent work on light-induced chloroplast movement and in the following section we describe the new type of chloroplast movement induced by mechanical stimulation. In the last section, we discuss the ecological significance of chloroplast movement.

## I. Introduction

Plants have acquired sophisticated mechanisms to monitor intensity, direction and wavelength of ambient irradiance in order to respond to fluctuating light conditions. Chloroplast photo-movement is one of the responses that optimizes photosynthetic light capture in weak light while avoiding damage to the photosynthetic apparatus in strong light. Under a low to moderate light intensity, chloroplasts accumulate along cell walls oriented perpendicular to the incident light (chloroplast accumulation response) and when light intensity is too strong, they move away from the face area to the profile area which is oriented parallel to the incident light (chloroplast avoidance response). These responses have been studied for over a century and are found in many species throughout the plant kingdom, from algae to angiosperms.

The effective wavelengths for inducing chloroplast movement are limited to the blue-light region in most plant species but in some ferns and mosses, red light is also effective for stimulating chloroplast accumulation and avoidance responses (Yatsuhashi *et al.*, 1985; Kadota *et al.*, 2000). Chloroplast movement is also found to be induced by mechanical stimulation in some ferns, mosses and at least one liverwort (Sato *et al.*, 1999, 2003a, b). Although the mechanism of the chloroplast photo- and mechano-relocation movement is not yet fully resolved, the photoreceptors controlling chloroplast movement have been identified not only in seed plants but also in ferns and mosses (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001, 2004; Sakai *et al.*, 2001; Kawai *et al.*, 2003; Kasahara *et al.*, 2004). Furthermore, a component involved in force generation of chloroplast movement has been recently identified from *Arabidopsis* mutant named chloroplast unusual positioning 1 (*chup1*) (Oikawa *et al.*, 2003). This chapter will focus exclusively on recent findings regarding chloroplast photo- and mechano-relocation response. Many valuable perspectives that cover additional aspects of chloroplast movement may be found in a series of excellent reviews (Haupt and Scheuerlein, 1990; Wada *et al.*, 1993, 2003; Yatsuhashi, 1996; Haupt, 1999; Wada and Kagawa, 2001; Kagawa and Wada, 2002).

*Abbreviations:* APM – amiprophosmethyl; BDM – 2, 3- butanedione monoxime; BTB/POZ – broad complex tramtrak and bric-a-brac/pox virus and zinc finger; CHUP chloroplast – unusual positioning; FMN – flavin mononucleotide; GFP – green fluorescent protein; LOV – light, oxygen, voltage; NPH – nonphototropic hypocotyl; NPL – nonphototropic hypocotyl like; PHOT – phototropin; PHY – phytochrome; PLC – phospholipase C; PRM – proline-rich motif; RPT – root phototropism.

## II. Light-Induced Chloroplast Movement

### A. Photoreceptors

The response of plants to a light signal starts with light perception by photoreceptors. Although early action spectra suggested that a flavoprotein might be the chloroplast movement photoreceptor, the chemical nature of the light-sensitive molecule remained a mystery. Recent genetic studies of *Arabidopsis* revealed that a novel family of blue-light photoreceptor proteins, the phototropins, is responsible for chloroplast movement induced by blue light (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). In *Arabidopsis*, two phototropins, designated as PHOT1 and PHOT2, regulate not only chloroplast movement but also stomatal opening and phototropic curvature (Huala *et al.*, 1997; Kinoshita *et al.*, 2001; Sakai *et al.*, 2001). These responses maximize photosynthetic activity by adjusting light absorption (via chloroplast movements and phototropism) and by promoting gas exchange (via stomatal control). Thus, it can readily be said that phototropins are the sensors of light direction and intensity to facilitate a photosynthetic activity.

#### 1. Phototropins are Light-Activated, FMN-Binding, Receptor Kinases

Briggs and colleagues first isolated a blue-light photoreceptor, designated nph1 (nonphototropic hypocotyl 1), from *Arabidopsis* mutants deficient in blue-light-induced phototropism (Huala *et al.*, 1997). Subsequently, on the basis of sequence similarity, a

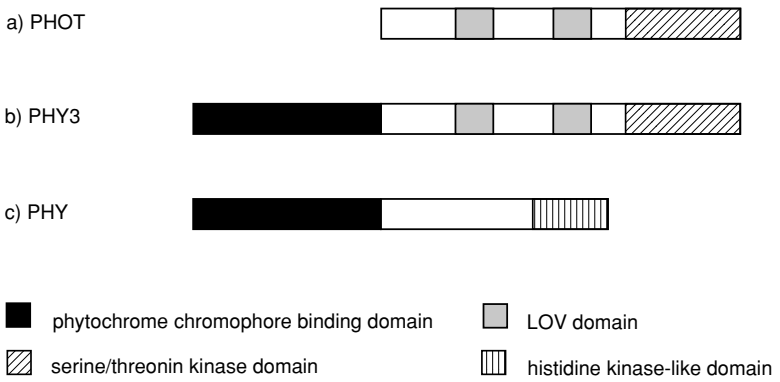


*Fig. 1.* Schematic structures of phototropin (PHOT), phytochrome 3 (PHY3) and conventional phytochrome (PHY). (a) PHOT is a classic serine/threonine kinase, which has two LOV domains as flavin-binding sites in the N-terminus. (b) PHY3 has a phytochrome chromophore-binding domain in the N-terminus followed by a complete PHOT domain in the C-terminus. (c) Conventional phytochrome has a phytochrome chromophore-binding domain and histidine kinase-like domain.

paralog of the *NPH1* gene, *NPL1* (NPH1 like 1) gene was isolated (Jarillo *et al.*, 1998). NPH1 and NPL1 were subsequently renamed phototropin1 (PHOT1) and phototropin 2 (PHOT2), respectively (Briggs *et al.*, 2001).

Phototropins contain two LOV (light, oxygen, or voltage-sensing) domains toward the N-terminus and a serine/threonine kinase domain in the C-terminus (Fig. 1a). The LOV domains of phototropins play a pivotal role in blue-light absorption. The binding of FMN (flavin mononucleotide) to each of the two LOV domains (LOV1 and LOV2) stimulates the phototropins' kinase activities (Liscum and Briggs, 1995; Christie *et al.*, 1998; Salomon *et al.*, 2000; Christie *et al.*, 2002). Genes encoding phototropins have also been found in numerous plant species from algae to angiosperms but are missing from organisms outside the plant kingdom such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, suggesting that phototropin genes evolved exclusively in the plant lineage.

## 2. Phototropin Regulation in Chloroplast Movement

Recent genetic analysis has shown that the two phototropin genes of *Arabidopsis*, *PHOT1* and *PHOT2*, overlap in function to mediate the blue-light-induced chloroplast accumulation movement (Sakai *et al.*, 2001). Plants impaired in *PHOT2* showed normal accumulation response at low fluence rate. However, at high fluence rates of up to 100 μmol $m^{-2}s^{-1}$ (blue light) chloroplasts retained accumulation response but failed to show the typical avoidance response (Jarillo *et al.*, 2001; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). In *phot1* deficient mutants, the accumulation response at moderate fluence rate and avoidance response at high fluence rate were not impaired but the fluence rate required for accumulation response was somewhat higher than in the wild type (Kagawa and Wada, 2000; Sakai *et al.*, 2001). In *phot1* and *phot2* double mutant plants, neither an accumulation nor an avoidance response was present under any light condition (Sakai *et al.*, 2001). These results suggest that under low fluence rate of blue light (0.4 to 1.0 μmol $m^{-2}s^{-1}$) only phot1 is active in mediating chloroplast accumulation response. Under moderate light conditions (2.0 to 32 μmol $m^{-2}s^{-1}$) both phot1 and phot2 mediate accumulation movement of chloroplasts. Finally, at strong light conditions (32 μmol $m^{-2}s^{-1}$ or higher) phot2 is the primary photoreceptor mediating chloroplast avoidance response (Fig. 2). In wild type plants, phot2 seems to activate the avoidance signal that works against phot1 action and it prevails over the accumulation signal produced under the high fluence rate condition.

## 3. Involvement of Phytochrome in Chloroplast Movement

In *Arabidopsis* leaves, red light alone is ineffective in inducing chloroplast movement (Trojan and Gabrys, 1996). However, subsequent study revealed that red light is necessary for inducing full response of blue-light directed accumulation movement, suggesting that red light is involved in the blue-light-induced chloroplast movement response (Kagawa and Wada, 2000). Recently, Hangarter and his colleagues examined the

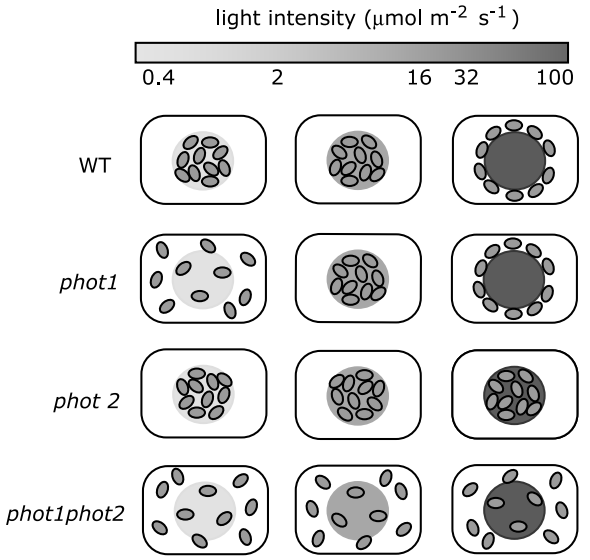


*Fig. 2.* See also Color Plate 7, p. xl. Blue light-induced chloroplast movement in *Arabidopsis* wild type (WT), single and double phototropin-deficient plants (*phot1*, *phot2* and *phot1phot2*) as analyzed by microbeam irradiation. The area of microbeam irradiation is indicated in a blue circle of each illustration of mesophyll cells.

involvement of phytochrome in blue-light-induced chloroplast movement using phytochrome deficient mutants (DeBlasio *et al.*, 2003). Spectroscopic detection of chloroplast relocation, measured by red-light transmission, showed no apparent difference in accumulation response between a *phyABD* mutant and wild type, suggesting that phyA, B, or D do not contribute the red-light-induced enhancement of chloroplast movement. However, they found that the avoidance response of chloroplasts at high fluence rate was enhanced in phyA or phyB deficient plants and that accumulation response at low fluence rate was enhanced in phyA or B overexpressing plants. Although the exact mechanism is not clear, they proposed that phyA and B might function as modulators of the response in the transition between accumulation and avoidance response.

In most plants, including *Arabidopsis*, it is unlikely that phytochrome is a primary photoreceptor for chloroplast movement because red light is not effective in inducing chloroplast movement. In some ferns, however, chloroplast movement is induced by both red and blue light (Yatsuhashi *et al.*, 1985). The red-light response was thought to be mediated by phytochrome because the response exhibits a typical red/far-red reversibility. Nozue *et al.* (1998) isolated a hybrid photoreceptor gene, *phytochrome3* (*PHY3*) that encodes fusion of phytochrome and phototropin in fern *Adiantum capillus-veneris*. Deduced 1465 amino acids of *PHY3* show striking similarity to phytochrome sequences at N-terminal 556 amino acids and to phototropin sequence at the following C-terminal region (Fig. 1b). It has been proposed that phy3 is a dual chromoprotein that is sensitive to both red/far red light and blue/UV-A light. Recently, Kawai *et al.* (2003) demonstrated that phy3 is a primary receptor in red-light-induced chloroplast movement and tropic response in *Adiantum*. *phy3* mutant plants exhibit no red-light-induced chloroplast movement or phototropism while the blue-light-induced responses are normal, probably because *Adiantum* has *PHOT1* and *PHOT2* genes and they mediate the blue-light response (Kagawa *et al.*, 2004). It would be an intriguing question whether phy3 is involved in the blue-light-induced chloroplast movement and tropic response. Red-light-induced chloroplast movement mediated by phytochrome was also reported in the moss *Physcomitrella patens* (Kadota *et al.*, 2000). Although four phytochrome genes, *Phypa: PHY; 1-4 (PP1-4)* were isolated in the moss, they are all conventional phytochromes (Fig. 1c). The ortholog genes of *PHY3* have been only found in some ferns but not in the mosses. How does the receptor system regulate the red-light-induced chloroplast movement in the moss? Mittmann *et al.* (2004) generated knockout strains of each phytochrome gene by using homologous recombination and analyzed the resulting phenotypes in response to red light. They reported that the *PP4* single knockout plant was impaired in polarized red-light-induced chloroplast movement but exhibited normal response to directional non-polarized red light, suggesting that *PP4* functions in red-light-induced chloroplast movement together with other phytochromes. Then, how do conventional phytochromes mediate chloroplast movement? Recently, fascinating results were provided by the analyses of phototropin-disruptants in the moss (Kasahara *et al.*, 2004). Phototropin functions in *Physcomitrella* show some parallels to that of *Arabidopsis* but some exciting differences also exist. Two classes of phototropin genes (*PHOTA1*, *A*2, *B*1 and *B*2) have been found in *Physcomitrella*. As in *Arabidopsis*, *Physcomitrella* phototropins function in chloroplast movements but both classes of genes, *PHOTA* and *PHOTB*, mediate avoidance response of chloroplasts. In *Arabidopsis*, only *PHOT2* regulates the latter, suggesting that the ancestral origin of phototropin genes in *Physcomitrella* belongs to *PHOT2* type. Interestingly, in the triple mutant plants of *photA2photB1photB2*, both red-light-and blue-light-induced movement were impaired, suggesting that the phototropin signaling pathway is required for phytochrome-mediated chloroplast movement in the moss. It will be interesting to elucidate the photoreceptor(s) and its functions

for chloroplast movement in the green algae *Mougeotia* since the responses are also induced by both blue and red light.

### 4. Localization of Phototropins

Physiological studies using polarized light revealed that chloroplast movement and phototropism in a protonemal cell of *Adiantum* show action dichroism, suggesting that the dipole moments of photoreceptors are arranged someway on the plasma membrane at a certain angle (Kadota *et al.*, 1985; Yatsuhashi *et al.*, 1985). Biochemical studies also demonstrated that the 120kD phototropin localizes in the plasma membrane fraction of dark grown monocots and dicots (Gallagher *et al.*, 1988; Hager and Birch, 1993; Palmer *et al.*, 1993; Short *et al.*, 1993; Salomon *et al.*, 1996; Sharma *et al.*, 1997). Furthermore, confocal laser microscopy also indicated that a phot1-GFP (green fluorescent protein) fusion protein is localized mainly on plasma membrane in dark-grown seedling of *Arabidopsis* (Sakamoto and Briggs, 2002). These results suggest that phototropins are directly or indirectly associated with the plasma membrane in the dark. Hydropathy profiles show no transmembrane domains in phototropins. After the blue-light irradiation, however, parts of phototropins dissociate from the plasma membrane and are released to the cytoplasm, although the significance of the redistribution is not known at the moment (Sakamoto and Briggs, 2002; Knieb *et al.*, 2004).

## B. Signal Transduction

### 1. Phototropin-Associated Protein

Although the precise downstream events following light perception by phototropin are unknown, two novel proteins, NPH3 and RPT2 have been identified as partners with phot1 in regulating the phototropic response and stomatal opening (Motchoulski and Liscum, 1999; Inada *et al.*, 2004). *NPH3* and *RPT2* genes both encode related proteins that have a BTB (broad complex tramtrak and bric-a-brac)/POZ (pox virus and zinc finger) domain and a coiled-coil protein interaction domain. They were found to directly bind to phot1 and were also shown to weakly interact each other. Thus, NPH3 and RPT2 have been thought to be the candidate molecules downstream of phototropins. Sakai and his colleagues examined this possibility and demonstrated that chloroplast accumulation and avoidance movements were not impaired in *rpt2-1*, *nph3-101* and *rpt2nph3* double mutant plants, suggesting that chloroplast photo-movement is not dependent on NPH3/RPT2 signaling pathway (Inada *et al.*, 2004). They mentioned the possibility that other members of *NPH3*/*RPT2* gene family are responsible for chloroplast movement since *Arabidopsis* has at least 32 genes in this family.

Another binding partner of phototropins are the 14-3-3 proteins that bind to their targets and regulate various kinds of signal transduction events in many organisms (Ferl, 1996). Shimazaki and his coworkers demonstrated that phototropin-mediated stomatal opening is dependent on the activation of the plasma membrane $H^+$-ATPase which binds 14-3-3 protein in its C-terminus (Kinoshita and Shimazaki, 1999; Emi *et al.*, 2001; Kinoshita *et al.*, 2001). Further, they found that the 14-3-3 protein binds phosphorylated phototropins in guard cells of *Vicia faba*. Phosphorylation of a serine residue between LOV1 and LOV2 domain is required for 14-3-3 protein binding (Kinoshita *et al.*, 2003). Although the physiological significance of the 14-3-3 binding to phototropins remains to be clarified, they proposed that the phenomenon would be common to phototropin-mediated responses such as phototropism, chloroplast movement and stomatal opening because the binding was evident both in etiolated seedlings and green leaves.

### 2. $Ca^{2+}$ as a Second Messenger

Phototropins may transmit the signal through the change of cytosolic $Ca^{2+}$ ion level by regulating a plasma membrane $Ca^{2+}$ channel. Blue-light-induced increase in cytosolic $Ca^{2+}$ concentration was reported in *Arabidopsis*, tobacco and *Physcomitrella* plants expressing aequorin (Russell *et al.*, 1998; Baum *et al.*, 1999; Harada *et al.*, 2003). Recently, phototropins have been shown to be involved in this phenomenon. Harada *et al.* (2003) demonstrated in *Arabidopsis* leaves that there is a partially functional redundancy between phot1 and phot2 in the regulation of $Ca^{2+}$ influx. They also revealed that phot1 and phot2 regulate $Ca^{2+}$ entry into the cytoplasm in a different manner. Cytosolic $Ca^{2+}$ elevation was strongly reduced by inhibitors of $Ca^{2+}$ influx through the plasma membrane such as $CoCl_2$, $LaCl_3$ and nefedipine in wild type, *phot1* and *phot2* mutant plants. However, PLC (phospholipase C) inhibitors such as neomycin and U-73122 did not significantly affect the $Ca^{2+}$ elevation in *phot2* mutant plants while they partially suppressed it in wild type and *phot1* mutant plants. These results suggest that both phot1 and phot2 induce $Ca^{2+}$ influx from the apoplast through the plasma membrane but that only phot2 induces $Ca^{2+}$ elevation through the release of

internal $Ca^{2+}$ storage, probably via the PLC-mediated inositol 1, 4, 5-triphosphate activation pathway. From these results, the authors proposed that a $Ca^{2+}$-induced $Ca^{2+}$ release is involved in the phot2-mediated response. Thus it has been established that the elevation of cytosolic $Ca^{2+}$ induced by blue light is mediated by phototropins. Furthermore, using electrophysiological, patch clamp methods, Stoelzle *et al.* (2003) showed that activation of $Ca^{2+}$-permeable channels in *Arabidopsis* mesophyll cells was regulated by phototropin. However, the causal relationship between the change in cytosolic $Ca^{2+}$ and chloroplast movement remains unclear at present. While blue-light-induced elevation of $Ca^{2+}$ was inhibited by inhibitors of $Ca^{2+}$ influx through the plasma membrane in all plant species tested, chloroplast photomovement was not impaired by these drugs (Sato *et al.*, 2001b, 2003b). Further studies will be required to determine whether cytosolic $Ca^{2+}$ really functions as a second messenger in chloroplast photo-movement.

## C. Motility System

### 1. Filament Tracks Responsible for Chloroplast Movement

Motility systems for chloroplast photo-movement have been studied extensively and have long been thought to be dependent on actin filaments and myosin motors. Chloroplast motility is suppressed by the actin depolymerizing drugs latrunculin B, cytochalasin B and D but not by the microtubule depolymerizing agents cholchicine and amiprophosmethyl (APM) (Kadota and Wada, 1992a; Tlalka and Gabrys, 1993; Malec *et al.*, 1996; Sato *et al.*, 1999). Further, myosin ATPase inhibitors such as *N*-ethylmaleimide and 2, 3-butanedione monoxime (BDM) inhibit chloroplast movement, although the myosins that regulate chloroplast movement have not yet been identified (Kadota and Wada, 1992a; Sato *et al.*, 1999). Immunohistochemical studies revealed that chloroplasts were closely associated with actin filaments but not microtubules in *Arabidopsis* (Kandasamy and Meagher, 1999). Recently, however, it was found that chloroplasts move along both tracks of actin filaments and microtubules and the use of these tracks was differentially regulated by light quality but not intensity in *Physcomitrella* (Sato *et al.*, 2001a). Red-light-induced chloroplast movement mediated by phytochrome was inhibited by the microtubule poisons cremart and oryzalin but not by cytochalasin B and latrunculin B. On the other hand, blue-light-induced movement mediated by phototropins was not blocked by the treatment with a single inhibitor for actin filaments or for microtubules. It was only blocked when both inhibitors were simultaneously treated. The rate of actin-based chloroplast movement in the cremart treated cells is close to that of *Adiantum* and *Arabidopsis* (0.3 to 0.5 μm $min^{-1}$), whereas the rate of microtubule-based movement in cytochalasin B treated cells is approximately five times faster (2.4 μm $min^{-1}$). These data suggest that red-light signals are transmitted to the microtubule system whereas blue-light signals activate both actin filament and microtubule systems. Immunohistochemical evidence that chloroplasts are associated with microtubules as well as with actin filaments is consistent with the pharmacological studies.

### 2. Rearrangement of Actin Filaments During Chloroplast Anchoring

It is well established that actin filaments change their organization between before and after chloroplast photo-relocation. In *Adiantum*, when a cell was partially irradiated with microbeam irradiation, a ring of actin filaments appeared along the edge of each photo-relocated chloroplast and disappeared before chloroplasts left the previously irradiated site when irradiation was stopped (Kadota and Wada, 1992b). Similarly, in mesophyll cells of *Vallisneria gigantea*, the honeycomb-like networks of actin filaments were found around photo-relocated chloroplasts and stability of these chloroplasts against centrifugal forces was increased in contrast to that of nonphoto-relocated chloroplasts in the dark (Takagi *et al.*, 1991; Dong *et al.*, 1998). These results suggest that the ring and honeycomb-like structures of actin filaments play a role in anchoring chloroplasts to the site.

### 3. The Actin Binding Protein Responsible for Chloroplast Positioning

Genetic studies using *Arabidopsis* have been unraveling not only photoreceptors but also the downstream components in the chloroplast photomovement. Chloroplast unusual positioning 1 (*chup1*) mutant was isolated in the same genetic screening as that used for *phot2* mutant isolation (Oikawa *et al.*, 2003). In *chup1* mutant plants, chloroplasts always gathered at the abaxial side of the cells and failed to show accumulation or avoidance movement in response to light conditions. Positioning of organelles other than chloroplasts, such as mitochondria and peroxisomes were not affected

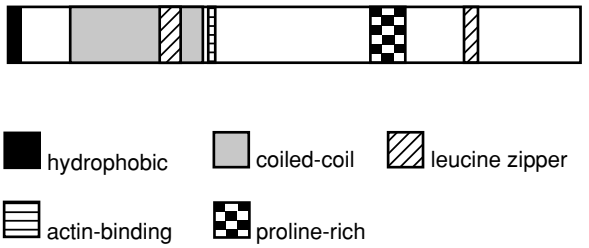


*Fig. 3.* A schematic structure of chloroplast unusual positioning 1 (CHUP1). The locations of hydrophobic region, coiled-coil domain, actin-binding motif, proline-rich motif and two leucine zipper domains are indicated.

in *chup1* mutant. Further, phototropic response induced by blue light was also not impaired. Thus, CHUP1 is suggested to be involved in the chloroplast photomovement downstream of phototropins.

*CHUP1* gene encodes a putative 112kD protein that contains a hydrophobic region, an actin-binding domain, a coiled-coil domain, a proline-rich motif (PRM) and two leucine zipper domains (Fig. 3). A short hydrophobic region at the N-terminal end is predicted to be localized in the membrane and in fact, transient expression of a GFP fusion with the N-terminus of *CHUP1* revealed the fluorescence on chloroplast membrane. An *in vitro* actin binding assay demonstrated that the actin-binding domain of CHUP1 bound F-actin. Thus, CHUP1 is considered to function in the interaction of chloroplasts with the actin filaments. A PRM domain of CHUP1 in the region from 648 to 705 amino acid residues seems to belong to PRM1 that associates with profilin and proteins containing PRM1 have been known to act on delivering actin monomers to specific cellular locations and on facilitating actin polymerization (Holt and Koffer, 2001). It should be analyzed whether CHUP1 initiates actin polymerization on the chloroplast surface. Further, since CHUP1 has protein–protein interaction domains including a coiled-coil and two leucine zipper domains, isolation of the binding partners is one of the urgent tasks to unravel the function of CHUP1 in the signaling pathway of chloroplast photomovement.

## III. Mechanical Stress-Induced Chloroplast Movement

### *A. Discovery of New Phenomenon on Chloroplast Motility*

Most studies on chloroplast movement have been performed from the viewpoint of the light signaling pathway. However, we demonstrated that intracellular distribution of chloroplasts is altered not only by light, but also by mechanical stimulation in fern protonemal cells (Sato *et al.*, 1999). When a part of the protonemal cell was touched by a rounded tip of capillary tube, chloroplasts exhibited directional movement away from the stimulated site. The mechano-avoidance movement of chloroplasts can be observed in all the fern protonemal cells tested including *Adiantum capillus-veneris, Dryopteris filix-mas, Onoclea sensibilis* and *Matteucia struthiopteris.* Subsequently, we also demonstrated mechanically-induced chloroplast movement in bryophyte cells, such as the mosses *Ceratodon purpureus* and *Physcomitrella patens* and the liverwort *Marchantia polymorpha* (Sato *et al.*, 2003b). However, chloroplasts in the moss and liverwort cells exhibit the opposite directional movement to that in fern cells. In these cells, chloroplasts move towards the stimulated site. Mechanically-induced chloroplast movement in pteridophytes and bryophytes is an excellent experimental system to elucidate the mechano-perception and—transduction pathways because the mechano-perception site is identified as a site of touching and thus mechano-signals spread from this point. It is also an advantage that we can compare the signaling cascades with those of light-induced chloroplast movement.

### *B. Mechano-Perception and the Signal Transduction*

Although even less is known about signal transduction pathways at present, mechano-movement is evoked in chloroplasts that have already had a photo-relocated position, suggesting that discrete mechano-specific signaling steps independent from those of light signaling are activated and dominate over the light signal (Sato *et al.*, 2001b). In fact, we demonstrated a different contribution of external $Ca^{2+}$ in the mechano- and photo-relocation of chloroplasts. Mechano-avoidance response in *Adiantum* was blocked by low concentrations of $Gd^{3+}$ (10 μM–a stretch-activated channel blocker) and $La^{3+}$ (100 μM–a plasma membrane $Ca^{2+}$ channel blocker) while photo-relocation movement was not impaired by these reagents even at higher concentrations of $Gd^{3+}$ (100 μM) and $La^{3+}$ (1 mM). Similar results were obtained in the mechano-accumulation response of *Physcomitrella* (Sato *et al.*, 2003b). These results suggest that the influx of external $Ca^{2+}$, probably through a stretch-activated channel, plays an essential role in regulating mechano-induced chloroplast movement. However, the same level of $Ca^{2+}$ influx from external source is not required for photo-relocation. The

plasma membrane $Ca^{2+}$ channel blocker also failed to inhibit chloroplast photo-movement in *Mougeotia* sp. and *Lemna trisulca* (Schonbohm *et al.*, 1990a, b; Tlalka and Gabrys, 1993; Tlalka *et al.*, 1999). On the other hand, chloroplast movement in these plants is induced by $Ca^{2+}$ ionophore A23187, which increases $Ca^{2+}$ influx through a plasma membrane (Serlin and Roux, 1984; Tlalka *et al.*, 1999). Taken together, these results suggest that chloroplast movement by artificial increase of $Ca^{2+}$ by A23187 may mimic mechano-relocation rather than photo-relocation.

### *C. Motility System*

In *Adiantum* protonemal cells, the speed of mechano-avoidance movement (0.8 μm $min^{-1}$) was close to that of photo-relocation which is dependent on actin filaments (Kadota and Wada, 1992a; Kagawa and Wada, 1996). Inhibitor studies indicated that the mechano-avoidance response of chloroplasts was also based on an actin-myosin system, since the response was suppressed by cytochalasin B and BDM but not by APM and cholchicine (Sato *et al.*, 1999). On the other hand, in *Physcomitrella*, the speed of mechano-accumulation movement was about 2.5 μm $min^{-1}$ which is similar to that of microtubule-based movement in blue-light-induced chloroplast movement (2.4 μm $min^{-1}$). Actually, mechano-accumulation response was suppressed by cremart but not by cytochalasin B, indicating that microtubule system was responsible for the response in the moss cell (Sato *et al.*, 2003b).

## IV. Ecological Meaning of Chloroplast Movement

Efficient photosynthesis is essential for plant survival. Chloroplast photo-movement is an evolutionally conserved character, implying that it provides some adaptive advantage to perform efficient photosynthesis for plants. Considering that plants cope with the ambient light condition, it would make sense that chloroplast movement is a cell autonomous and reversible phenomenon. However, no causal link has been established between chloroplast movement and its advantage for plants. Recently, the physiological importance of the chloroplast avoidance response was demonstrated in *Arabidopsis* using *phot2* and *chup1* mutant plants that are defective in photo-avoidance movement, showing the compelling evidence that chloroplast avoidance response is crucial for plants to survive under high fluence rate condition (Kasahara *et al.*, 2002). The mutants were more susceptible to damage under strong light than wild type and photodamage of the mutants was so severe that bleaching was apparent 24 h after onset of strong light treatment. These data suggest that chloroplast avoidance response reduces photodamage on photosynthetic pigments. It is also of significance that by the evolution of *PHOT2*, plants acquired the ability to sense strong light and develop a strategy to survive under excess light conditions. On the other hand, the accumulation response of chloroplasts has long been accepted as a means of maximizing light capture for photosynthesis but the extent of contribution of this potential advantage to plants remains to be elucidated. No convincing explanation for the ecological meaning is given at the moment for chloroplast mechano-relocation and on the dark positioning of chloroplasts. Furthermore, in leaf spongy mesophyll cells, it has been known that chloroplasts are absent on the cell region adjacent to another cell and instead, they position around the cell area facing the intercellular air space (Evans and Von Caemmerer, 1996). It would be conceivable that chloroplasts move towards the position of higher $CO_2$ concentration for efficient photosynthesis, but it has not been shown whether $CO_2$ functions as a trigger of chloroplast movement.

In some cryptogam plants, phytochrome as well as phototropin mediates chloroplast movement. Is there any adaptive advantage to use red light for chloroplast movement? The answer to this question is probably "yes". In the fern *Adiantum* sporophytes, *PHY3* impaired mutant, *rap2* (red-light aphototropism 2), exhibit much lower photosensitivity to low fluence rate unilateral white light compared with the wild type, indicating that phy3 enhances light sensitivity under dim light conditions (Kawai *et al.*, 2003). Furthermore, Smith (2000) concluded that phytochromes are proximity sensors and modify the growth and development in plants because "Changes in the red: far-red ratio are much more reliable indicators of the proximity of potentially competing neighbors than the concomitant reductions in the total amount of light penetrating the canopy".

## V. Conclusions and Future Prospects

Identification of molecular components controlling chloroplast movement—the photoreceptor, phototropin and the possible component in motile system, CHUP1—represents major advances in recent studies. However, much remains to be elucidated on the

signaling pathways, especially those connecting the two elements. We have to identify the interacting partner of phototropins that is responsible for chloroplast photo-movement. CHUP1 has many functional domains but little is known about its real function except for the evidence of F-actin binding. In addition to the yeast two-hybrid interaction assays, genetic screening of mutants in chloroplast movement and their analyses will continue to help us understand the components that participate in regulating chloroplast movement. However, it is not our goal to merely list the component molecules involved in chloroplast movement but the question is how, when and where these components work in the regulation of chloroplast movement. GFP-technology for observing protein dynamics in living cells will help us to understand this question. Elucidation of the signaling pathway downstream of phototropins that is specific to chloroplast movement will be expected soon.

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**Note added in proof:** While this chapter was in production, Suetsugu *et al.* (2005) reported chimeric photoreceptor genes between phytochrome and phototropin, resembling *Adiantum PHY3*, in *Mougeotia scalaris*. They named these chimeric photoreceptors as Neochromes (MsNEO1 and MsNEO2), demonstrating that these have bilin-binding activity and red/far-red photoreversibillity and revealing that these function in red light-induced chloroplast movement.

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# Chapter 27

# Oxygen Metabolism and Stress Physiology

Barry A. Logan*
*Biology Department, Bowdoin College, Brunswick, ME 04011, U.S.A.*

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## Summary

Plants in nearly all growth environments absorb more light energy than they can utilize in support of photosynthetic $CO_2$ assimilation. This "excess light" is problematic because it can lead to the formation of unstable forms of oxygen known as reactive oxygen species (ROS), including superoxide and singlet $O_2$. ROS damage to chloroplast macromolecules contributes to light-mediated decreases in photosynthetic capacity. The rate of ROS formation increases during exposure to environmental stresses such as chilling, since such conditions exacerbate the imbalance between light absorption and light use by inhibiting Calvin-Benson cycle activity. Plants minimize oxidative damage caused by ROS primarily via two mechanisms, antioxidation and energy dissipation. In this chapter, I review attempts to quantify the rate of ROS formation, the molecular mechanisms of antioxidation and energy dissipation as well as their acclimation to the growth environment. I also survey recent attempts to employ molecular genetic techniques to confer greater stress tolerance to plants via manipulation of the production of proteins involved in antioxidation and energy dissipation.

## I. Introduction

It has been known for some time that molecular oxygen ($O_2$) is capable of accepting electrons from the photosynthetic electron transport chain (Mehler, 1951; Mehler and Brown, 1952). Photoreduction of $O_2$ in the so-called "Mehler reaction" yields superoxide, a species with considerable reactivity and the ability to damage cellular macromolecules (Halliwell and Gutterridge, 1999). Molecular oxygen can also be converted to singlet $O_2$, another highly reactive species, via interaction with long-lived, triplet-excited-state forms of chlorophyll (Chl) (Foote, 1976). Collectively, singlet $O_2$, superoxide and the two- and three-electron products of $O_2$ reduction ($H_2O_2$ and the hydroxyl radical, respectively) are referred to as "active" or "reactive" oxygen "intermediates" or "species" (abbreviated variously as: AOI, AOS, ROI, or the term I shall use throughout this chapter, ROS). A large body of evidence suggests that abiotic environmental stresses that perturb the balance between light absorption and photosynthetic light utilization in favor of excess light absorption increase the rate of chloroplastic ROS

*Author for correspondence, email: blogan@bowdoin.edu

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generation. Furthermore, molecular damage caused by ROS likely plays a role in slowly reversible, stress-induced loss of photosynthetic capacity, which is commonly referred to as photoinhibition (Allen, 1995; Niyogi, 1999).

Plants are not completely at the mercy of ROS and the damage they can render; they possess an integrated array of biochemical mechanisms that both proactively prevent the formation of ROS and also detoxify those ROS that are formed. These mechanisms fall under the general heading "photoprotection" and include energy dissipation, which safely converts absorbed light energy to heat (Demmig-Adams and Adams, 1996; Niyogi, 1999), and the low-molecular weight and enzymatic antioxidants that operate in concert to reduce superoxide to $H_2O_2$ and ultimately to water (Alscher and Hess, 1993; Asada, 1996, 1999; Logan *et al.*, 1999a). Research into photoprotective processes has flourished over the last two decades, revealing much about their molecular mechanics and ecophysiology. Furthermore, with the arrival of molecular genetic techniques, the discipline has expanded from examining wild-type plants to manipulating components of photoprotection in transgenic plants in an attempt to improve performance and stress tolerance.

In this chapter, I will describe the current state of knowledge of the mechanisms of energy dissipation and chloroplastic antioxidation, the role they play in the regulation of chloroplast metabolism, and the manner in which these processes acclimate to the growth environment. In addition, I will survey the results of recent attempts to enhance plant stress tolerance via transgenic upregulation of proteins involved in photoprotection.

## II. The Size of the $O_2$ Photoreduction "Sink"

Molecular oxygen can be reduced by several components of the photosynthetic electron transport chain, however reduction by Fe-S clusters of photosystem (PS) I appears to predominate (Asada, 1999; Badger *et al.*, 2000). The overall size of the electron "sink" represented by $O_2$ reduction via the Mehler reaction is an area of active research, with different experimental approaches often yielding profoundly divergent results that range from negligible to ca. 30% of total photosynthetic electron transport (reviewed in Badger, 1985; Osmond and Grace, 1995; Badger *et al.*, 2000). Quantifying $O_2$ photoreduction via leaf $O_2$ uptake is complicated by the oxygenase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) as well as by simultaneous $O_2$ production by the Oxygen Evolving Complex (OEC) of PSII. Some of the first attempts to quantify the Mehler reaction took advantage of the fact that $O_2$ uptake and efflux can be distinguished by mass spectrometry in the presence of isotopically labeled $^{18}O_2$ since the $O_2$ produced by the OEC derives from water. Under high [$CO_2$] to inhibit Rubisco oxygenase activity, significant electron flow to $O_2$ has been observed, often between 10 and 30% of total electron transport (e.g. Canvin *et al.,* 1980; Furbank *et al.*, 1982). The magnitude of the Mehler reaction also can be quantified from the relationship between rates of photosynthetic electron transport estimated from Chl fluorescence versus those estimated from $O_2$ evolution. $O_2$ uptake via the Mehler reaction will "silence" a portion of photosynthetic $O_2$ evolution and thereby influence the slope of this relationship. Using this method the Mehler reaction has been shown to account for as much as 30% of total electron transport at light saturation in tropical trees (Lovelock and Winter, 1996).

Recently, transgenic tobacco expressing an antisense construct against the small subunit of Rubisco has been employed to examine photosynthetic $O_2$ reduction (Badger *et al.,* 2000; Ruuska *et al.*, 2000a). These plants possess reduced Rubisco activities and greatly depressed steady-state rates of $CO_2$ assimilation without concomitant reductions in electron transport capacity. Since reductions in Rubisco oxygenase activity parallel reductions in carboxylase activity in the transgenic plants, comparison with wild-type plants enables one to parse photorespiratory $O_2$ consumption from contributions of the Mehler reaction. Using simultaneous measurements of Chl fluorescence and gas exchange, the authors showed that the relationship between the rate of photosynthetic electron transport and the rate of $CO_2$ assimilation was linear across a range of $CO_2$ and $O_2$ concentrations and that this relationship was similar in transgenic and wild-type plants (Ruuska *et al.*, 2000a). Therefore, electron flow to the Mehler reaction was not appreciable, even in transgenic plants with strongly reduced photosynthetic light utilization. These observations were confirmed with measurements of $^{18}O_2$ uptake (Ruuska *et al.*, 2000a).

*Abbreviations:* APX – ascorbate peroxidase; Asc – ascorbate; DHA – dehydroascorbate; DHAR – dehydroascorbate reductase; Fd – ferredoxin; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; MDA – monodehydroascorbate radical; MDAR – monodehydroascorbate reductase; OEC – oxygen evolving complex; SOD – superoxide dismutase.

All of the techniques used to date to quantify photosynthetic $O_2$ reduction via the Mehler reaction come with attendant complications. Isotopic labeling/mass spectrometry and determinations of photosynthetic $O_2$ evolution require that measurements be performed under physiologically unrealistic concentrations of $O_2$ and/or $CO_2$. Estimates of whole leaf electron transport from Chl fluorescence suffer from the possibility that fluorescence emission overemphasizes the response of the upper layer of photosynthetic cells, which may not be representative of the leaf as a whole. Antisense transgenic plants might exhibit pleiotropic effects that could alter chloroplastic function or regulation. Whether superoxide formation via the Mehler reaction is, itself, a large sink for reducing equivalents (and hence, absorbed light energy) under steady-state conditions remains to be resolved. Multiple methods do agree, however, that significant electron flow to $O_2$ occurs during photosynthetic induction after a period of prolonged darkness (Neubauer and Yamamoto, 1992; Ruuska *et al.*, 2000b). Even if the Mehler reaction is ultimately shown to be a relatively minor sink at steady state, superoxide production and detoxification have profound effects on the balance between reduction and oxidation of key chloroplast constituents (i.e. the "redox state" of the chloroplast) and the response to stress. An overwhelming body of evidence, albeit indirect, of the induction of superoxide-scavenging antioxidants under conditions of stress strongly suggests that environmental conditions influence the rate of chloroplastic superoxide production and that protecting against ROS-induced molecular damage is of paramount importance for stress tolerance.

## III. The Water-Water Cycle

In the chloroplast, superoxide is detoxified by a complex and, in places, redundant series of reactions that leads to the formation of water using reducing power derived from photosynthetic electron transport. This series of reactions has acquired several different names, some of which honor important contributors to its understanding, others recognize important components, still others attempt to do both. The "Mehler-peroxidase pathway," "Ascorbate-glutathione cycle," "Halliwell-Asada pathway (or cycle)," "Foyer-Halliwell-Asada cycle" and the name I shall use through this chapter, the "Water-Water cycle" are among those commonly used. Some authors restrict the use of some of these names to narrowly defined portions of the reaction series (e.g. Asada, 1999; Mittler, 2002), however this practice is by no means uniformly applied across the literature. The name "Water-Water cycle" was coined by Kozi Asada (Asada, 1999) and recognizes the fact that water is both the original reducing agent (via water-splitting by the Oxygen Evolving Complex associated with PSII) as well as the final product of superoxide reduction. Hence, the Water-Water cycle is a series of reactions that produces nothing except a sink for photosynthetically-generated reducing equivalents (Fig. 1).

The first step in the detoxification of superoxide is its disproportionation (i.e. dismutation) to $H_2O_2$ and $O_2$. This reaction can occur non-enzymatically, but is greatly accelerated by the enzyme superoxide dismutase (SOD; EC 1.15.1.1) (McCord and Fridovich, 1969), for which there are stromal and thylakoid-associated isoforms employing various metal cofactors, including a thylakoid-associated CuZn-SOD and a stromal Fe-SOD (Kurepa *et al.*, 1997; Asada, 1999). Hydrogen peroxide, although less reactive than superoxide, must be removed from the chloroplast nonetheless, as it may disrupt photosynthesis by deactivating certain Calvin-Benson cycle enzymes, such as the reductively-activated *bis*phosphatases (Charles and Halliwell, 1981). In addition, $H_2O_2$ can decompose into the hydroxyl radical via the Fenton reaction, if superoxide is available to reduce local transition-metal cations, such as $Fe^{3+}$. The hydroxyl radical is a very powerful oxidizing agent and the most reactive ROS (Halliwell and Gutteridge, 1999); it has been described as reacting at "diffusion-controlled" rates. The area around PSI would seem acutely vulnerable to hydroxyl radical attack, as PSI binds several Fe-S clusters and is also the principal site for superoxide generation. Catalase, the antioxidant enzyme principally responsible for $H_2O_2$ detoxification in peroxisomes and other cellular compartments (Halliwell and Gutteridge, 1999), is not found in chloroplasts at appreciable activities. Instead chloroplasts dispose of $H_2O_2$ via ascorbate-specific peroxidases (APX; EC 1.11.1.11) (Jablonski and Anderson, 1982). APX catalyzes the two-electron reduction of $H_2O_2$ to water using ascorbate as a reductant and generating two monodehydroascorbate radicals, the one-electron oxidation product of ascorbate, as by-products. Several isoforms of APX are found in the chloroplast, including stromal and thylakoid-associated forms and perhaps also a lumenal isoform that has been putatively identified via analysis of the arabidopsis chloroplast proteome (Peltier *et al.*, 2002).

CuZn-SOD and APX are found in the chloroplast at approximately equimolar concentrations with P700 of PSI (Miyake and Asada, 1992; Asada, 1996).

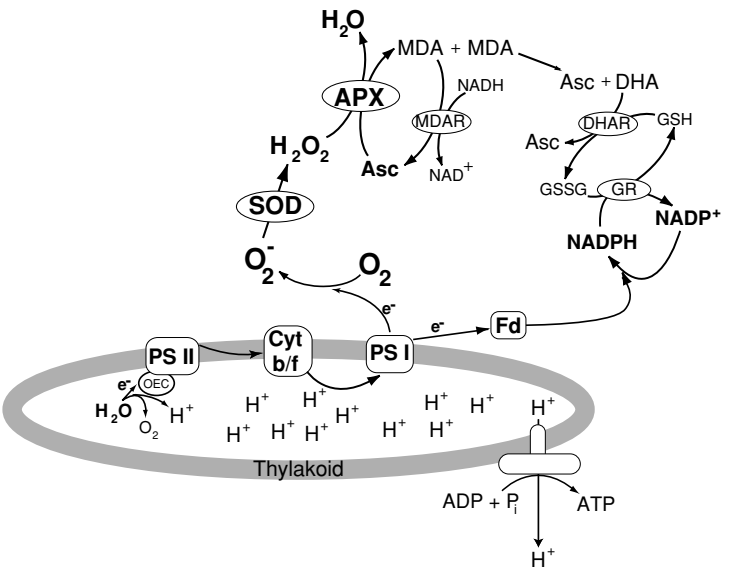


*Fig. 1.* A schematic depiction of electron flow through the water-water cycle. APX = ascorbate peroxidase; Asc = ascorbate, DHA = dehydroascorbate, DHAR = dehydroascorbate reductase, Fd = ferredoxin, GR = glutathione reductase, GSH = reduced glutathione, GSSG = oxidized glutathione, MDA = monodehydroascorbate radical, MDAR = monodehydroascorbate reductase, OEC = oxygen evolving complex, SOD = superoxide dismutase.

Immunogold labeling experiments suggests that thylakoid-associated and even stromal forms of APX are found predominantly in close association with PSI. These observations led Asada to propose the existence of a thylakoid super-enzyme complex that includes PSI, SOD and APX (Asada, 1996). If such a complex exists, it could greatly minimize the potentially harmful effects of ROS generation by catalyzing superoxide detoxification in an assembly-line fashion, thus limiting ROS escape.

Three known mechanisms re-reduce monodehydroascorbate back to ascorbate. Monodehydroascorbate can be photoreduced directly, a reaction that is thought to occur at either the cytochrome $b_6/f$ complex or at PSI (Miyake and Asada, 1992; Grace *et al.*, 1995). It can also be reduced via the activity of monodehydroascorbate reductase (MDAR), which utilizes NADH (and to a lesser extent NADPH) as a reductant (Hossain *et al.*, 1984) (Fig. 1). Additionally, two monodehydroascorbate radicals can disproportionate to form ascorbate and the two electron oxidation product of ascorbate, dehydroascorbate, which can be recycled back to ascorbate via dehydroascorbate reductase utilizing reduced glutathione (GSH) as a reductant (Hossain and Asada, 1984). Finally, oxidized glutathione (GSSG) is re-reduced enzymatically by glutathione reductase (GR; EC 1.6.4.2) utilizing NADPH as a reductant (Smith *et al.*, 1989) (Fig. 1).

In addition to the largely enzyme-driven reaction sequence described above, an ROS detoxification/ascorbate regeneration pathway that is non-enzymatic, with the exception of GSSG reduction via GR, appears to be chemically feasible (Kornyeyev *et al.*, 2003b). Ascorbate, which can be found in greater-than 10 mM concentrations in the chloroplast (Foyer, 1993), can quench superoxide non-enzymatically (Halliwell and Gutteridge, 1999) and dehydroascorbate can be reduced by GSH non-enzymatically in the alkaline conditions that one would expect to find in the stroma during illumination (Foyer and Halliwell, 1976; Winkler *et al.*, 1994). While the relative contributions of various enzymatic versus non-enzymatic reactions to oxidative metabolism are the subject of debate (see Asada, 1999; Polle, 2001), the role of non-enzymatic steps might be greatest during exposure to chilling when the demand for GSH should be high and low temperatures limit enzyme activities.

### *A. The Response of the Water-Water Cycle to Environmental Stress*

The activities/contents of antioxidants that participate in the water-water cycle undergo large changes in response to growth conditions. Acclimation can take place over the course of days to weeks after transitions in light or temperature regimes (Logan *et al.*, 1998b, 2003). The acclimation of antioxidants

to environmental stresses can be understood in terms of the effects these stresses have upon the balance between light energy absorption by the photosystems and light energy utilization via the Calvin-Benson cycle. It appears that ROS production correlates well with the level of excess light absorption (i.e. absorbed light that exceeds the capacity of photosynthetic utilization). Exposure of a broad taxonomic array of plant species to high light intensities (Gillham and Dodge, 1987; Mishra *et al.*, 1993, 1995; Logan *et al.*, 1996; Grace and Logan, 1996) results in several-fold increases in SOD, APX and GR activities and ascorbate and glutathione contents. The linkage between excess light (and not simply light intensity) and the levels of antioxidants is exemplified by the observation that *Vinca major*, a slow-growing ornamental with low capacities for photosynthesis, possesses greater foliar antioxidant activities than pumpkin, a fast-growing crop with high photosynthetic capacities, when both are raised under full-sunlight (Logan *et al.*, 1998a) (Fig. 2A, E-F). Although overall light inputs are equivalent in this experiment, greater photosynthetic light utilization by pumpkin resulted in lower levels of excess light absorption and consequently a lesser need to maintain ROS scavenging systems. Spinach raised hydroponically at either ~400 or 800 μmol photons $m^{-2}$ $s^{-1}$ possess similar SOD, APX and GR activities (B. Logan, T. Rosenstiel, B. Demmig-Adams and W. Adams, unpublished data),

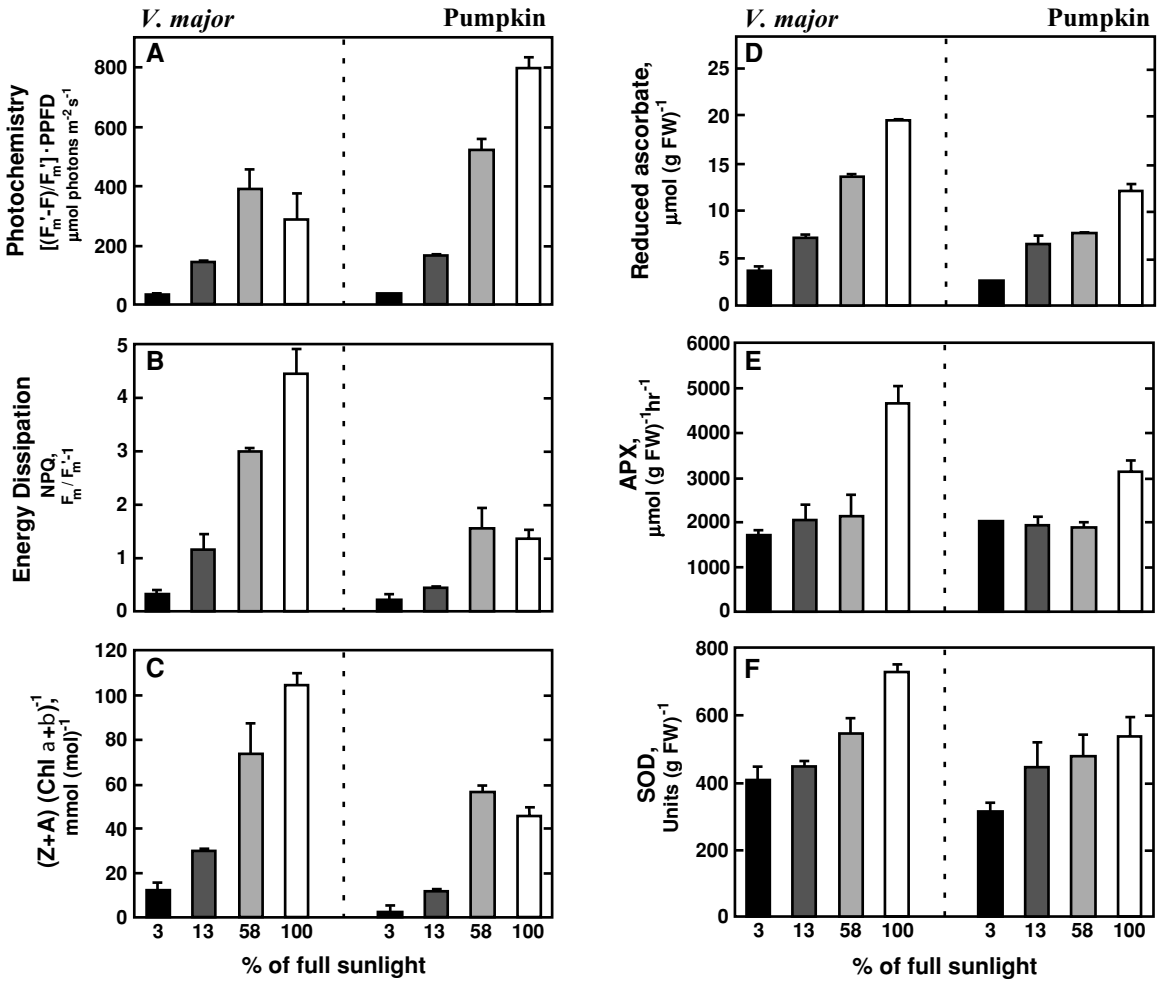


*Fig. 2.* Midday rate of photochemistry (a), energy dissipation (as nonphotochemical quenching) (b), the content of zeaxanthin and antheraxanthin (Z + A) per total Chl $a + b$ (c), reduced ascorbate content (d), ascorbate peroxidase (APX) activity (e) and superoxide dismutase (SOD) activity (e) for *Vinca major* and pumpkin acclimated to four different growth light intensities in the field and measured in their respective growth light environments. Growth light environments were achieved using neutral density shade cloth of various weaves. Values are means of three measurements from separate leaves. Error bars represent standard deviations. Redrawn from Logan *et al.* (1998a).

probably because the additional light absorbed at the higher intensity could be accounted for fully by photosynthetic utilization and therefore did not result in increased excess light absorption.

Chilling temperatures suppress Calvin-Benson cycle enzyme activity, but have little effect on the biophysical process of light absorption (Wise, 1995). Consequently, seasonally colder temperatures or experimentally-imposed chilling can greatly increase excess light absorption, even during exposure to moderate light intensities. Furthermore, chilling temperatures can also suppress antioxidant enzyme activities, further exacerbating the potential for oxidative damage. In many plant species, acclimation to chilling has been shown to involve profound increases in the activities of antioxidants (Schöner and Krause, 1990; Anderson *et al.*, 1992; Mishra *et al.*, 1993; Logan *et al.*, 1998c, 2003). The increase in antioxidant activity of white pine needles from summer to winter can exceed one hundred-fold (Anderson *et al.*, 1992). In addition to up-regulation in overall activity, some species have been shown to respond to chilling with preferential expression of antioxidant enzyme isoforms with lower temperature optima and other biochemical features that would favor activity at colder temperatures (Guy and Carter, 1984).

The response of antioxidants to drought varies. Experimental drought of wheat has been shown to bring about a short-term rise in SOD activity followed by a depression (Zhang and Kirkham, 1994), while GR activities were unaffected by drought in a field experiment (Gamble and Burke, 1984). In peas, SOD and APX activities increased as stomatal conductance fell after the onset of drought in a study by Mittler and Zilinskas (1994), whereas Moran *et al.* (1994) reported drought-induced decreases in APX, GR, ascorbate and glutathione. Some of the seeming contradictions in the findings above are likely to be the result of the many ways in which water stress can be imposed experimentally. It may also be that leaf wilting and drought-induced decreases in leaf Chl content may reduce overall light absorption and thus also reduce excess light absorption in some plant species.

Nitrogen limitation leads to depressed photosynthetic activities, as the demand for photosynthate falls with whole-plant growth and less nitrogen is available for maintenance of the photosynthetic apparatus. The resultant decrease in photosynthetic light use may be expected to lead to a compensatory increase in excess light absorption. However, plants acclimate to limiting nitrogen availability by strongly decreasing leaf Chl contents (Verhoeven *et al.*, 1997; Logan *et al.*, 1999b), and in doing so, can effectively limit excess light absorption. Thus when the antioxidant enzyme activities (SOD, APX and GR) of nitrogen-limited spinach are compared to those of nitrogen-replete plants, they do not differ statistically when expressed on a per Chl basis and are actually significantly lower on a leaf area basis. This means of coping with excess light minimizes the nitrogen investment in Chl, antioxidant enzymes and glutathione.

Little is known about the response of antioxidants to growth at elevated $CO_2$. This is remarkable given the substantial attention paid to the effects of elevated $CO_2$ on many aspects of plant biology and the relevance of this abiotic perturbation to global change. Schwanz and Polle (2001) examined the foliar antioxidants of pendunculate oak (*Quercus robur*) and maritime pine (*Pinus pinaster*) raised at high $CO_2$ (either 700 or 1200 ppm). Plants of each species under elevated $CO_2$ possessed lower SOD activities, which is consistent with the hypothesis that elevated $CO_2$ increased light utilization for photosynthesis, thereby reducing excess light absorption and ROS generation.

Cross-study comparisons of the response of antioxidants to stress can be complicated by the use of multiple bases for expression for enzyme activity. The choice of a reference basis of expression for enzyme activities (e.g. per fresh weight, per protein, per leaf area) is arbitrary to a certain degree, but it can profoundly affect the nature of the trends apparent in the data. Knowledge of (and publication of) the effect of stress factors such as drought on the basis of expression, itself, is essential if one is to evaluate data relying upon it.

### *B. Oxygen Metabolism and the Regulation of Chloroplast Redox State*

While $O_2$ photoreduction may have arisen as an unavoidable consequence of photosynthetic electron transport in an $O_2$-rich atmosphere, it now appears that superoxide production and scavenging have been co-opted into the regulatory mechanisms that minimize photoinhibition by maintaining low reduction states among electron carriers such as $Q_A$, the primary quinone acceptor of PSII. PSII centers with $Q_A$ in the reduced state are more vulnerable to photoinhibitory damage because they are more likely to undergo charge recombination as electrons get "backed up" during electron transfer. Charge recombination brings about the formation of triplet-excited Chl, a long-lived excited state that can sensitize singlet $O_2$ formation (Melis, 1999). Singlet $O_2$, in turn, can irreversibly damage Chl and proteins via oxidation.

The reduction state of $Q_A$ is determined by the balance between light energy inputs into PSII and downstream electron flow. Any process that consumes reducing equivalents and thereby increases downstream electron flow will serve to lower the reduction state of $Q_A$ and lower its vulnerability to photoinhibition. Under conditions of stress, where the ability of Calvin-Benson cycle activity to consume reducing equivalents is compromised, electron flow through the Water-Water cycle may serve in this capacity. This effect has been demonstrated in a series of experiments examining the performance of transgenic cotton with elevated activities for chloroplastic antioxidant enzymes. In comparison to the wild type, plants that overproduce either GR or APX maintain higher rates of electron transport through PSII, lower $Q_A$ reduction states and sustain less PSII photoinhibition during exposure to chilling in the light at 10°C and 500 μmol photons $m^{-2}$ $s^{-1}$ (Kornyeyev *et al.*, 2003a,b). The protective effect of APX or GR overproduction on PSII function is abolished when electron transfer from PSII is inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) (Kornyeyev *et al.*, 2001). Thus, the protection conferred by antioxidant overexpression is not due to the direct effects of enhanced ROS scavenging, instead it is due to the effect this enhancement has upon the redox state of PSII. Antioxidant enzyme overproduction is not likely to affect the rate of $O_2$ photoreduction (the Mehler reaction). Rather, it increases the rate of superoxide scavenging and in doing so increases the demand for reducing equivalents to recycle ascorbate and glutathione. This is supported by the observation that during chilling, the transgenic genotypes maintain their ascorbate and glutathione pools in more highly reduced states than wild type (Kornyeyev *et al.*, 2003a,b).

The ability of antioxidant overproduction to improve the stress-tolerance of cotton is confined to moderately chilling temperatures (10 to 15°C). During exposure to 500 μmol photons $m^{-2}$ $s^{-1}$ at 5°C, electron flow falls to near zero as PSII is almost completely reduced in wild type and transgenic cotton alike. All genotypes are equally sensitive to photoinhibition under this extreme stress. At warm temperatures (i.e. 20 to 30°C) antioxidant overproduction also brings about no enhancement in resistance to photoinhibition. It is likely that at warm temperatures the relative contribution of the Water-Water cycle to overall electron flow declines as Calvin-Benson cycle activity consumes a greater share of reducing equivalents and as low temperature restrictions on native GR activity are lifted (Kornyeyev *et al.*, 2003b, 2005).

## IV. Dissipation of Excess Absorbed Energy

In addition to the antioxidants of the Water-Water cycle, which can be thought of as reactive in their protection against oxidative damage because they detoxify ROS after they are formed, chloroplasts possess an exquisitely responsive photoprotective process that minimizes damage by proactively preventing the formation ROS altogether. This process, which is referred to as "energy dissipation," safely converts absorbed light energy into heat before it can potentiate singlet $O_2$ formation. Energy dissipation is regulated over time-scales ranging from seconds to seasons to remove excess light without compromising light use for $CO_2$ assimilation.

The biochemical reactions that comprise the photosynthetic pathway generally exhibit saturation at light intensities well below full sunlight (which is ~2000 μmol photons $m^{-2}$ $s^{-1}$) in C3 plants. In contrast, the biophysical process of light absorption by Chl saturates well in excess of full sunlight. Therefore, with the exception of those under deep, continuous shade, plants growing in the field commonly absorb light energy that exceeds their capacity for photosynthetic light utilization. Environmental stresses such as drought and chilling exacerbate the absorption of excess light because they further limit the photosynthetic pathway without exerting similar effects of light absorption (Wise, 1995).

Excess light absorption can have dangerous consequences. Chl, like most molecules, is singlet in the ground state, meaning that all electrons are found in pairs with opposing spins. When Chl absorbs a photon, it is converted to a singlet-excited state. If the energy of this singlet-excited molecule is not trapped by charge separation in the reaction center, Chl has a low, but significant, probability of undergoing a "spin flip" referred to as an intersystem crossing, which results in the formation of triplet-excited Chl (Foote, 1976). Triplet-excited Chl possesses two unpaired electrons and, like all triplet molecules, earns its name from the fact that it yields a three-line spectrum in electron paramagnetic resonance spectroscopy (Turro, 1978). Triplet-excited Chl possesses a spin restriction on energy decay back to the ground state and therefore is longer-lived than singlet-excited Chl. Triplet-excited Chl is also poised to react with ground state $O_2$ because $O_2$ is unusual in that it is triplet in the ground state (Halliwell and Gutteridge, 1999). Energy transfer between triplet excited Chl and ground-state triplet $O_2$ results in the formation of singlet $O_2$, a dangerous ROS (Asada, 1996; Niyogi,

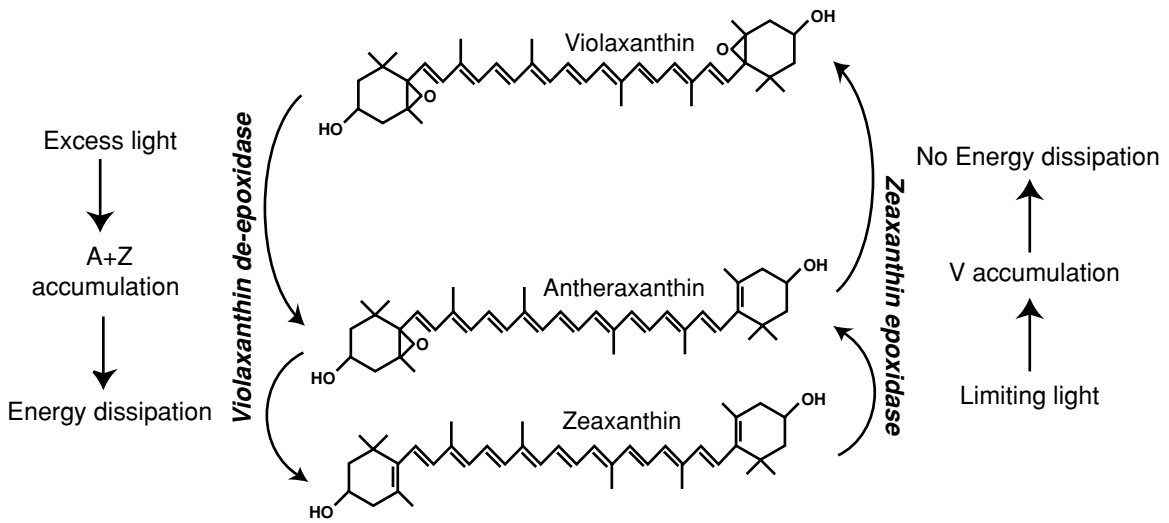


*Fig. 3.* The carotenoids of the xanthophyll cycle and the enzymes involved in xanthophyll cycle conversions.

1999). Under conditions that lead to excess light absorption, the lifetime of singlet-excited Chl in the light harvesting complexes lengthens since light energy trapping by reaction centers is saturated. The extended lifetime of singlet-excited Chl increases the probability that intersystem crossing leading to triplet-excited Chl will occur.

Energy dissipation interrupts the formation of singlet $O_2$ by converting the energy of singlet-excited Chl to heat, which is readily exchanged with the surroundings across the leaf lamina. Energy dissipation requires the presence of either zeaxanthin (Z) or antheraxanthin (A) (Gilmore and Yamamoto, 1993a, 1993b; Demmig-Adams and Adams, 1996) and a low pH in the thylakoid lumen (Fig. 3). When energy dissipation is invoked in leaves exposed to excess light, A and Z are created from the de-epoxidation of violaxanthin (V) in a reaction catalyzed by violaxanthin de-epoxidase (VDE), an enzyme localized to the thylakoid lumen. Upon return to less stressful conditions, plants reverse this reaction and reform V from Z and A via the activity of zeaxanthin epoxidase, which is localized to the chloroplast stroma. The three carotenoids and the enzymes that catalyze their interconversions are referred to as the "xanthophyll cycle." The precise role that xanthophylls play in energy dissipation remains the subject of debate and active research; however, evidence is building in favor of a direct role involving energy or electron transfer from Chl to Z (Frank *et al.*, 1994; Owens *et al.*, 1997; Ma *et al.*, 2003; Holt *et al.*, 2005). Z appears to possess an $S_1$ excited state that is lower in energy than Chl, thus making energy transfer from Chl to Z thermodynamically feasible. Alternatively, absorption of light by chlorophyll has been shown to lead to the formation of a carotenoid radical under conditions that bring about high rates of energy dissipation (Holt *et al.*, 2005). This suggests that the molecular mechanism of energy dissipation involves energy transfer to a chlorophyll-zeaxanthin dimer, which undergoes charge separation followed by recombination (Holt *et al.*, 2005).

Violaxanthin de-epoxidation is a reductive process and VDE utilizes ascorbic acid, and not the ascorbate anion, as a source of electrons (Bratt *et al.*, 1995). As a result, V de-epoxidation has a relatively low pH optimum. This biochemical feature of Z and A formation can be thought of as part of the regulation of energy dissipation, since VDE is localized to the thylakoid lumen, a compartment that one would expect to become more acidic under conditions of excess light absorption, as electron transport augments the proton gradient faster than it is utilized for ATP formation.

Energy dissipation occurs on a protein subunit of PSII known as PsbS (Li *et al.*, 2000), which has been referred to also as CP22 (Funk *et al.*, 1994). This was revealed by analyses of npq4-1, a null mutant of Arabidopsis PsbS, which is deficient in energy dissipation but possesses a fully functional xanthophyll cycle (Li *et al.*, 2000). Subsequently, transgenic plants that overproduce PsbS have been shown to exhibit higher than wild type levels of energy dissipation after abrupt transfer from darkness to intense light (Li *et al.*, 2002a). PsbS belongs to the Light Harvesting Complex protein superfamily, and has four (rather than the more

common three) trans-membrane helices (Li *et al.*, 2000). Loops of PsbS that connect trans-membrane helices and project into the thylakoid lumen possess conserved glutamic acid residues that are required for energy dissipation, as demonstrated by site-directed mutagenesis (Li *et al.*, 2002b). A compelling model is emerging from this research in which acidification of the thylakoid lumen under conditions of excess light absorption brings about the protonation of critical glutamic acid residues of PsbS, which alters the conformational structure of the protein in a way that leads to Z binding or brings previously-bound Z into an alignment with Chl to permit energy dissipation. This model is consistent with the requirement for a low lumenal pH not only for VDE activity, but also for energy dissipation itself.

Plants alter the de-epoxidation state of their xanthophyll cycle on a minute-to-minute basis throughout the day to ensure adequate protection against the damaging effects of excess light, while at the same time leaving productive use of absorbed light energy for carbon assimilation uncompromised (Adams and Demmig-Adams, 1992; Demmig-Adams and Adams, 1992a, 1996). Thus, this proactive photoprotective mechanism is exquisitely responsive and efficient, requiring only relatively rapid and energetically inexpensive catalytic conversions among pigments instead of far slower and more costly de novo synthesis. Plants acclimate on longer time scales (weeks to seasons) to prevailing conditions and the potential for excess light absorption by adjusting the size of their xanthophyll cycle pool and also the expression of PsbS. In numerous field, greenhouse and growth chamber studies, plants acclimated to more intense light environments possess larger xanthophyll cycle pools (Fig. 4) and convert a greater fraction of their xanthophyll cycle pool to Z and A at midday (e.g. Demmig-Adams and Adams, 1992b; Grace and Logan, 1996; Logan *et al.*, 1998a,b,c). When raised under similar light environments, plants with a greater capacity to utilize light for photosynthetic carbon assimilation possess smaller xanthophyll cycle pools (Logan *et al.*, 1998a) (Fig. 2A-C). Environmental stresses such as wintertime cold temperatures or low soil nitrogen availability lead to lower photosynthetic capacities. Plants have been shown to adjust Chl contents downwards in response to these environmental stresses and also to increase their xanthophyll cycle pool size (when expressed per unit Chl) (Adams and Demmig-Adams, 1994; Verhoeven *et al.*, 1996, 1997; Logan *et al.*, 1998c; Burkle and Logan, 2003). Studies of the acclimation of PsbS expression to environmental stress have just begun, however increased

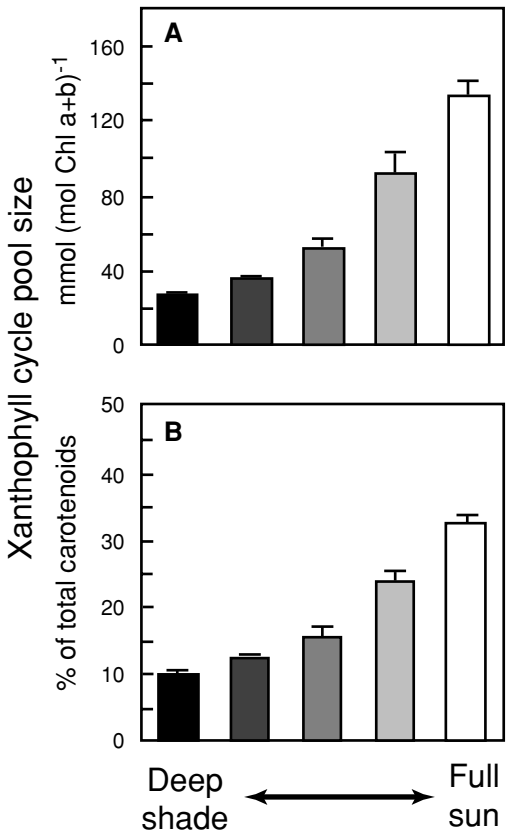


*Fig. 4.* Xanthophyll cycle pool sizes expressed per total Chl $a + b$ (a) and as a percentage of the total carotenoid pool (b) for plants collected from 5 sites representing a continuum from deeply shaded to full sun environments. Sites were located in Dorrigo National Park, a subtropical rainforest in New South Wales, Australia. Error bars represent standard error of the mean. n = 10, 12, 7, 4, and 8 different plant species for sites from deeply shaded to full sun, respectively. Redrawn from Logan *et al.* (1996).

expression (relative to the expression of PSII core proteins) has already been demonstrated in response to seasonally colder temperatures in pine (Ottander *et al.*, 1995; Ebbert *et al.*, 2005) and high light in Arabidopsis (B. Logan, K. Niyogi, unpublished data) and *Monstera deliciosa* (V. Ebbert, B. Demmig-Adams and W. Adams, unpublished data).

Highly dynamic light environments such as the forest understory present unique challenges to plants because they experience rapid, at-times large, and unpredictable fluctuations in light intensity. Sunflecks nearing the intensity of full sunlight can penetrate the overstory canopy and abruptly strike understory leaves that would otherwise be deeply shaded. Changes in light intensity can occur more quickly than plants can respond to via enzyme-catalyzed interconversions among

xanthophyll cycle pigments. Instead, plants have been shown to de-epoxidize V in response to the first sunflecks experienced each day and retain Z between sunflecks (Logan *et al.*, 1997; Adams *et al.*, 1999). The level of energy dissipation is then presumably rapidly modulated via the strength of the trans-thylakoid proton gradient and its effects on lumenal pH.

## V. Transgenic Manipulations of Photoprotection

The observation that the response to many environmental stresses, particularly chilling, includes upregulation of antioxidant systems has led plant geneticists to attempt to improve the stress tolerance of some crop species by transforming plants with genes for chloroplast-targeted antioxidant enzymes (for reviews see Foyer *et al.*, 1994; Allen, 1995). Such attempts have met with mixed results. Various degrees of protection from chilling-induced photoinhibition at high PPFD were reported for poplar overproducing chloroplastic GR (Foyer *et al.*, 1995) and MnSOD (Foyer *et al.*, 1994) and tobacco overproducing Cu/ZnSOD (Sen Gupta *et al.*, 1993a,b). In contrast to these studies, little protection was conferred by overproduction of chloroplastic FeSOD in poplars, cytosolic GR in tobacco exposed to high light (Tyystjärvi *et al.,* 1999), or chloroplastic MnSOD in cotton exposed to high light at cold temperatures (Payton *et al.* 1997).

The studies mentioned above and most others seeking to examine the stress tolerance of transgenic plants employ abruptly imposed, severe stress exposures. Typically, leaf tissues are detached from plants raised under relatively benign conditions and placed under stresses that far exceed those that the species under study might encounter in the field. Experiments of this sort have yielded important insights into the mechanisms of chilling tolerance and the regulation of oxidative metabolism, however the enhanced stress tolerance that is occasionally observed under such artificial conditions may not be predictive of enhanced stress tolerance under field conditions. For example, when leaf discs of warm-grown transgenic cotton that possess 30- to 40-fold higher chloroplastic GR activities were abruptly exposed to 10°C at 500 μmol photons $m^{-2}$ $s^{-1}$, they sustained approximately 28 and 20% lower levels of PSII and PSI photoinhibition, respectively, in comparison to wild type (Kornyeyev *et al.*, 2001, 2003b) (Fig. 5 left panel). However, chilling tolerance was not enhanced when this same cotton genotype was raised in a growth chamber in which temperatures were lowered from 28 to 14°C over 9 days and held at for a subsequent 9-day period at 14°C (Logan *et al.*, 2003) (Fig. 5 right panel). The absence of an effect of GR overproduction under longer-term, gradually imposed chilling may be

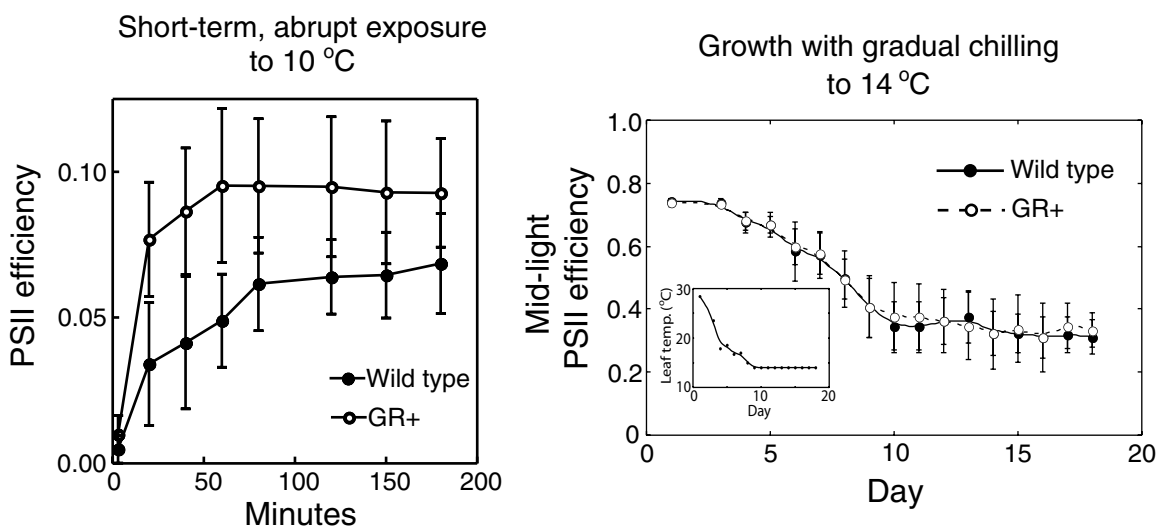


*Fig. 5.* The effect of two different chilling regimes, short-term abrupt exposure to 10°C (left panel) and growth with gradual chilling to 14°C (right panel), on the photosystem II efficiency ($[F_m'-F]/F_m'$) of wild-type cotton (closed circles) and transgenic cotton exhibiting a 30 to 40-fold overproduction of chloroplastic glutathione reductase (GR+; open circles). Error bars represent standard deviation, n = 10 − 13 for the short-term exposure and 8–16 for the gradual chilling. Redrawn from Kornyeyev *et al.* (2003) and Logan *et al.* (2003).

explained, in part, by the fact that wild-type cotton acclimated to this chilling regime by upregulating native GR activity two-fold (Logan *et al.*, 2003).

The recent discovery that PsbS is required for energy dissipation has yielded a means of transgenically enhancing capacities for energy dissipation. Arabidopsis plants that have been transformed to overproduce PsbS possess correspondingly higher capacities for energy dissipation (Li *et al.*, 2002a). In comparison to wild-type plants, PsbS overproducers sustained slightly, but significantly, less photoinhibition when raised in a greenhouse (B. Logan and K. Niyogi, unpublished data). Interestingly, however, no differences in photoinhibition were observed when these same genotypes were raised under continuous light of 1500 μmol photons $m^{-2}$ $s^{-1}$ in a growth chamber. Although the growth chamber provided much higher total daily photon fluxes than the greenhouse, the greenhouse light environment included rapid and large fluctuations in light intensity. Taken together, these results suggest that energy dissipation may be most effective at managing light stress under fluctuating light intensities.

Clearly, the nature and timing of the stress profoundly influences the response of transgenic plants with increased ROS scavenging capacity or levels of energy dissipation. This should be taken into account when assessing the utility of manipulating photoprotective processes as a strategy for developing more stress-tolerant crop varieties for agricultural use and underscores the need to design experiments that examine the performance of transgenic genotypes under realistic conditions of stress.

## VI. Extra-Chloroplastic Photoprotection

Photoprotection is not confined to chloroplast biochemistry; plants have evolved biochemical, ultrastructural and anatomical means of reducing chloroplastic light stress that reside beyond the bounds of the chloroplast envelope. These include ROS scavenging in other cellular compartments, chloroplast movements and a host of leaf surface features and morphological adjustments aimed at reducing excess light absorption.

Superoxide does not readily cross membranes and is sufficiently unstable that it is likely to react very near to its source. Hydrogen peroxide, on the other hand, is less reactive and capable of passing through membranes. This introduces the possibility that photogenerated $H_2O_2$ might diffuse out of the chloroplast and render its effects in other cellular compartments. Light stress has been shown to lead to the up-regulation of cytosolic isoforms of APX (Karpinski *et al.*, 1997; Yoshimura *et al.*, 2000). In addition, light stress very often induces the accumulation of phenolic compounds (Grace *et al.*, 1998; Grace and Logan, 2000). Certain phenolics act as efficient reductants for vacuolar guaiacol peroxidase (Yamasaki *et al.*, 1997; Yamasaki and Grace, 1998). Since phenolics tend to be concentrated in the vacuole and the vacuole occupies most of the cell volume, phenolic-assisted reduction of $H_2O_2$ to $H_2O$ might be a significant pathway for ROS detoxification.

Chloroplasts are closely associated with actin filaments of the cytoskeleton, which can control organelle position within the cell. When exposed to low light intensities, chloroplasts organize along the upper and lower planes of the cell in order to maximize light interception. However, exposure to high light intensities leads chloroplasts to migrate to the lateral walls of cells to maximize self-shading and thereby minimize light interception (Haupt and Scheuerlein, 1990; Brugnoli and Björkman, 1992). Such movements probably serve to optimize light absorption under low irradiance conditions (Williams *et al.*, 2003) and may also reduce photodamage at high irradiance (Park *et al.*, 1996; Kasahara *et al.*, 2002).

Many plants acclimated to full sunlight develop leaves with steep angles (relative to horizontal orientation) in order to reduce sunlight exposure (e.g. Mooney *et al.*, 1977). Some plant species are capable of leaf movements that influence light interception (Koller, 1990). *Oxalis oregana*, an understory herb in redwood forests of the northwestern United States, folds it leaflets downwards within ~five minutes of exposure to bright sunflecks (Powles and Björkman, 1989). Leaflets that were experimentally restrained in the horizontal position suffered almost twice as much sunfleck-induced photoinhibition (Powles and Björkman, 1989). During drought, the legume *Macroptilium atropurpureum* orients its leaves parallel to the sun's rays in order to minimize light stress and photodamage, a response referred to as paraheliotropism (Ludlow and Björkman, 1984). At the other end of the spectrum, some desert plants exhibit diaheliotropism, leaf movements that maintain leaf lamina perpendicular to the sun's rays, after periods of rainfall in order to maximize their potential for photosynthetic carbon gain during the transient period when adequate water supplies permit increased rates of evapotranspiration (Ehleringer and Forseth, 1980). Under low nitrogen availability, soybean employs a combination of midday paraheliotropism and afternoon diaheliotropism to orient leaves such that they are experiencing the light intensity where electron transport and the

Calvin-Benson cycle co-limit photosynthetic activity, thereby maximizing their return on investment of nitrogen into the photosynthetic machinery and reducing light stress (Kao and Forseth, 1992).

Leaves of various species possess highly reflective surface waxes (Barker *et al.*, 1997) or pubescence (Ehleringer and Björkman, 1978) to minimize light absorption. *Mahonia repens*, a broad-leafed evergreen native to the western United States, accumulates high concentrations of anthocyanins (which are red in color) in its upper epidermis in winter, only to "regreen" during the following growing season (Grace *et al.*, 1998). Anthocyanins may serve as a sunscreen in winter when photosynthetic light use is greatly suppressed by low temperatures. *Atriplex hymenelytra*, an evergreen desert shrub, possesses bladder-like trichomes on leaf surfaces, which are filled with salty water (Mooney *et al.*, 1977). During the moist season, when photosynthetic light use is greatest, these bladders remain hydrated and are optically transparent. In the dry season, when photosynthetic light use falls with intensifying drought, these bladders collapse, leaking their contents onto the leaf surface, where the water quickly evaporates and leaves behind a highly reflective layer of crystalline salt that reduces photodamage to mesophyll cells below (Mooney *et al.*, 1977).

## VII. Concluding Remarks

Chloroplasts are uniquely vulnerable to oxidative damage because they are the site of both $O_2$ production and of energy and electron transfer reactions that can potentiate ROS formation. Research over the last two decades has established the importance of photoprotection in maintaining a functional photosynthetic apparatus. In fact, as the elegance and intricacies of photoprotective mechanisms become more apparent, we are growing to appreciate that photosynthesis and photoprotection are tightly interwoven. Evolution has established a role for $O_2$ photoreduction and subsequent ROS scavenging in the regulation of photosynthetic electron transfer and chloroplast redox state. Antioxidants can no longer be viewed simply as "mop-up" agents that eradicate toxic ROS. ROS themselves have been shown to influence cellular signal transduction pathways that alter gene expression. Future research into the subtle interplay between the potentially harmful and the regulatory aspects of chloroplast oxidative metabolism may lead to novel strategies for employing transgenic technologies to improve the stress tolerance of crops. Or, perhaps, it will lead us simply to marvel further at the balancing act between autotrophic light use and protection against oxidative damage that plants accomplish.

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