Advances in Photosynthesis and Respiration 36
Including Bioenergy and Related Processes

Basanti Biswal
Karin Krupinska
Udaya C. Biswal *Editors*

Plastid Development in Leaves during Growth and Senescence

Springer

Plastid Development in Leaves During Growth and Senescence

This book deals with *plastid development in leaves during growth and senescence*. Leaves undergo various phases during their development finally leading to senescence and death. Deciduous trees form new leaves in spring, a process paralleled by development of photosynthetically active chloroplasts. In autumn, leaves undergo spectacular changes in colour ranging form yellow, red up to brown, a process paralleled by degradation of chlorophyll and dismantling of chloroplasts. We introduce this book with the above photographs taken by Karin Krupinska, showing at the left emerging and young leaves of *Tilia europaea* and at the right senescing leaves of *Acer tataricum* L.

Advances in Photosynthesis and Respiration
Including Bioenergy and Related Processes

VOLUME 36

The book series Advances in Photosynthesis and Respiration *Including Bioenergy and Related Processes* provides a comprehensive and state-of-the-art account of research in photosynthesis, respiration and related processes. Virtually all life on our planet Earth ultimately depends on photosynthetic energy capture and conversion to energy-rich organic molecules. These are used for food, fuel, and fiber. Photosynthesis is the source of almost all bioenergy on Earth. The fuel and energy uses of photosynthesized products and processes have become an important area of study, and competition between food and fuel has led to resurgence in photosynthesis research. This series of books spans topics from physics to agronomy and medicine; from femtosecond processes through season-long production to evolutionary changes over the course of the history of the Earth; from the photophysics of light absorption, excitation energy transfer in the antenna to the reaction centers, where the highly-efficient primary conversion of light energy to charge separation occurs, through the electrochemistry of intermediate electron transfer, to the physiology of whole organisms and ecosystems; and from X-ray crystallography of proteins to the morphology of organelles and intact organisms. In addition to photosynthesis in natural systems, genetic engineering of photosynthesis and artificial photosynthesis is included in this series. 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 and related energy processes. The purpose of this series is to improve understanding of photosynthesis and plant respiration at many levels both to improve basic understanding of these important processes and to enhance our ability to use photosynthesis for the improvement of the human condition.

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Plastid Development in Leaves During Growth and Senescence

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This book is dedicated to the memory of

Prasanna Mohanty (1934–2013)

A dear friend, an eminent plant biologist, a pioneer of photosynthesis research, and a loving teacher

From the Series Editors

Advances in Photosynthesis and Respiration Including Bioenergy and Related Processes

Volume 36: Plastid Development in Leaves During Growth and Senescence

We are delighted to announce the publication of Volume 36 in this series. The series *Advances in Photosynthesis and Respiration* was updated in Volume 35 to include the subtitle: *Including Bioenergy and Related Processes*. The front cover, which had a distinctive white background and a colored illustration, was changed to a web-friendly green background. Further, the series publisher, Springer, has made the front matter of all of the volumes freely available online. Links to each volume are given below. Readers may also notice that this volume and the past few volumes have had color figures integrated into the chapters, instead of being collected in one section of the book. This improvement was possible because of changes in the method of book production. Another change is that references to chapters in books will soon be tracked by bibliographic services. This will help authors provide evidence of the importance of their work. We hope that these updates will maintain the importance of these edited volumes in the dissemination of the science of photosynthesis and bioenergy.

This Book

Volume 36 deals with *Plastid Development in Leaves During Growth and Senescence*. We refer the readers to the excellent Preface (see pp. xxvii–xxix) by three outstanding editors of this book: Basanti Biswal (Sambalpur University, Jyoti Vihar, Odisha, India), Karin Krupinska (University of Kiel, Kiel, Germany), and Udaya Biswal (Sambalpur University, Jyoti Vihar, Odisha, India). These three, which include the wife (Basanti) and husband (Udaya) team of the Biswals and Karin Krupinska, are established authorities in the field of plant biology (see their biographies on pp. xxxi–xxxvi). An understanding of this aspect of plant life is important for all projects dealing with the production of increased biomass and bioenergy. Chloroplast development is a key process in the life of a plant. Recent advances in plant biology reveal that chloroplasts also determine the development, the structure, and the physiology of the entire plant. A number of books have emphasized the biogenesis of the chloroplast, but few have dealt with the events associated with the transformation of a mature chloroplast into a gerontoplast during senescence. This book, which has 28 chapters, is unique because it describes the process of chloroplast maturation and its subsequent transformation into a gerontoplast during senescence, a process required for nutrient recycling in plants. The book includes a state-of-the-art survey of the current knowledge of the regulation and mechanisms of chloroplast development. Authors critically discuss the signaling process, the expression potential of plastid DNA, the interaction of cellular organelles, and the molecular mechanisms associated with the assembly and the disassembly of organellar complexes. Finally, how chloroplast development is modulated by environmental

signals is discussed. We hope the readers will find this volume not only enlightening and fascinating but of practical use in their own endeavors. We are grateful to Basanti, Udaya, and Karin for their timely submission of this 28-chapter book and to all the 57 authors who contributed to this outstanding book in an area that had been somewhat neglected.

Authors

The current book contains 28 chapters written by 57 authors from 13 countries (Argentina (5); Croatia (3); France (2); Germany (8); Hungary (1); India (14); Italy (1); Japan (8); Spain (3); Sweden (2); Switzerland (2); UK (6); and USA (2)). We thank all the authors for their valuable contribution to this book; their names (arranged alphabetically) are listed below:

Mats X. Andersson (Chap. 8); Henrik Aronsson (Chap. 3); Basanti Biswal (Chaps. 1, 2, and 28); Udaya C. Biswal (Chaps. 1 and 2); Maryse Block (Chap. 7); Thomas Börner (Chap. 11); Naini Burman (Chap. 25); Cristian A. Carrion (Chap. 18); Maria L. Costa (Chap. 18); Vijay Dalal (Chap. 27); Emmanuelle Dubots (Chap. 7); Hrvoje Fulgosi (Chap. 26); Facundo M. Gomez (Chap. 18); John C. Gray (Chap. 9); Juan J. Guiamet (Chap. 18); Yukako Hihara (Chap. 5); Stefan Hörtensteiner (Chap. 16); Hiroyuki Ishida (Chap. 19); Paul Jarvis (Chap. 12); Padmanava Joshi (Chap. 28); Kengo Kanamaru (Chap. 10); Yusuke Kato (Chap. 20); Renu Khanna-Chopra (Chap. 17); Jitendra P. Khurana (Chap. 25); Karin Krupinska (Chaps. 1 and 14); Hartmut K. Lichtenthaler (Chap. 15); Karsten Liere (Chap. 11); Qihua Ling (Chap. 12); Nikola Ljubesic (Chap. 26); Eric Maréchal (Chap. 7); Mercedes Martín (Chap. 23); Dana E. Martínez (Chap. 18); Karin Meierhoff (Chap. 4); Bijaya K. Mishra (Chap. 2); Amarendra N. Misra (Chap. 28); Maria Mulisch (Chap. 14); Sergi Munné-Bosch (Chap. 22); Lalitendu Nayak (Chap. 28); Larry D. Noodén (Chap. 13); Kamlesh Kant Nutan (Chap. 17); Ashwani Pareek (Chap. 17); Matthew Paul (Chap. 24); Thomas Pfannschmidt (Chap. 22); Mukesh K. Raval (Chap. 2); Harry Roy (Chap. 6); Bartolomé Sabater (Chap. 23); Wataru Sakamoto (Chap. 20); Katalin Solymosi (Chap. 3); Kintake Sonoike (Chap. 5); Mamoru Sugita (Chap. 10); Baishnab C. Tripathy (Chap. 27); Raphael Trösch (Chap. 12); Shinya Wada (Chap. 19); Peter Westhoff (Chap. 4); Astrid Wingler (Chap. 24); Mercedes Wrischer (Chap. 26); and Michela Zottini (Chap. 21).

Our Books: 35 Volumes

We list below information on all the 35 volumes that have been published thus far (see http://www.springer.com/series/5599). We are pleased to note that Springer, our publisher, is now producing complete Tables of Contents of these books. Electronic access to individual chapters depends on subscription (ask your librarian), but Springer provides free downloadable front matter as well as indexes. As of July 2011, Tables of Contents are available for all the volumes. The website URLs of the books in the series are listed below.

- **Volume 35 (2012)** ***Genomics of Chloroplasts and Mitochondria***, edited by Ralph Bock and Volker Knoop, both from Germany. Nineteen chapters, 475 pp, Hardcover, ISBN: 978-94-007-2919-3 (HB) ISBN978-94-007-2913-0 (e-book) (http://www.springerlink.com/content/978-94-007-2919-3/)
- **Volume 34 (2012) Photosynthesis – Plastid Biology, Energy Conversion and Carbon Assimilation**, edited by Julian Eaton-Rye, Baishnab C. Tripathy, and Thomas D. Sharkey, from New Zealand, India, and USA; 33 chapters, 854 pp, Hardcover, ISBN 978-94-007-1578-3 (HB) ISBN 978-94-007-1579-0 (e-book) (http://www.springerlink.com/content/978-94-007-1578-3/)
- **Volume 33 (2012): Functional Genomics and Evolution of Photosynthetic Systems**, edited by Robert L. Burnap and Willem F.J. Vermaas, from USA; Fifteen chapters, 428 pp, http://www.springer.com/life+sciences/

book/978-94-007-1532-5 (http://www.springerlink.com/content/978-90-481-1532-5/)

- **Volume 32 (2011): C4 Photosynthesis and Related CO_2 Concentrating Mechanisms,** edited by Agepati S. Raghavendra and Rowan Sage, from India and Canada. Nineteen chapters, 425 pp, Hardcover, ISBN 978-90-481-9406-3 (http://www.springerlink.com/content/978-90-481-9406-3/)
- **Volume 31 (2010): The Chloroplast: Basics and Applications,** edited by Constantin Rebeiz (USA), Christoph Benning (USA), Hans J. Bohnert (USA), Henry Daniell (USA), J. Kenneth Hoober (USA), Hartmut K. Lichtenthaler (Germany), Archie R. Portis (USA), and Baishnab C. Tripathy (India). Twenty-five chapters, 451 pp, Hardcover, ISBN:978-90-481-8530-6(http://www.springerlink.com/content/978-90-481-8530-6/)
- **Volume 30 (2009): Lipids in Photosynthesis: Essential and Regulatory Functions,** edited by Hajime Wada and Norio Murata, both from Japan. Twenty chapters, 506 pp, Hardcover, ISBN: 978-90-481-2862-4; e-book, ISBN: 978-90-481-2863-1 (http://www.springerlink.com/content/978-90-481-2862-4/)
- **Volume 29 (2009): Photosynthesis In Silico: Understanding Complexity from Molecules,** edited by Agu Laisk, Ladislav Nedbal, and Govindjee, from Estonia, The Czech Republic, and USA. Twenty chapters, 525 pp, Hardcover, ISBN: 978-1-4020-9236-7 (http://www.springerlink.com/content/978-1-4020-9236-7/)
- **Volume 28 (2009): The Purple Phototrophic Bacteria,** edited by C. Neil Hunter, Fevzi Daldal, Marion C. Thurnauer and J. Thomas Beatty, from UK, USA and Canada. Forty-eight chapters, 1053 pp, Hardcover, ISBN: 978-1-4020-8814-8 (http://www.springerlink.com/content/978-1-4020-8814-8/)
- **Volume 27 (2008): Sulfur Metabolism in Phototrophic Organisms**, edited by Christiane Dahl, Rüdiger Hell, David Knaff and Thomas Leustek, from Germany and USA. Twenty-four chapters, 551 pp, Hardcover, ISBN: 978-4020-6862-1 (http://www.springerlink.com/content/978-1-4020-6862-1/)
- **Volume 26 (2008): Biophysical Techniques Photosynthesis**, Volume II, edited by Thijs Aartsma and Jörg Matysik, both from The Netherlands. Twenty-four chapters, 548 pp, Hardcover, ISBN: 978-1-4020-8249-8 (http://www.springerlink.com/content/978-1-4020-8249-8/)
- **Volume 25 (2006): Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications,** edited by Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger, and Hugo Scheer, from Germany and Australia. Thirty-seven chapters, 603 pp, Hardcover, ISBN: 978-1-40204515-8 (http://www.springerlink.com/content/978-1-4020-4515-8/)
- **Volume 24 (2006): Photosystem I: The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase,** edited by John H. Golbeck, from USA. Forty chapters, 716 pp, Hardcover, ISBN: 978-1-40204255-3 (http://www.springerlink.com/content/978-1-4020-4255-3/)
- **Volume 23 (2006): The Structure and Function of Plastids**, edited by Robert R. Wise and J. Kenneth Hoober, from USA. Twenty-seven chapters, 575 pp, Softcover, ISBN: 978-1-4020-6570-6; Hardcover, ISBN: 978-1-4020-4060-3 (http://www.springerlink.com/content/978-1-4020-4060-3/)
- **Volume 22 (2005): Photosystem II: The Light-Driven Water: Plastoquinone Oxidoreductase**, edited by Thomas J. Wydrzynski and Kimiyuki Satoh, from Australia and Japan. Thirty-four chapters, 786 pp, Hardcover, ISBN: 978-1-4020-4249-2 (http://nwww.springerlink.com/content/978-1-4020-4249-2/)
- **Volume 21 (2005): Photoprotection, Photoinhibition, Gene Regulation, and Environment,** edited by Barbara Demmig-Adams, William W. Adams III and Autar K. Mattoo, from USA. Twenty-one chapters, 380 pp, Hardcover, ISBN: 978-14020-3564-7 (http://www.springerlink.com/content/978-1-4020-3564-7/)
- **Volume 20 (2006): Discoveries in Photosynthesis**, edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen, from USA, Canada and UK. One hundred and eleven chapters, 1304 pp, Hardcover, ISBN:978-1-4020-3323-0(http://www.springerlink.com/content/978-1-4020-3323-0/)

- **Volume 19 (2004): Chlorophyll *a* Fluorescence: A Signature of Photosynthesis**, edited by George C. Papageorgiou and Govindjee, from Greece and USA. Thirty-one chapters, 820 pp, Hardcover, ISBN: 978-1-4020-3217 (http://www.springerlink.com/content/978-1-4020-3217-2/)
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- **Volume 14 (2004): Photosynthesis in Algae**, edited by Anthony W. Larkum, Susan Douglas and John A. Raven, from Australia, Canada and UK. Nineteen chapters, 500 pp, Hardcover, ISBN: 978-0-7923-6333-0 (http://www.springerlink.com/content/978-0-7923-6333-0/)
- **Volume 13 (2003): Light-Harvesting Antennas in Photosynthesis**, edited by Beverley R. Greenand William W. Parson, from Canada and USA. Seventeen chapters, 544 pp, Hardcover, ISBN: 978-07923-6335-4 (http://www.springerlink.com/content/978-0-7923-6335-4/)
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- **Volume 10 (2001): Photosynthesis: Photobiochemistry and Photobiophysics,** authored by Bacon Ke, from USA. Thirty-six chapters, 792 pp, Softcover, ISBN: 978-0-7923-6791-8; Hardcover: ISBN: 978-0-7923-6334-7 (http://www.springerlink.com/content/978-0-7923-6334-7/)
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Further information on these books and ordering instructions can be found at http://www.springer.com/series/5599. Contents of Volumes 1–31 can also be found at http://www.life.uiuc.edu/govindjee/photosynSeries/ttocs.html.

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

Future Advances in Photosynthesis and Respiration and Other Related Books

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

- The Structural Basis of Biological Energy Generation (Editor: Martin Hohmann-Marriott)
- Photosynthesis in Bryophytes and Early Land Plants (Editors: David T. Hanson and Steven K. Rice)
- Canopy Photosynthesis: From Basics to Applications (Editors: Kouki Hikosaka, Ülo Niinemets and Niels P.R. Anten)
- Non-Photochemical Quenching (NPQ) and Energy Dissipation in Plants, Algae and Cyanobacteria (Editors: Barbara Demmig-Adams, Gyozo Garab, William W. Adams III, and Govindjee)
- Microbial BioEnergy: Hydrogen Production (Editors: Davide Zannoni and Roberto De Philippis)
- Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling (Editors: William Cramer and Toivo Kallas)

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

- Algae, Cyanobacteria: Biofuel and Bioenergy
- Artificial Photosynthesis
- ATP Synthase and Proton Translocation
- Bacterial Respiration II
- Biohydrogen Production
- Carotenoids II
- Cyanobacteria II
- Ecophysiology
- Evolution of Photosynthesis
- FACE Experiments
- Global Aspects of Photosynthesis
- Green Bacteria and Heliobacteria
- Hydrogen Evolution
- Interactions Between Photosynthesis and Other Metabolic Processes
- Limits of Photosynthesis: Where Do We Go from Here
- Photosynthesis, Biomass and Bioenergy
- Photosynthesis Under Abiotic and Biotic Stress
- Plant Respiration II

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

In view of the interdisciplinary character of research in photosynthesis and respiration, it is

our earnest hope that this series of books will be used in educating students and researchers not only in Plant Sciences, Molecular and Cell Biology, Integrative Biology, Biotechnology, Agricultural Sciences, Microbiology, Biochemistry, Chemical Biology, Biological Physics, and Biophysics, but also in Bioengineering, Chemistry, and Physics.

Acknowledgments

We take this opportunity to thank and congratulate Basanti Biswal, Karin Krupinska, and Udaya Biswal for their outstanding editorial work and for their highly professional dealing with the reviewing process; they have done a fantastic job not only in editing but also in organizing this book for all of us. We thank all the 57 authors of this book (see the list above); without their authoritative chapters, there would be no such volume. We give special thanks to Marjorie Ann P. Pacleb, of SPi Global, for directing the typesetting of this book; her expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Ineke Ravesloot, and André Tournois (of Springer) thanks for their friendly working relation with us that led to the production of this book.

January 26, 2013 **Govindjee**

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Series Editors

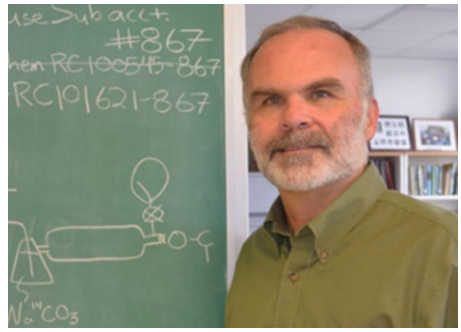
A 2012 Photograph of Govindjee. On a motorboat in Cayuga lake, Ithaca, New York. Photo by Morten Christiansen.

Govindjee, who uses one name only, was born on October 24, 1932, in Allahabad, India. We celebrated his 80th birthday last year. Since 1999, he has been Professor Emeritus of Biochemistry, Biophysics, and Plant Biology at the University of Illinois at Urbana-Champaign (UIUC), Urbana, IL, USA. He obtained his B.Sc. (Chemistry and Biology) and M.Sc. (Botany: Plant Physiology) in 1952 and 1954 from the University of Allahabad. He had studied at Allahabad under Shri Ranjan, who had been a student of F.F. Blackman in UK. Govindjee studied "Photosynthesis" at the UIUC under two pioneers of photosynthesis, Robert Emerson and Eugene Rabinowitch, obtaining his Ph.D. in 1960, in Biophysics. He is best known for his research on excitation energy transfer, light emission, primary photochemistry, and electron transfer in "Photosystem II" (PS II, water-plastoquinone oxidoreductase). His research, with many collaborators, has included the discovery of a short-wavelength form of chlorophyll (Chl) *a* functioning in the Chl*b*-containing system, now called PS II; of the two-light effect in Chl*a* fluorescence; and, with his wife Rajni Govindjee, of the two-light effect (Emerson enhancement) in NADP reduction in chloroplasts. His major achievements, together with several other researchers, include an understanding of the basic relationships between Chl*a* fluorescence and photosynthetic reactions; a unique role of bicarbonate/carbonate on the electron acceptor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants, algae and cyanobacteria; the first picosecond measurements on the primary photochemistry of PS II; and the use of Fluorescence Lifetime Imaging Microscopy (FLIM) of Chl*a* fluorescence in understanding photoprotection, by plants and algae, against excess light.

His current focus is on the "History of Photosynthesis Research," on "Photosynthesis Education," and on the "Possible Existence of Extraterrestrial Life." He has served on the faculty of the UIUC for approximately 40 years. Govindjee's honors include: Fellow of the American Association of Advancement of Science (AAAS); Distinguished Lecturer of the School of Life Sciences, UIUC; Fellow and Lifetime Member of the National Academy of Sciences (India); President of the American Society for Photobiology (1980–1981); Fulbright Scholar, Fulbright Senior Lecturer and Fulbright Specialist; Honorary President of the 2004 International Photosynthesis Congress (Montréal, Canada); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology, 2006; Recipient of the Communication Award of the International Society of Photosynthesis Research, 2007; and the Liberal Arts & Sciences Lifetime Achievement Award of the UIUC, 2008.

Further, Govindjee was honored **(1)** in 2007, through two special volumes of *Photosynthesis Research*, celebrating his 75th birthday and for his 50-year dedicated research in "Photosynthesis" (Guest Editor: Julian Eaton-Rye); **(2)** in 2008, through a special International Symposium on "Photosynthesis in a Global Perspective," held in November 2008 at the University of Indore, India; **(3)** in 2012, through dedication to him of Volume 34 of this series, celebrating his 80th year; and **(4)** through a book *Photosynthesis (Overviews on Recent Progress and Future Perspectives)* (edited by S. Itoh, P. Mohanty, and K.N. Guruprasad, released in 2012, and published by IK International Publishers, New Delhi, India), honoring him for *his outstanding research and teaching of photosynthesis and for being a global leader for stimulating photosynthesis research throughout the world*.

Govindjee is coauthor of *Photosynthesis* (John Wiley, 1969) and editor of many books, published by several publishers including Academic Press and Springer. Since 2007, each year a Govindjee and Rajni Govindjee Award for Excellence in Biological Sciences is given to graduate students by the Department of Plant Biology at the UIUC. For further information on Govindjee, see his website http://www.life.illinois.edu/govindjee.



A 2012 photograph of Thomas D. Sharkey standing next to the chalk board in his laboratory at Michigan State University.

Thomas D. (Tom) **Sharkey** obtained his Bachelor's degree in Biology in 1974 from Lyman Briggs College, a residential science college at Michigan State University, East Lansing, Michigan. After working for 2 years as a research technician, Tom entered a Ph.D. program in the federally funded Plant Research Laboratory at Michigan State University under the mentorship of Professor Klaus Raschke and graduated in 1980. Postdoctoral research was carried out with Professor Graham Farquhar at the Australian National University, in Canberra, where he coauthored a landmark review on photosynthesis and stomatal conductance that continues to receive much attention 30 years after its publication. For 5 years, Tom worked at the Desert Research Institute together with Professor Barry Osmond and then for 20 years as Professor of Botany at the University of Wisconsin in Madison. In 2008, he became Professor and Chair of the Department of Biochemistry and Molecular Biology at Michigan State University. Tom's research interests center on the biochemistry and biophysics of gas exchange between plants and the atmosphere. Photosynthetic gas exchange, especially carbon dioxide uptake and use, and isoprene emission from plants are the two major research topics of his laboratory. Among his contributions are measurements of the carbon dioxide concentration inside leaves, studies of the resistance to diffusion of carbon dioxide within the mesophyll of leaves of C_3 plants, and an exhaustive study of short-term feedback effects on carbon metabolism. As part of the study of short-term feedback effects, Tom's research group demonstrated that maltose is the major form of carbon export from chloroplasts at night and made significant contributions to the elucidation of the pathway by which leaf starch is converted to sucrose at night. In the isoprene research field, Tom is recognized as the leading advocate for thermotolerance of photosynthesis as the explanation for why plants emit isoprene. In addition, his laboratory has cloned many of the genes that underlie isoprene synthesis, and he has published many papers on the biochemical regulation of isoprene synthesis. Tom has coedited three books: (1) *Trace Gas Emissions from Plants* (T.D. Sharkey, E.A. Holland, and H.A. Mooney (eds.), Academic Press, San Diego, CA, 1991); (2) Volume 9 of this series; and (3) Volume 34 of this series. Tom joined the founder of this Series Govindjee as Coeditor from Volume 31. He is a "highly cited researcher" according to the Thomson Reuters Institute for Scientific Information. Tom is currently the Chairperson of the Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan. For further information, see his web page at http://www.bch.msu.edu/faculty/sharkey.htm.

Contents

Part II: Chloroplast Biogenesis During Leaf Development

Preface

Chloroplasts, the green plastids, harvest sunlight to convert CO_2 to organic carbon while producing oxygen from water. In addition, they manufacture phytohormones, amino acids, lipids, and thiol compounds. Because of their metabolic network and interaction with other cellular organelles, they play an important role in plant growth and development. The development of chloroplasts from proplastids is tightly coordinated with plant growth, whereas dismantling of chloroplasts in senescing leaves (chloroplast-to-gerontoplast transition) is an intrinsic feature of plant senescence, the terminal phase of plant development. Although studies on chloroplast development have mostly focused on photosynthesis, it is known to be also important for regulating plant growth and development. Another significance of chloroplast development, during the life of the plant, is the response to environmental stress. The chloroplast is known to be a sensor of changes in the environment. For example, some of the intermediates of biosynthetic pathways of pigments, specifically of chlorophylls, are photodynamic in nature and therefore make plants sensitive to abiotic stresses. Moreover, energy distribution within the photosynthetic apparatus and redox changes of the components involved in electron transport are sensitive toward the continuously changing environment including abiotic and biotic stresses. The sensitivity toward changes in environmental factors is likely to change with development. Possible changes in redox homeostasis, energy imbalance, and perturbation in metabolic networks during biogenesis and senescence of chloroplasts are likely to contribute to stress responses and adaptation of the whole plant.

Although chloroplast development is recognized as one of the major events in the development of plants, only few books have addressed this topic in the last decade. The books by J.T.O. Kirk and R.A.E. Tilney-Bassett (*The Plastids: Their Chemistry, Structure, Growth and Inheritance*, 1967, Freeman, London); by N.R. Baker and J. Barber (*Chloroplast Biogenesis*, 1984, Elsevier, Amsterdam); and by J.R. Ellis (ed.) (*Chloroplast Biogenesis*, 1984, Cambridge University Press, Cambridge and New York) are now outdated. Volume 23 of this series, *The Structure and Function of Plastids* (edited by R.R. Wise and J.K. Hoober, 2007), covers only a few topics (Chaps. 2, 7, 9, 15, 17, and 22) on chloroplast development, obviously not the focus of that volume. The books published thus far have emphasized primarily chloroplast biogenesis (buildup) during the greening process, but the events associated with yellowing at the end of leaf development have generally been neglected. Chloroplast development during leaf senescence when mature chloroplasts are transformed into senescing chloroplasts (gerontoplasts) had been de-emphasized for historical reasons. The process of senescence was ill-defined for a long time, but the advances made in plant molecular biology in the recent past have facilitated expansion of the knowledge of this process, of its mechanism, as well as its regulation. The availability of genome sequences of plants, specifically that of Arabidopsis, and extensive molecular genetic studies with it as a model system have allowed us to address several complex problems associated with the conversion of mature chloroplasts to gerontoplasts. A large volume of literature on the genetic program of leaf development and senescence, including gene expression and regulation, signaling systems, regulated dismantling of cellular fabrics, and molecular disassembly of the chloroplast during senescence, is now available. The progress in the field of plant senescence during the last decade has been discussed in several books and reviews. These include *Plant Cell Death Processes* (edited by L. D. Noodén, 2004, Academic

Press/Elsevier, San Diego, CA), *Senescence Processes in Plants* (edited by S. Gan, 2007, Wiley), and *Senescence Processes and Their Regulation* (edited by K. Krupinska and K. Humbeck, 2008, in *Plant Biology*) (Special Issue: Plant Senescence), German Botanical Society and The Royal Botanical Society of the Netherlands. They provide extensive information on the molecular mechanisms and regulation of the catabolic processes associated with chloroplast senescence and death. A broader view on chloroplast biogenesis and development was presented in the book *Chloroplast Biogenesis: From Proplastid to Gerontoplasts*, authored by U.C. Biswal, Basanti Biswal, and M.K. Raval (2003, Springer).

In the current volume, we present an updated and comprehensive overview of the knowledge on plastid biology during the entire life span of the leaf, ranging from chloroplast biogenesis to the transition into gerontoplasts. This volume, with 28 chapters, is unique in the series Advances in Photosynthesis and Respiration *Including Bioenergy and Related Processes* since the chapters describe the way the photosynthetic machinery (the green chloroplast) is built and subsequently transformed into another machinery (the yellow chloroplast/the gerontoplast) responsible for nutrient recycling. Authors (see List of Contributors, pp. xxxvii–xl) of the chapters (see Table of Contents, pp. xvii–xxv) are established scientists from different countries working in the area.

This book is divided into six parts. Part I, with two chapters, provides an introduction to chloroplast development, with a brief description of developmental transients, mechanisms, signaling, and regulation of the process. Chapter 1 outlines not only the current knowledge of chloroplast development but includes a discussion of the dynamic role of the organelle in integrating plant growth and development. In spite of the massive use of molecular biological tools, many questions concerning organelle development remain unanswered, suggesting limitations of these tools. It is time now to look for answers beyond the genetic basis of chloroplast development. Chapter 2 includes a discussion of the limitations of genomics, proteomics, and metabolomics and of the application of thermodynamic principles to understanding the developmental process.

The development of tools of bioinformatics and the rapid progress in plant molecular biology have significantly contributed to the expansion of knowledge in the area of biogenesis of the organelle during leaf development. The recent crystallographic studies of chloroplast complexes provide the molecular framework and geometry of the association of components of the complexes that have facilitated studies in elucidating the structural assembly of organelle complexes. In fact, the recent study of the structure of the intermediates of Rubisco (see Chap. 6) at atomic resolution during the biogenesis of this enzyme complex is a big step forward in our understanding of the assembly processes of multimeric protein complexes. The development of proplastids and etioplasts, which are the precursors of the mature organelle, into chloroplasts is discussed in Part II in seven chapters (Chaps. 3, 4, 5, 6, 7, 8, and 9) that critically review the current literature on the synthesis, trafficking, assembly, and regulation of thylakoid complexes and Rubisco. In Part III, Chaps. 10, 11, and 12 focus on the dynamic nature, expression, regulation, and stability of the plastid genome during its development as well as on targeting of nuclear-encoded plastid proteins.

Genetically programmed dismantling of chloroplast during leaf senescence, the regulated transformation of a mature chloroplast to a gerontoplast, constitutes one of the major current areas of study of plastid biology. Part IV contains eight chapters (Chaps. 13, 14, 15, 16, 17, 18, 19, and 20) that deal with the definition and concept of plant senescence, ultrastructural changes of thylakoid membranes, degradation of proteins and pigments, and their degradation pathways, mechanisms, and regulation. Part V includes four chapters (Chaps. 21, 22, 23, and 24) that describe the organellar control of chloroplast development and the role of chloroplasts in

regulating leaf senescence involving interorganellar signaling. Finally, the last part (Part VI) includes four chapters (Chaps. 25, 26, 27, and 28) that deal with responses of the developing chloroplast to environmental signals. These chapters critically dissect the mechanisms and control of the modulation of chloroplast biogenesis and of senescence during leaf growth and development in different environmental settings.

We thank all our contributors and reviewers of the different chapters of this volume and hope that this volume will be a valuable source of information and reference for those working in photosynthesis and plant developmental biology in general. As the book consists of extensive illustrations and graphics, it may also be used as a textbook by graduate and advanced undergraduate students.

We are grateful to Govindjee, who appreciated our comprehensive concept of chloroplast biology and invited us to edit this volume of the series, and to Thomas D. Sharkey, who is his co-series editor. Govindjee's valuable comments, continuous support, and timely advice during the preparation of this volume were of great help and inspiration to us.

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The Editors

Basanti Biswal was born in Parlakhemundi in the Gajapati district of Odisha, India. During 1956–1966, she attended schools in Cuttack and Bhubaneswar, Odisha, and then joined Buxi Jagabandhu Bidyadhar (BJB) College, Bhubaneswar. She received her B.Sc. (major in Botany) in 1970 from Khallikote College, Berhampur, and her M.Sc. (Botany) in 1973 from Berhampur University, Odisha. She was awarded a Ph.D. in 1984 and a D.Sc. in 2010 from Sambalpur University, Odisha, for her work on "Chloroplast Development and Senescence." Before obtaining her Ph.D., under the supervision of Professor Udaya Chand Biswal, Basanti had worked as an Associate Scientist in 1981 with Professor Hans Mohr at the University of Freiburg, Germany; there, she had worked on "Chlorophyll Biosynthesis in Pinus." In 1985, she was a Research Associate of the Council of Scientific and Industrial Research (CSIR), New Delhi, and then in 1986, she became a Lecturer in the School of Life Sciences, Sambalpur University, where subsequently she was promoted to a Reader and finally a Professor in 2007. At Sambalpur University, she has been involved in teaching and research in developmental biology, plant physiology, biochemistry, and photobiology. Her research work has significantly contributed to an understanding of the mechanism of regulation of chloroplast development and senescence. Her early work in the 1980s, when the regulation of senescence was not clearly understood, provided important information on the nuclear control of chloroplast demolition during senescence. She has also demonstrated the role of photoreceptors, such as the phytochrome and blue light receptors, in modulating leaf senescence, both in lower and higher plants. Currently, Basanti is working on "Stress Modulation of Leaf Senescence and Photosynthetic Response to Environmental Stress." She has had extensive collaboration with several laboratories in India and abroad. In 1990, she was a Visiting Professor in the Department of Chemistry, Nebraska University, Lincoln, USA, and has worked with Professor Pill-Soon Song on the involvement of G-proteins in phytochrome-mediated signaling in plants. While on a Commonwealth Fellowship (1992–1993), she collaborated with Dr. Howard Thomas of the Institute of Grassland and Environmental Research, Aberystwyth, UK, and with Professor Lyndon J. Rogers of the Department of Biochemistry, University

of Aberystwyth, UK, on the stability of the D1 protein of Photosystem II of chloroplasts during leaf senescence of *Festuca pratensis*. In 1994, she visited Professor Horst Senger's laboratory in Philipps University, Marburg, Germany, supported by a DAAD (Deutscher Akademischer Austauschdienst) visiting fellowship program. In 1997, Basanti was invited by Professor Akira Watanabe of the Department of Biological Sciences, University of Tokyo, to work in the area of molecular biology of plant development with the support of a Japanese Society for the Promotion of Science (JSPS) fellowship. She worked there on the involvement of sugar and calcium signaling in regulating the expression of senescence-associated genes. In 2007 and again in 2009, she visited the University of Kiel in Germany for collaborative research work with Professor Rüdiger Schulz. In 2011, she visited the University of Alcala in Spain for collaborative research with Professor Bartolomé Sabater. In addition to many original research papers, Basanti has written several invited reviews and book chapters and has coauthored a research monograph *Chloroplast Biogenesis: From Proplastid to Gerontoplast* (Springer). She has mentored several Ph.D. and M.Phil. students at Sambalpur University. Basanti has served as a member of the editorial board of the *Journal of Photochemistry and Photobiology B: Biology* (Elsevier). She is also acting as the Editor-in-Chief of the Indian Photobiology Society Newsletter, Kolkata, India. Currently, Basanti is one of the consulting editors of the series Advances in Photosynthesis and Respiration *Including Bioenergy and Related Processes* (Springer). In 1997, Basanti received the prestigious Samant Chandra Sekhar Award of the Government of Odisha and is now a Fellow of the National Academy of Sciences, India.

Karin Krupinska was born in Kassel, Germany. She now lives close to Kiel, Germany, where she is a Professor of Plant Cell Biology in the Institute of Botany at the Christian-Albrechts-University. She received her doctoral degree in 1984 from Philipps University in Marburg, Germany, under the supervision of Horst Senger. Her dissertation addressed the thylakoid membrane organization in chloroplasts from synchronous cultures and pigment mutants of the green alga *Scenedesmus obliquus*. In 1986, Karin received an EMBO (European Molecular Biology Organisation) fellowship for postdoctoral research in the laboratory of Diter von Wettstein at the Carlsberg Research Centre in Copenhagen, Denmark. During her stay in Carlsberg, she identified the cytochrome b_{559} genes in the barley plastid genome and was able to overexpress them. After 2 years in Copenhagen (1986–1987), she went to Kiel, where she studied light- and age-dependent changes in the transcription of plastid genes in barley (*Hordeum vulgare* L.) as a postdoctoral research associate in the laboratory of Klaus Apel at the Institute of Botany. Three years later, she accepted an appointment as a Junior Research Leader at the Center of Applied Plant Molecular Biology in the Institute of Botany at the University of Hamburg, Germany, where she remained for 5 years (1990–1995). During these years, she studied functional and molecular changes occurring during senescence of flag leaves of barley under field conditions. In 1995, she accepted her first appointment as a Professor of Botany at the University of Cologne (*Köln*), Germany, where she stayed until 1998. Since 1998, she has been a Professor in the Institute of Botany at the University of Kiel, where she has served for 5 years (2000–2005) as Chair of the institute. Since 2000, she has been the Head of the Central Microscopy research facility of the Faculty of Mathematics and Natural Sciences at the Christian-Albrechts-University of Kiel. From 2002 to 2008, she was the leader of the Research Training Group on Natural Antioxidants. Her area of focus in this project was the functional significance of tocopherols during leaf senescence. Karin has organized several symposia on plant senescence processes, for example, the 2007 Third European Workshop on Plant Senescence (Salzau, Germany); participants of this meeting contributed to a special issue of *Plant Biology* coedited by Klaus Humbeck and herself (*Plant Biology* 10, Supplement 1, 2008). Since January 2011, Karin has been the leader of the European Marie Curie Initial Training Network CropLife that includes eleven research groups from six European countries (Denmark, France, Germany, United Kingdom, Poland, and Switzerland). Within this project, the important impact of leaf senescence on crop productivity is being

investigated. Karin has served as a cochair of the Gordon Research Conference on Plant Senescence (held in Stonehill College near Boston, Massachusetts, USA, July 2012). Besides leaf senescence, her long-term research concerns the function and molecular composition of plastid nucleoids. A milestone of her research was the discovery that the plastid nucleoid–associated protein WHIRLY1 is located in both the chloroplasts and the nucleus within the same cell. A major focus of her research is the role of WHIRLY1 in plastid-to-nucleus signaling which might involve translocation of the protein from plastids to the nucleus. Further current research activities of Karin include identification of transcription factors involved in the regulation of senescence in barley leaves and their distribution within the cell.

Udaya Chand Biswal, after his school education, joined, in 1962, Ravenshaw College, now Ravenshaw University, Odisha, India. He obtained his bachelor degree in 1966 with Honors in Botany. Subsequently, during his 2-year M.Sc. program, Professor Gadadhar Misra, a renowned photobiologist, introduced him to the biology of yellow leaves (senescing leaves) for his research project. This work, which he had conducted for his M.Sc. project, was published in 1973 in the Botanical Gazette (G. Misra and U.C. Biswal (1973) Factors concerned in leaf senescence. I. Effects of age, chemicals, petiole, and photoperiod on senescence in detached leaves of Hibiscus rosa-sinensis L, Bot Gazette 134(1) 5–11.) at a time when leaf senescence and chloroplast dismantling during the process did not draw serious attention by plant biologists. The publication cited above, however, inspired Udaya to work in this area in subsequent years of his scientific career. In 1971, he joined as a Lecturer in the Department of Botany at Berhampur University, Odisha, India. He then went for his doctoral studies to the School of Life Sciences at the Jawaharlal Nehru University (JNU) in New Delhi, India. In 1977, he was awarded the Ph.D. degree from JNU for his work on aging-induced alterations in the structure and function of chloroplasts of detached barley leaves under the joint guidance of Professors Prasanna Mohanty and Gauri S. Singhal. During 1980–1982, Udaya worked with Professor Hans Mohr at the University of Freiburg in Germany. With Mohr, he worked on the changes in the pigment composition and ultrastructure of chloroplasts during senescence of the cotyledons of *Sinapis alba* and their regulation by phytochrome. This collaborative work was sponsored by the Humboldt Foundation of Germany. From 1984 until his retirement in 2005, Udaya served as a Professor in the School of Life Sciences, Sambalpur University, Odisha. At Sambalpur University, he developed an active group with research scholars from Physics, Chemistry, and Biological Science background to study photosynthesis with an interdisciplinary approach. The group has contributed significantly to the knowledge of the alterations in primary photochemical reactions of thylakoids of higher plants during senescence and stress response. Udaya's group has demonstrated that Photosystem II (PS II), specifically the oxygen-evolving complex (OEC), is the major target of both senescence and stress. In the early 1980s, his laboratory proposed theoretical models on the structure of PS II, when the structural details of the photosystem at atomic resolution were not available. After his retirement from professorship, he has continued his research in collaboration with Basanti Biswal (coeditor of this volume)

on the photosynthetic response of senescing plants to UV radiation, cell wall catabolism, and sugar signaling involved during senescence and stress response. Udaya has worked with Professor Rüdiger Schulz of the University of Kiel, Germany, on "Quinol Oxidase of Thylakoid Membranes of *Synechocystis* PCC 6803," during his visits there in 2007 and 2009; this collaboration was supported by the Humboldt Foundation. In addition to his many research publications, Udaya has authored several book chapters, reviews, as well as a research monograph *Chloroplast Biogenesis*. In 1989, he, along with G. Britton, edited the book *Trends in Photosynthesis Research* (Agro Botanical Publishers, India). Udaya was on the editorial board of the *Journal of Photochemistry and Photobiology B: Biology* (Elsevier). He has served as the President of the Indian Photobiology Society, Kolkata (2005–2007), and then joined as the Vice-Chancellor of Sambalpur University in 2007. For his achievements in the field of research in plant sciences, he received the Samant Chandra Sekhar Award in Life Sciences in 1995 and the prestigious Prana Krushna Parija National Award in 2008. He is a Fellow of the National Academy of Sciences, India.

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Part I

General Aspects of Chloroplast Development

Chapter 1

The Dynamic Role of Chloroplasts in Integrating Plant Growth and Development

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Summary

This chapter refers to the entire book and uses the information presented in the individual chapters to provide a brief survey of the current knowledge of plastid development and chloroplast biology, which reaches far beyond the photosynthetic function of the organelle. The organelle significantly modulates plant growth, development and senescence. The development of chloroplasts is closely associated with the development of the whole plant. Its development involves both nuclear and plastid gene expression and environmental modulation. Although the levels of transcriptional and post-transcriptional control of gene expression and the import of nuclear encoded proteins into the organelle have been studied intensively, the coordinated assembly of the multimeric complexes, required for chloroplast function, still remains a mystery. New ideas are emerging on the expression potential of plastid DNA, its stability and regulation during development of the organelle. The regulation of chloroplast development involves interactions of cellular organelles, exchange of metabolites, participation of phytohormones, reactive oxygen species (ROS) and intensive cross-talk with the nucleus (anterograde and

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retrograde signaling). Chloroplast development begins with proplastid-to-chloroplast transformation that involves coordinated synthesis of lipids, proteins and pigments. In multimeric protein complexes bound to thylakoids or located in the stroma, the proteins and cofactors assemble in sequence with a definite stoichiometry. On the other hand, transformation of mature chloroplasts to gerontoplasts during leaf senescence causes regulated disassembly of the structural fabric of the organelle with loss in photosynthesis. The mechanisms of senescence induced degradation of pigments, proteins and lipids follow distinct pathways. The enzymes involved in the degradation are largely known. The degradation pathways occur inside and outside of plastids; the latter is mediated by autophagy, participation of senescence associated vacuoles (SAVs) and Rubisco containing bodies (RCBs). The process is associated with expression of senescence associated genes (*SAGs*). Chloroplasts, both during biogenesis and senescence, respond to the environmental changes and adapt with appropriate modifications, in response to the changes. Finally, in this chapter we have raised several unanswered questions to be addressed in the future and have provided a critical discussion on the direction of further research in the area.

I. Introduction

Types of plastids and diversities in their structure, function and location have been reviewed, in 2006, in volume 23 of this series (*Advances in Photosynthesis and Respiration Including Bioenergy and Other Processes*); this book was edited by Robert Wise and Kenneth Hoober. Photosynthetically active chloroplasts, located in all plant and algal cells, are the best characterized plastids. Chloroplasts develop from either proplastids in meristematic cells or from etioplasts formed in the dark. During senescence chloroplasts are transformed into gerontoplasts, a term first coined by Sitte (1977). Proplastids are the precursors of all plastid forms including chloroplasts. The development of chloroplasts from proplastids and their subsequent transformation into gerontoplasts is tightly coupled to plant development (Fig. 1.1).

Examples for the coupling of plastid development and leaf expansion are monocot leaves, having meristematic cells at the base, and the oldest cells at the tip (Boffey et al. 1980; Biswal et al. 2003). Although the primary role of chloroplasts is to harvest sunlight and to convert it to organic carbon and subsequently to other essential metabolites, their role further extends to numerous aspects of whole-plant growth and maturation (Lopez-Juez and Pyke 2005; Lopez-Juez 2007; van Doorn and Yoshimoto 2010). Chloroplasts, and their biogenesis in particular, signal and modulate several crucial plant developmental processes like embryogenesis and leaf growth as well as plant stress responses and plant microbial interactions (Inaba and Ito-Inaba 2010).

The developmental stage of plastids is not only an excellent reporter for the developmental stage of the cell in a given tissue, but has an impact on the overall development of the plant. Analyses of Arabidopsis mutants with deletions of genes encoding plastid targeted proteins revealed severe disturbances in overall plant development (Inaba and Ito-Inaba 2010). This shows that plastids play an essential role for plant development far beyond their fundamental roles in the photosynthetic production of metabolites and ATP (Fig. 1.1).

Abbreviations: ABA – Abscisic acid; ER – Endoplasmic reticulum; GFP – Green fluorescent protein; JA – Jasmonic acid; NEP – Nuclear encoded phage-type RNA polymerase; PEP – Plastid encoded RNA polymerase; PSI – Photosystem I; PSII – Photosystem II; RCBs – Rubisco containing bodies; ROS – Reactive oxygen species; Rubisco – Ribulose bis phosphate carboxylase oxygenase; SA – Salicylic acid; *SAGs* – Senescence associated genes; SAVs – Senescence associated vacuoles; TIC – Translocon of the inner envelope membrane of chloroplast; TOC – Translocon of the outer envelope membrane of chloroplast; T6P – Trehalose 6-phosphate

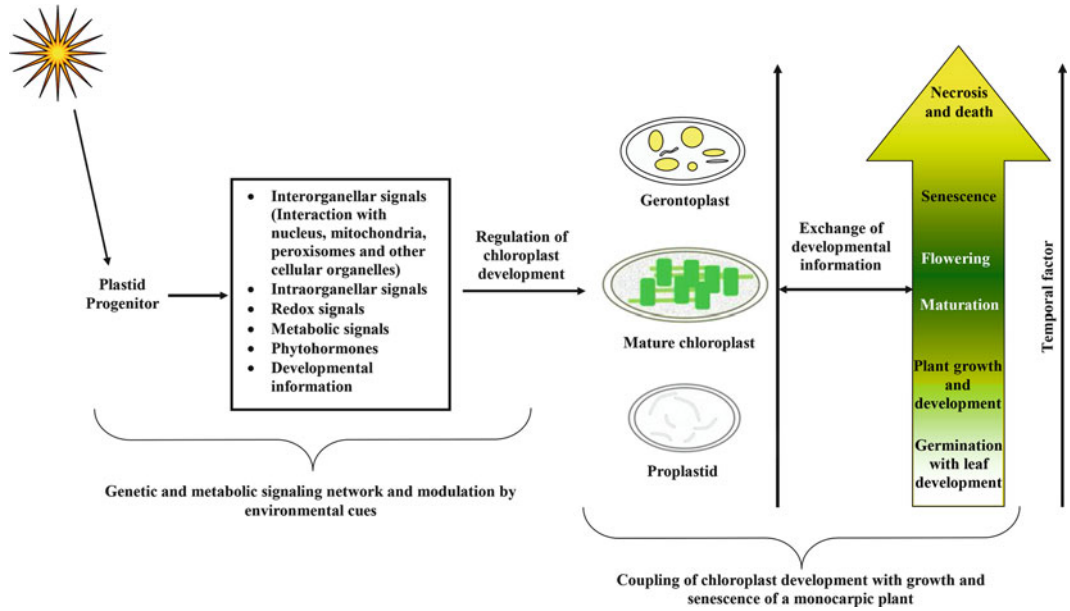

Fig. 1.1. Role of plastid development in integrating growth, maturity and senescence of a monocarpic plant. Proplastid to chloroplast transition associated with seedling growth and maturity and formation of gerontoplasts during leaf senescence indicate close association between the plastid and whole plant development. These developmental transitions involve interactions of several internal and environmental factors. Light is considered as one of the major factors initiating developmental signal cascades and regulating complex molecular and metabolic networks for plastid development from the progenitor, the proplastid. In addition, the temporal factor and exchange of developmental information play important roles in coordination of plastid development and whole-plant development. The scheme presented in the figure was designed on the basis of the current literature available in the field and primarily from the reviews of Inaba and Ito-Inaba (2010) and Pogson and Albrecht (2011).

Based on all the chapters of the book, Chap. 1 presents a survey of the current knowledge of chloroplast development during plant growth being of pivotal importance for plant productivity. It gives an overview of the classical topics of plastid research such as the regulation of the genome copy number and the expression of plastid genes during chloroplast development as well as the biosynthesis of pigments and proteins and their assembly into functional complexes of the thylakoid membrane and stromal components such as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase). Furthermore, this chapter includes a discussion of recent genetic research on the importance of plastids for overall plant development and on interaction of plastids with other compartments, in particular, the nucleus and the mitochondria. All aspects of plastid research are discussed with respect to plastid development and its environmental modulation.

To understand the role of plastids in overall plant development, an interdisciplinary approach including biochemistry, physiology, molecular biology, genomics, proteomics and biophysical methodology and also thermodynamic aspects is required (Raval et al., Chap. 2).

II. The Plastid Genome: Gene Dosage and Gene Expression During Development

Plastids contain numerous copies of their genome organized in structures called nucleoids (Sakai et al. 2004). The numbers of nucleoids and plastid DNA copies show substantial increase at early stages of chloroplast development. This ensures, on one hand, that plastids don't lose their genome during division, but, on the other hand, high genome copies meet the increasing demand for plastid

gene products during chloroplast development (Liere and Börner, Chap. 11). Although the copy number of the plastid genome increases during early chloroplast development, it decreases during leaf senescence. The fate of plastid DNA during senescence seems to be regulated in a species specific manner whereby in some species a considerable decline in the plastid DNA level was observed. In Chap. 11, Liere and Börner discuss the implications of preservation and degradation of plastid DNA during plastid development.

Plastids have a highly complex pattern of transcripts which differs between different types of plastids (Zhelyazkova et al. 2012) and also during chloroplast development. Although the major control of plastid gene expression is exerted at the post-transcriptional level (Meierhoff and Westhoff, Chap. 4), transcription is also involved in the developmental control of gene expression. Likewise as the multimeric complexes of the thylakoid membrane, the transcriptional apparatus of plastids is of dual genetic origin. Plastids possess a plastid encoded RNA polymerase (PEP) and a nuclear encoded phage-type RNA polymerase (NEP). PEP requires for functionality, in addition to the plastid-encoded components, one of several nuclear-encoded sigma factors (Kanamaru and Sugita, Chap. 10). *Arabidopsis thaliana* has six sigma factors which differ in abundance during chloroplast development, in different organs and cell types. Investigations with sigma factor mutants revealed that transcriptional regulation is important for fine-tuned plastid gene expression (Chap. 10).

Most plastid proteins are encoded in the nuclear genome and are post-translationally imported into the organelle (Ling et al., Chap. 12). The current knowledge on the components and structure of the complex canonical plastid translocon system, consisting of TOC and TIC (translocon of the outer/inner envelope membrane of chloroplasts) is mainly based on biochemical analyses with mature chloroplasts and has been summarized in various reviews (Soll and Schleiff 2004; Jarvis 2008; Li and Chiu 2010). TOC and TIC are hetero-oligomeric protein complexes in the outer and inner envelope membranes that incorporate functional receptors, channels, motors and regulators. In 1998, the stromal processing peptidase mediating cleavage of the N-terminal transit sequence was identified (Richter and Lamppa 1998). Information on the complete genome of *Arabidopsis thaliana* and studies with mutants lacking one or the other component of the TOC/TIC system have shown that the apparatus is much more complex and heterogeneous than anticipated. Multiple TOC receptors enable the operation of different import pathways with different substrate preferences. These are likely to play important roles in the differentiation of plastids (Chap. 12). Recently, Ling et al. (2012) have shown that chloroplast development is regulated by remodeling of the protein import machinery. They have detected a RING-type ubiquitin E3 ligase which targets components of the plastid protein import machinery. In an Arabidopsis mutant lacking this ligase, chloroplast biogenesis and chloroplast-to-gerontoplast transformation were shown to be impaired. This study shows that plastid development is under the direct control of the ubiquitin-proteasome system. The development associated changes in the plastid proteome are in addition caused by changes in the targeting destinations of certain plastid proteins which might be regulated by posttranslational modifications of the precursor proteins. It has become increasingly apparent that a significant number of proteins has more than one destination. The most common form of dual targeting is transport to both plastids and mitochondria (Small et al. 1998; Carrie et al. 2009). The most striking example reported for dual targeting occurs among the aminoacyl-tRNA-synthetases, where 15 out of 24 organellar proteins are targeted to plastids and mitochondria (Duchene et al. 2005). Dual targeting was also observed for few proteins transported to either plastids or mitochondria and the nucleus (Krause and Krupinska 2009). One example is the WHIRLY1 protein being located in both plastids and the nucleus (Grabowski et al. 2008). Recently, it has been shown to be retranslocated from plastids to the nucleus (Isemer et al. 2012).

Studies on the proteome of plastids have shown that not all proteins use the canonical TOC/TIC import system, but alternative routes as well (Chap. 12). One of these passes through the endoplasmic reticulum (ER) and the Golgi apparatus. An example of a plastid protein targeted to the organelle via ER derived vesicles is the carbonic anhydrase (Villarejo et al. 2005). Another example is a cysteine protease synthesized in the ER which has been identified in the stroma proteome of chloroplasts (Bayer et al. 2011).

III. Chloroplast Biogenesis Through Different Routes

In the light, chloroplasts develop from proplastids. This route of chloroplast development is best investigated in monocot leaves where a gradient of cells of different age with basal meristematic cells containing proplastids, and mesophyll cells at the tip containing fully differentiated chloroplasts are found (Boffey et al. 1980). In this book the very fast light-dependent etioplast-to-chloroplast development is discussed as a paradigm for chloroplast development (Solymosi and Aronsson, Chap. 3). Etioplasts have a unique inner membrane consisting of the paracrystalline prolammellar body (PLB) and lamellar prothylakoids. The factors affecting the formation of these structures and their molecular compositions are also reviewed by Solymosi and Aronsson (Chap. 3). Their specific lipid and protein compositions are prerequisites for the very fast formation of photosynthetically active chloroplasts in the light. Block and co-workers (Chap. 7) review the biosynthesis of galactolipids as the characteristic lipids of chloroplasts, as well as their significance for photosynthesis. Lipids synthesized at the plastid envelope might get transported out of plastids under conditions of phosphate limitation (Andersson, Chap. 8). The observation suggests that a plant cell has mechanisms to mediate bidirectional transport of highly hydrophobic molecules between plastids and the ER. The identification of envelope associated components of a putative complex for the transport of precursor lipids (Xu et al. 2003) and the detection of strong attracting forces at membrane contact sites between ER and chloroplasts (Andersson et al. 2007) provide the basis for further investigations on ER-chloroplast contacts in the plant cell. Physical contacts between ER and plastids might change during development; the amount of ER co-purified with plastids was shown to decrease during development (Chap. 8). Thylakoid biogenesis requires the coordinated biosynthesis of lipids (Block et al., Chap. 7), proteins and co-factors such as chlorophyll. To avoid production of reactive oxygen species, biosynthesis of chlorophyll must be tightly coordinated with the biosynthesis of the proteins binding chlorophyll (Tripathy and Dalal, Chap. 27). During the last decades, thylakoid biogenesis has been studied mainly in Arabidopsis by taking advantage of the large collection of mutants impaired in different steps of thylakoid membrane biogenesis. The multimeric complexes making up the photosynthetic apparatus are of dual genetic origin and their biogenesis is a complicated and highly regulated process. The structural details of these complexes at atomic resolution provide their molecular framework (Amunts et al. 2010; Bracher et al. 2011; Umena et al. 2011), and with the combined approach of biochemistry and molecular biology, an in depth study of the complex assembly process has become possible. Increased research activities are required to answer the following questions:

1. How is the synthesis of plastid and nucleus encoded plastid proteins coordinated?
2. How do these proteins, in association with other factors including redox cofactors and pigments, assemble with a definite stoichiometry into functionally active complexes?
3. By which mechanism is the assembly of supramolecular complexes regulated in response to environmental cues? And,
4. What role do the auxiliary proteins play in the assembly process?

Numerous studies with higher plants and with algae such as Chlamydomonas have contributed to the basics of our understanding of the

complex assembly process during chloroplast biogenesis (Lopez-Juez 2007; Ozawa et al. 2009; Nixon et al. 2010; Rochaix 2011).

In Chap. 4, Meierhoff and Westhoff present an excellent review on the control of Photosystem II (PSII) biogenesis as a case study. By mutant analyses, numerous nuclear-encoded auxiliary factors involved in the coordination of plastid and nuclear genes encoding components of the PSII complex have been identified. Moreover these studies have shown that the expression of plastid genes is regulated mainly at the post-transcriptional level, i.e., the processing of transcripts and translation. The crystal structure of Photosystem I (PSI) has provided detailed insight into organization of the proteins and cofactors within this multimeric complex (Amunts et al. 2010). For the true understanding of PSI functionality, it is important to elucidate the dynamic assembly and the degradation of the complex as emphasized by Hihara and Sonoike (Chap. 5). Schöttler et al. (2011) have critically described the details of the PSI structure, its sequential assembly, role of auxiliary proteins, and insertion of cofactors into the photosystem and finally protein-protein interactions during the process of PSI assembly in higher plants.

Besides thylakoid biogenesis, photosynthesis requires the assembly of the stromal enzymes for CO_2 fixation. Biogenesis of Rubisco has been intensively investigated as a model system for understanding the coordinated formation of chloroplast enzymes consisting of plastid and nucleus encoded components. Intensive research on the rather complex process of Rubisco biogenesis in higher plants is presented in Chap. 6 by Roy. $RBCX_2$, an assembly chaperone has been shown to facilitate the assembly of the large subunit of Rubisco (RbcL) (Saschenbrecker et al. 2007; Liu et al. 2010). Importantly, Bracher et al. (2011) have obtained the crystal structure at 3.2 Å atomic resolution of an assembly intermediate of RbcL subunits of cyanobacterial Rubisco associated with $RBCX_2$ molecules. Analysis of this intermediate has significantly contributed to our understanding of the complex assembly of RbcL subunits and the role of chaperones in modulating their correct association. Binding of the small subunit of Rubisco (RBCS) then displaces $RBCX_2$ from this intermediate complex, and finally the stably assembled Rubisco is obtained (see Fig. 6.1 by Roy, Chap. 6). The presence of homologs of cyanobacterial RBCX in plants suggests that the mechanism of Rubisco assembly is highly conserved in different organisms (Saschenbrecker et al. 2007).

IV. New Insights into Contacts of Plastids to Other Compartments of the Plant Cell

Novel cell biology approaches using fluorescent proteins have revolutionized the understanding of the living cell. Plastids transformed with constructs directing the green fluorescent protein (GFP) to the stroma enabled the detection of highly dynamic long and thin stroma-filled tubules named stromules (Gray, Chap. 9). These tubules were shown to interconnect plastids, can serve for exchange of stromal proteins and nucleic acids between different plastids and possibly also between plastids and other compartments of the cell. GFP fluorescence facilitated observation of stromules (Köhler et al. 1997) and confirmed the earlier observations on protrusions emanating from the surface of plastids (Esau 1944; Wildman et al. 1962). Mutants impaired in stromule formation have not yet been detected. Stromule formation is considered to be essential for the plant cell (Chap. 9). It was shown to depend on environmental factors such as temperature and hormones (Gray et al. 2011). Stromules might play a role in the distribution of proteins within a cell; they have been observed to contain abscisic acid (ABA) dependent RNA binding proteins (Raab et al. 2006) and the HSP70 chaperone as well as a virus movement protein (Krenz et al. 2010).

Tight interconnections between plastids and other compartments of the cell are documented by early ultrastructural studies. Cisternae of the ER were frequently observed

to lie adjacent to plastids; also, they may be continuous with the nuclear envelope (Wooding and Northcote 1965; Gibbs 1981; Whatley et al. 1991). Sometimes, connections by short cisternae were observed between the outer plastid envelope and the ER (Abreu and Santos 1977; Gibbs 1981). Direct continuities between the plastid outer membrane with the ER, the plasma membrane as well as with the tonoplast have been shown (Crotty and Ledbette 1973). Wellburn and Hampp (1976) proposed that metabolites during early chloroplast development can be transported in a continuity compartment formed between the perimitochondrial and the periplastidial space. Temporal continuities between the outer envelope membrane and the ER have also been observed at an early stage of amyloplast development (Whatley et al. 1991). The continuities between plastids and other compartments could be the structural advantage for changes in the competence of plant cells to react to certain stimuli.

V. Leaf Senescence and Chloroplast Dismantling

For a long time, senescence in plants was not clearly defined and was considered as the deteriorative passive process leading to death of an organ or organism. Against the background of the rapid expansion of knowledge and large accumulation of data during the last 30 years in the area of molecular biology, the concept of senescence has been changing with emerging new ideas and terminology (Thomas et al. 2003; van Doorn and Woltering 2004; see Chap. 13 by Noodén). The precise definition of programmed cell death (PCD) and necrosis have contributed to a better understanding of senescence processes, which are reversible in contrast to the aforementioned processes. Chapter 13 describes characteristic features of senescence and cell death in photosynthetic tissues.

Senescence in green plants is characterized by leaf yellowing, the visible symptom of the process caused by the loss of chlorophyll (Lim et al. 2007). The process brings about controlled dismantling of the cellular fabric; it includes disorganization of the chloroplast, the organelle that exhibits the first signs of structural dismantling and stays alive until very late stages of senescence allowing a reversal of the process (Biswal and Biswal 1988; Fulgosi et al., Chap. 26). Electron microscopy has revealed sequential degradation of the chloroplast structure with unstacking of thylakoid membranes and their subsequent degradation with concomitant formation of plastoglobuli (Lichtenthaler 1969). With the progress of senescence, these plastoglobuli significantly increase in size and often also in number (Mulisch and Krupinska, Chap. 14; Lichtenthaler, Chap. 15). The structural disorganization of thylakoids is accompanied by a decline in the primary photochemical reactions and in the efficiency of the Calvin-Benson cycle enzymes including Rubisco (Biswal et al. 2003). During senescence, the components of the photosynthetic apparatus are degraded for remobilization of nitrogen to younger still growing leaves as well as to seeds and fruits. Up to 70% of the chloroplast nitrogen is bound in Rubisco. Another major fraction of the nitrogen is bound in the apoproteins of the light-harvesting complexes of the thylakoid membrane. Proteolytic activity for protein degradation is required for organelle biogenesis and senescence (Eckardt 2007; Kato and Sakamoto 2010). A large number of proteases participate in protein targeting and assembly of multimeric protein complexes and also in the degradation of macromolecules during senescence. Degradation of plastid proteins during senescence follows different pathways involving plastid located proteases, and extraplastidial proteolytic systems (Kato and Sakamoto, Chap. 20).

As far as the degradation of Rubisco is concerned, increasing evidence indicates that the enzyme is mainly degraded outside the organelle and that its degradation involves two kinds of structures, the Rubisco containing bodies (RCBs) (Wada and Ishida, Chap. 19) and the senescence associated vacuoles

(SAVs) (Costa et al., Chap. 18). The large subunit of Rubisco was shown to be oxidized inside the organelle before it gets degraded. The group of Mae was the first to report on an export of Rubisco in vesicles budding off from the chloroplasts (Chiba et al. 2003). Ultrastructural analyses showed that these structures are surrounded by two envelope membranes. In addition to the large subunit of Rubisco they include other stromal proteins such as glutamine synthetase II. It seems that these vesicles derive from stromules which by light microscopy were observed to be disintegrated into vesicles (Gunning 2005). Biochemical studies dating back to the 1970s have shown that cysteine proteases are likely to be involved in Rubisco degradation (for a review see Feller et al. 2007). Most cysteine proteases thus far characterized reside in the vacuole and are active at low pH. Guiamet and his co-workers (Otegui et al. 2005) have shown that in senescing leaves, mesophyll cells contain many tiny compartments having a pH lower than the central vacuole. These senescence associated vacuoles are enclosed by a single membrane. The group of Guiamet showed that these vacuoles might contain Rubisco and other stromal proteins as well as chlorophyll binding proteins of PSI (Chap. 18). The content of thylakoid derived proteins distinguishes SAVs from RCBs which are devoid of chlorophyll. Because SAVs were also reported to contain proteins synthesized at the ER (the precursors have a signal peptide), they were suggested to be derived from the ER (Chap. 18). Considering, however, that chloroplast proteins are included in SAVs, it is possible that these vacuoles originate from plastids. Ultrastructural investigations on plastids in senescing leaves have revealed the presence of numerous vesicles between plastids and the closely adjacent ER (see Chap. 14). It is, however, not possible to distinguish vesicles derived from plastids on one hand and vesicles transported to plastids on the other hand. It is possible that at a late stage of senescence, when the outer plastid membrane gets disintegrated, vesicles excluded from plastids only have a single membrane envelope. Both structures, SAVs and RCBs, share Rubisco. Their potential relationship remains to be elucidated.

Pigment binding proteins of the thylakoid membrane cannot be degraded before chlorophylls get detached from the proteins and are catabolized (Hörtensteiner, Chap. 16). Chlorophyll degradation requires at least six enzymatic and one non-enzymatic reactions. Catabolites are finally transported out of plastids into the vacuole where they are deposited. The pathway seems to be, however, more complicated than originally anticipated (Hörtensteiner and Kräutler 2011) and some of the catabolites might have specific biological roles. Several mutants impaired in chlorophyll breakdown have been described (Hörtensteiner 2007). They can be categorized in functional and non-functional "stay-green" mutants (cosmetic "stay-green" mutants). The *SGR* gene responsible for the "stay-green" phenotype of a group of non-functional "stay-green" mutants has been identified, and the gene product seems to be involved in the destabilization of chlorophyll binding proteins, thereby regulating the accessibility of the substrates by degrading enzymes (Chap. 16). Several mutations in genes encoding components of chlorophyll catabolism were shown to have consequences not only for senescence but also for stress responses, notably hypersensitivity towards pathogens (Ougham et al. 2008; Mur et al. 2010).

VI. Plastid Control of Plant Development and Senescence

Plastids are unique compartments tightly integrated in the metabolism of the plant cell. They share several biosynthetic pathways with mitochondria and have intensive metabolite exchange with them (Zottini, Chap. 21) and other compartments. There is evidence that the numbers of plastids and mitochondria per cell are also co-ordinately regulated (Chap. 21). Padamasree et al. (2002) have observed that the ratio of mitochondria to chloroplasts increases with the rate of photo-

synthesis, because oxidative electron transport in mitochondria might protect chloroplasts against reactive oxygen species produced by photosynthesis.

Plastid function and differentiation are controlled by nuclear factors during all the phases of plant development. These controls are exerted at different levels ranging from plastid gene transcription (Chap. 10), transcript processing and translation until the complete assembly of supramolecular complexes (Chap. 4). On the other hand it is well established that plastid signaling also affects gene expression in the nucleus (Pfannschmidt and Munné-Bosch, Chap. 22; Sabater and Martin, Chap. 23).

Chloroplasts during senescence are in a dilemma situation. They are the sites of photosynthesis for energy production and at the same time they are degraded (Krupinska and Humbeck 2004). To adjust the senescence process to the functionality of the chloroplast and, vice versa, to keep photosynthesis going during dismantling of the organelle, an intensive cross-talk between plastids and the nucleus is required. Plastid signals include redox changes and reactive oxygen species, and the latter are assumed to induce cell death processes and senescence (Chap. 22). Sabater and Martin (Chap. 23) proposed that the upregulation of genes for NDH subunits and the downregulation of genes for chloroplast superoxide dismutase together lead to a feed-forward increase in ROS, which might be crucial for senescence initiation. Chloroplasts not only produce ROS, but the components of the organelles are also major targets for damage by ROS, and Rubisco degradation has been shown to begin with an oxidative modification (Khanna-Chopra et al., Chap. 17).

During senescence, the classical portfolio of plastid signals requires extension. Plastid signaling during senescence involves not only ROS and sugars (Wingler and Paul, Chap. 24), but also hormones such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) that are partly or fully synthesized in plastids. Indeed, signaling from plastids was shown to vary during development. Pfannschmidt and Munné-Bosch (Chap. 22) present the current knowledge on plastid signaling with regard to the developmental stage of the plastids ranging from proplastids to gerontoplasts. Classical nuclear target genes of plastid factors such as *LHCB1* and *RBCS* are important for building up the photosynthetic apparatus during chloroplast development. Target genes affected at other stages of plastid development have barely been studied with regard to plastid control. The plastid derived hormones JA, SA and ABA are known to play roles in the acceleration of senescence. They affect the expression of many genes reported to be up- or downregulated during senescence. The genes responding to these hormones are target genes of plastid signals during senescence. It is hence important to study the hormones and intermediates of their biosynthesis in plastids with respect to plastid signaling (Chap. 22). So far the extensive cross-talk between mitochondria and chloroplasts has been largely neglected. Their metabolic inter-dependence and inter-organellar signaling could be at least in part mediated by physical interactions (Chap. 21). Already in the 1960s membrane continuities were reported to appear between chloroplasts and mitochondria (Wildman et al. 1962) and were proposed to form a peri-organellar (perimitochondrial, perichloroplastidial) space for metabolite transport (Wellburn and Hampp 1976). Application of novel in vivo fluorescence imaging techniques in cell biology revealed that the different compartments of the plant cell might not physically be as isolated as anticipated. In particular, fluorescence imaging microscopy has shown that plant cells possess a complex endomembrane network frequently forming transient contacts (Mathur et al. 2010), and strong attractive forces have been shown to occur between the outer membrane of chloroplasts and the ER (Andersson et al. 2007). Considering the evolutionary relationship and the physical interactions of organelles and the endomembrane system, it is not surprising that the endomembrane system plays an important role in providing plastid proteins during chloroplast development

(Chap. 12) and in dismantling of chloroplasts during the period of senescence (Chap. 14).

Photosynthesis of chloroplasts leads to the biosynthesis of sugars which are not only carbon sources but are also involved in signaling (Chap. 24). Sugar signals can originate from plastids but they can also have an extraplastidial origin. Trehalose 6-phosphate (T6P), synthesized as a precursor of trehalose in the cytoplasm, has been shown to act as a high carbon signal to activate starch biosynthesis in chloroplasts (Wingler et al. 2012). T6P was also shown to regulate plant development including a role in floral initiation as well as in senescence (Chap. 24).

VII. Environmental Modulation of Chloroplast Development

Plants adapt differentially to different ecological settings in nature. The structural and functional adaptive characteristics of the plant cell depend largely on the functionality of chloroplasts in response to environmental signals. The development of chloroplasts follows short term or long term adaptations depending on the developmental stage and environmental conditions.

When chloroplasts are fully mature, the response of the organelle to environmental signals is primarily restricted to the metabolic adjustments required for the maintenance of energy and redox homeostasis along with readjustment of cellular metabolites to sustain essential metabolic networks (Anderson et al. 1995; Ensminger et al. 2006; Biswal et al. 2011). The responses of plants to environmental changes involve changes in the biosynthesis of pigments (Chap. 27) and proteins resulting in appropriate changes in the composition and stoichiometries of the structural components of plastids, in particular of the supramolecular complexes of the photosynthetic machinery. Light with variation in quality, quantity and duration can significantly modulate organelle development (Burman and Khurana, Chap. 25; Joshi et al., Chap. 28). The activities of photomorphogenic receptors including phytochrome and blue light receptors have a remarkable impact on plastid biogenesis and senescence (Biswal et al. 2003). Well studied sun type and shade type chloroplasts with distinct compositional and structural characteristics are typical examples demonstrating the flexibility of developing chloroplasts to variations in light intensity (Chap. 15). Many abiotic stress factors were shown to induce extensive structural alterations during proplastid to chloroplast transformation as revealed by electron microscopy (Kwak et al. 2011, Chap. 28). Similarly, several environmental stress factors are reported to induce and accelerate the senescence process, which is also regarded as a stress escape response of green plants. These factors cause transformation of chloroplasts to gerontoplasts with consequent loss in photosynthesis, which is likely to bring about imbalances in metabolic networks including changes in cellular sugar status, known to initiate a metabolic signal cascade for senescence regulation (Wingler et al. 2006). Changes in environmental factors were even shown to revert the process of senescence resulting in regreening of senescing leaves (Chap. 26).

VIII. Conclusions and Open Questions

The literature available on plastid development and senescence in green plants is rich. With the tremendous progress in plant molecular biology and the continuous development of new techniques, many complex events associated with the biogenesis of the chloroplast and dismantling of the organelle during senescence have been elucidated. There are, however, many questions that still remain unanswered and need to be addressed in the future. A few specific questions and perspectives in this area of research are highlighted below:

1. Much is known about the biogenesis of thylakoids and the assembly of thylakoid complexes during proplastid to chloroplast transformation. The biogenesis of thylakoid and stromal multimeric protein complexes requires the coordinated synthesis and spatio-temporal assembly of several factors. Many factors, not necessarily associated with the mature complexes, are also reported to facilitate the assembly process. So far it is, however, largely unknown how these factors act mechanistically, and how their functions are modulated by environmental factors.
2. The formation of chloroplasts from gerontoplasts during regreening of yellow leaves needs serious attention because the pathways from disorganized structures of the organelles to organized ones during retransformation of gerontoplasts to chloroplasts are not well defined, and the signaling systems associated with the reversibility of senescence are unknown.
3. Several proteases have been identified and are known to be associated with quality control, maturation and senescence of chloroplasts during leaf development. However, the specific substrates and their recognition largely remain unclear. Similarly many questions regarding the synthesis and degradation of chlorophylls during plastid development are not satisfactorily answered.
4. The mechanism of signal perception, its processing and the downstream regulation of gene expression for induction, progression and completion of the senescence program require further studies. Using forward and reverse genetic tools, several regulating factors for induction of senescence have been proposed (Lim et al. 2007). But a clear picture on the integration of the factors and the mechanism of induction of the process has not yet emerged. Plastid-to-nucleus signaling (Chaps. 22 and 25) and inter-organellar signaling between plastids and mitochondria (Chap. 21) are areas of increasing research. Hitherto, the molecular mechanisms of signal transfer between the compartments and the potential changes of the communication mechanisms during chloroplast biogenesis and senescence (Chap. 23) remain to be elucidated.
5. Finally, a molecular road map of chloroplast biogenesis and senescence during leaf development remains to be drawn in order to get a complete understanding of the developmental flexibility of the organelle. Unfortunately, plastid systems biology still remains in its infancy (Lopez-Juez 2007). It may be possible, in the future, to identify the regulatory genes and their roles in an integrated genetic network that underlies chloroplast development in green plants. This area of research would benefit from bioinformatic analyses of the results obtained by combined research of genetics, physiology and biochemistry including microarray analyses of global gene expression and proteomic studies on plastids of different developmental stages.

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Chapter 2

Chloroplast Development: Time, Dissipative Structures and Fluctuations

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Summary

Chloroplast development describes the life cycle of plastids from the proplastid to the mature chloroplast, which is subsequently transformed to a gerontoplast and finally to a necrotic plastid. Similar to any living system, the chloroplast may be defined as an open thermodynamic system far away from equilibrium. It has self-organized dissipative structures, namely, metabolome and genome, which fluctuate with development. The proplastid grows to become a mature chloroplast with self-organizing metabolic networks consisting of core, plastic, and signaling subsystems. The major function of the chloroplast is photosynthesis. Light induces redox reactions resulting finally into the synthesis of sugars. The photoelectron transport systems and sugars are not only two components of the core metabolic network, but these are also elements of signaling subsystems. The signaling regulatory and metabolic networks associated with chloroplast development are complex in nature and therefore are not fully understood. Many experimental data in the area remain to be explained without ambiguity. Examination of chloroplast development with respect to time, structure and fluctuations under the lens of non-equilibrium thermodynamics may contribute to our understanding of the process.

I. Introduction

The chloroplast is an important organelle in mesophyll tissues of plants. It absorbs solar radiation thereby capturing energy for plants as well as other living systems on the planet Earth. Therefore, the study of structure and function of chloroplasts has become one of the thrust areas of research in plant science. Structure and function of chloroplasts can be well understood through the study of development of the organelle. The transformation of proplastids to chloroplasts and subsequently chloroplasts to gerontoplasts (senescing chloroplasts) are linked to leaf development and senescence (Taylor 1989; Kutik 1998). These transformations during development find their expression in the change in relative concentrations of green and yellow pigments in the organelle and changes in the color of leaves from almost colorless to green to yellow and finally to reddish brown. The colorful panorama of landscapes along with the seasonal changes serves as a feast to the eyes of the beholder (Fig. 2.1). The aesthetic sense associated with an important change on Earth's surface not only inspires authors and artists but also greatly enhances the inquisitiveness among the researchers to translate the overwhelming emotions into the scientific explanation of the phenomena and their implications on the planet Earth (Fig. 2.2). The importance of this study is reflected in the accumulation of a large number of research papers in the area along with reviews discussing several dimensions of development of chloroplasts. Most of the reviews on organelle development focus on morphology, biochemistry, metabolism, and genetics (see e.g., Thomson and Whatley 1980; Bauer et al. 2001; Biswal et al. 2003; Stern et al. 2004; Biswal 2005; López-Juez and Pyke 2005; Møller 2005; Kessler and Schnell 2009; Inaba and Ito-Inaba 2010; Adam et al. 2011; Nickelsen et al. 2011; Pogson and Albrecht 2011). The tools and databases in the post-genomic era have provided an elaborate and deep insight into the molecular scenario of the development of the organelle from the proplastid to the

Abbreviations: ATP – Adenosine 5′-triphosphate; CES – Control by epistasy of synthesis; CF_0 – Coupling factor intrinsic component; CF_1 – Coupling factor extrinsic component; Cyt b/f – Cytochrome b/f complex; MEP – Maximum entropy production; MS – Mass spectroscopy; NADPH – Nicotinamide adenine diphosphate (reduced); PSI – Photosystem I; PSII – Photosystem II; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase

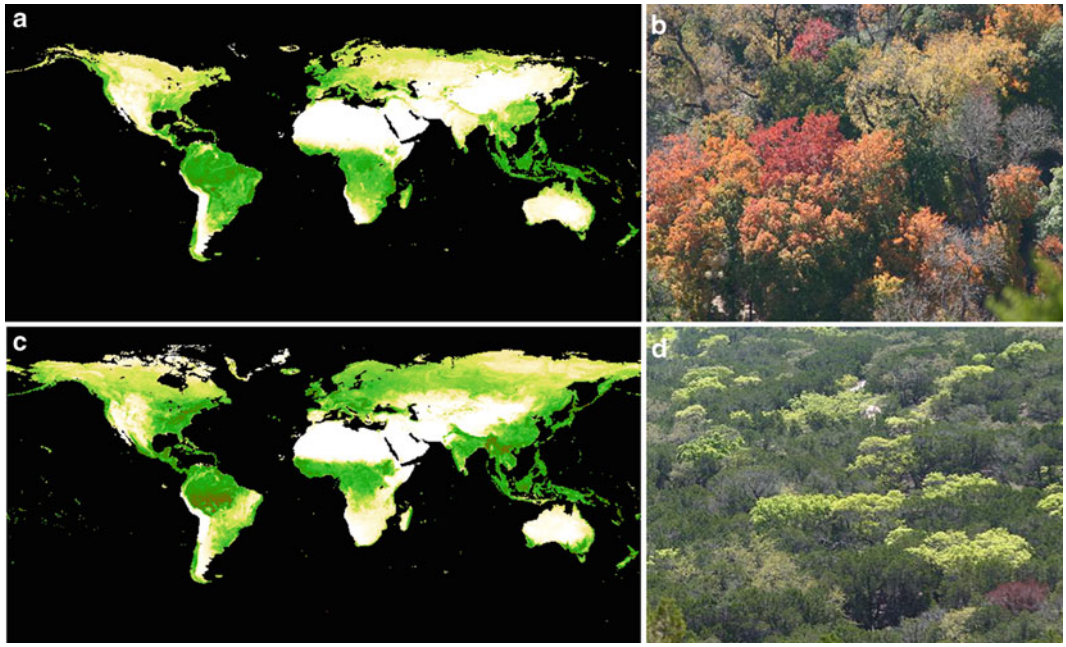

Fig. 2.1. World map showing the fraction of absorbed photosynthetically active radiation (FAPAR) in April 2006 (**a**), and September 2005 (**c**) (From http://fapar.jrc.ec.europa.eu; Gobron et al. 2006). Panorama of deciduous forest landscapes in autumn (**b**) and in spring (**d**) (http://en.wikipedia.org/wiki/File:Aerial_View_of_Autumn_Forest_Colors.jpg (b); http://en.wikipedia.org/wiki/File:Spring_Forest_Leaves_in_Texas_Hill_Country.jpg (d)). The *green* color of the vegetation manifests the *green* color of chlorophylls in the leaves. The colorful strokes in the landscape reflect the colors of degraded chlorophyll species and dominating carotenoid pigments in senescing or dead leaves.

gerontoplast. Metabolic networks, gene expression, cross-talk between nuclear and plastid genomes and signaling processes are targets of study to understand the developmental process at the molecular level. However, the role of the chloroplast is not limited to photosynthesis and production of metabolites; it further extends to numerous aspects of plant growth and development as a whole (López-Juez and Pyke 2005; López-Juez 2007; Van Doorn and Yoshimoto 2010). Further, development of chloroplasts influences embryogenesis, leaf development, reproductive systems, stress response, and plant-microbe interactions (Inaba and Ito-Inaba 2010). In addition to photosynthesis, the impact of chloroplast development reaches out to the biotic and abiotic systems of the planet Earth. Biological tools are not sufficient to understand the wide pervading effect of chloroplast development. The study of chloroplast development needs a wide angle lens, which can include the entire planetary system. One such discipline is thermodynamics. So far, little effort has been made to review the chloroplast development from the perspective of thermodynamics. An attempt is made in this chapter to consolidate and interpret the reports on chloroplast development in terms of dissipative structures and fluctuations in the thermodynamic time-space.

A. Biosystem: A System in Thermodynamics

Living organisms appear to be far away from physical and chemical inanimate systems. However, when we put a biosystem into the frame of thermodynamics, the hard boundary between animate and inanimate worlds melts down. A biosystem is as good as any other physico-chemical system. It is an open thermodynamic system away from the

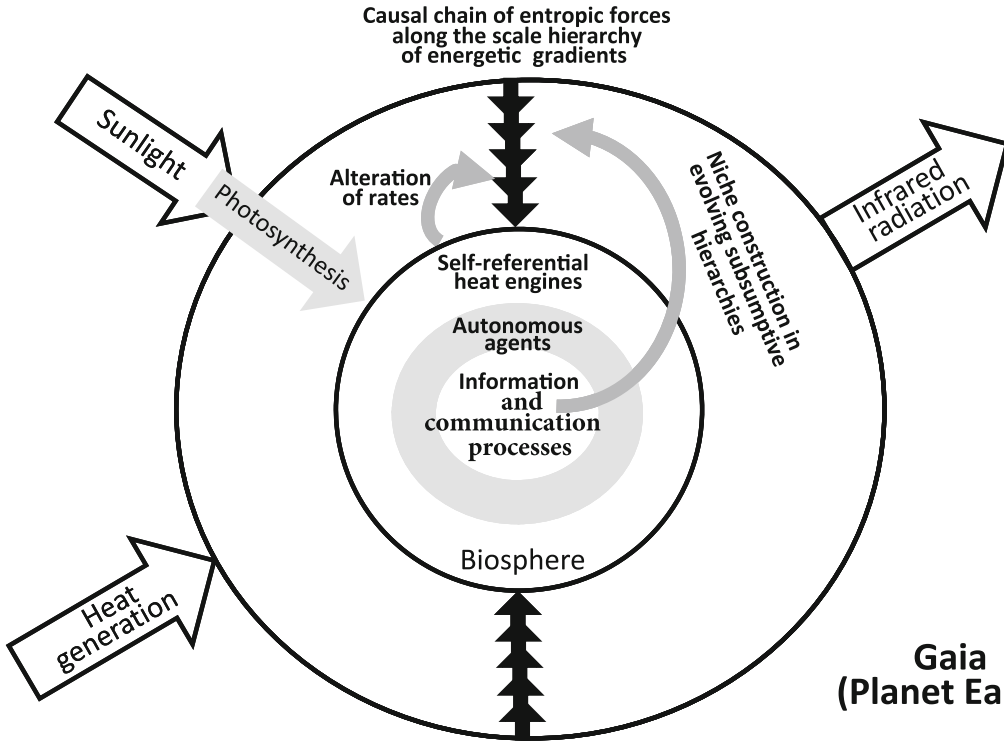

Fig. 2.2. Representation of Gaia (planet Earth). Energy fluxes, biotic and abiotic components, self-referential heat engines, information and communication processes, and the causal chain of entropic processes along the hierarchy scale of the energetic gradient are depicted (modified after Herrmann-Pillath 2011).

equilibrium. A biosystem exchanges mass and energy with its environment. Hence, thermodynamically it is an open system. Moreover, the fluxes of matter and energy take it to a non-equilibrium region. A system in the non-equilibrium region maintains gradients away from the equilibrium. The system resists and dissipates externally applied gradients; as a result it moves away from the equilibrium. Hence order emerges from disorder in the formation of dissipative structures (Prigogine 1967).

A self-organized structure is supposed to have the following characteristics (Pulselli et al. 2009):

1. A boundary that physically separates a system from its environment. The boundary can generate reactant gradients and flows. Compartmentalization within the plasmamembrane is an important characteristic of higher life. Internal compartments caused by sub-cellular organelles, e.g., mitochondria and plastids, are the main contributors to the characteristic low entropy of living cells (Marín et al. 2009). The entropy associated with the compartmentalization of components in eukaryotic cells, as a function of cell and compartment volumes, and of the concentration of solutes, has been estimated to be approximately -14.4×10^{-14} J K^{-1} cell^{-1} (-0.7 J K^{-1} L^{-1}) in the case of *Saccharomyces cerevisiae*, a typical eukaryotic cell, and approximately -49.6×10^{-14} J K^{-1} cell^{-1} (-1.0 J K^{-1} L^{-1}) in the more complex cell of *Chlamydomonas reinhardtii* (Marín et al. 2009). Compartmentalization is believed to be an essential development that significantly decreases the entropy of living cells during biological evolution in comparison to other potential contributing factors, e.g., the informational entropy of DNA and the conformational entropy of proteins (Marín et al. 2009).

2. Inflow-outflow of energy, matter and information: The system must be open and exist along with an environment, far from equilibrium (Prigogine 1978; Pulselli et al. 2009).
3. Other characteristics include non-linear interactions, e.g., feedbacks, autocatalysis or autoinhibiting loops between the elements within the system (Prigogine 1978; Pulselli et al. 2009).

As the system moves away from equilibrium, it takes advantage of all available means to resist externally applied gradients and emerges as a highly ordered system at the cost of increasing disorder at the higher level in the system's hierarchy (Schneider and Kay 1994).

Boltzman (1886) realized that the solar energy gradient drives the living process and stated:

> "The general struggle for existence of animate beings is therefore not a struggle for raw materials – these, for organisms, are air, water and soil, all abundantly available – nor for energy which exists in plenty in anybody in the form of heat (albeit unfortunately not transformable), but a struggle for entropy, which becomes available through the transition of energy from the hot sun to the cold earth."

Schrödinger (1944) suggested that an organism stays alive by importing energy from the environment and degrading it to maintain the organizational structure of the system:

> "Thus the device by which an organism maintains itself stationary at a fairly high level of orderliness (= fairly low level of entropy) really consists in continually sucking orderliness from its environment. …plants can still make use of it. (These, of course have their most powerful supply of 'negative entropy' in the sunlight.)"

Biosystems utilize free energy for metabolism and to do work (grow, move, reproduce, etc.). The most common sources of free energy are geologic chemical compounds (chemotrophs), direct sunlight (phototrophs), or organic material (heterotrophs) (Kleidon and Lorentz 2005). Thus biosystems are far from equilibrium dissipative systems and have great potential for reducing radiation gradients on Earth (Kay 1984; Ulanowicz and Hannon 1987).

According to the Gaia concept, the "Earth" system is considered to be an integrated complex system, within which the living subsystem evolves in a way to maintain the biogeochemical, climatic, and other physical conditions necessary for its viability (Lovelock and Margulis 1974; Lovelock 1990). A Gaia system is depicted in Fig. 2.2 with biotic and abiotic components and energy flux. Photosynthesis serves as a major process to funnel energy flux into the Gaia. The original concept suggests that the Gaia system maintains homeostasis at the equilibrium. However, it is observed that Gaia is a non-equilibrium system evolving its own self-organized structures. The Earth system is a non-equilibrium system, which is driven by external energy flux from the solar radiation and by the endogenous processes that have resulted from the emergence of life on Earth (Kleidon 2009, 2010a, b). The non-equilibrium systems follow the path of maximum entropy production (MEP) to reach a steady state. It is the export of entropy that matters, not the status of the exporting system. The entropy of the system decreases at the cost of an increase in the entropy of the environment (Dewar 2003; Kleidon 2010a, b; Herrmann-Pillath 2011). Two entropic time arrows, distinguishable from the general entropic time arrow of the universe have been proposed by Sabater (2009): one for survival of biological systems and the other for aging and evolution. However, there are several reports suggesting the MEP principle, which governs the cosmos, as the doctrine for evolution and survival of the biosystems (Dewar 2003; Kleidon 2010a, b; Herrmann-Pillath 2011). Hence, the time arrows associated with survival, aging and evolution of biosystems are integral parts of cosmological time arrows. Kauffman (2000) wrote:

> "Biospheres and the universe create novelty and diversity as fast as they can manage to do so without destroying the accumulated propagating organization that is the basis and nexus from which further novelty is discovered and incorporated into the propagating organization."

The above statement is referred to as Kauffman's version of the so-called fourth law of thermodynamics. It explains the accumulation and build up of information to a higher order of complexity as the system evolves.

Life is viewed as the emergence of structures which increase the speed and efficacy of entropy production. This view conforms to non-equilibrium thermodynamics. It also adds to the perspective of information and communication among the elements of the system (Herrmann-Pillath and Salthe 2011). It provides scope to understand the correlation between the dissipation of energy and the accumulation of information.

All systems, biotic or abiotic, evolve through a spontaneous conversion of environmental potential energy into some less available form – "heat", according to the general principles of thermodynamics (Schrödinger 1944; Kleidon 2004). Such a system is known as heat engine or self-referential heat engine (Herrmann-Pillath 2011). An evolving system and its environment, separated by a permeable interface (membrane in case of a living system) at constant temperature (T_S) and pressure (P) constitute a simple self-referential heat engine (Garrett 2011). The interface maintains a Gibb's energy potential, ΔG (T_S, P), so that the system as a whole is able to convert available energy into work (W) within a time frame (t) at a rate *w*,

$$w = \mathrm{d}(\Delta \mathrm{G}) / \mathrm{dt} \tag{2.1}$$

$$a = \alpha \Delta \mathrm{G} \tag{2.2}$$

where, *a* is the rate of consumption of energy to do work at a rate *w*, α is an engine-specific constant coefficient.

The efficiency, ε, is given by

$$\epsilon = w / a \tag{2.3}$$

The heat (Q) produced at a rate ($a - w$) is transferred to the colder surroundings at temperature T. The system with positive feedback loop in which, through work, *a* and ΔG evolve logarithmically at rate

$$\mathrm{d}\ln a / \mathrm{dt} = \mathrm{d}\ln(\Delta \mathrm{G}) / \mathrm{dt} = \eta = \epsilon\alpha \tag{2.4}$$

where, η can be considered as a feedback efficiency or rate of return.

B. The Chloroplast System

Chloroplasts are responsible for transducing the majority of the energy flux into the Gaia (planet Earth). They absorb light and convert light energy into chemical energy by photoredox reactions. The reactions result in the production of oxygen from water, synthesis of ATP, and reduction of NADP to NADPH. The chemical potential thus stored in these chemical species is utilized to carry out several biological functions: biosynthesis of sugars from carbon dioxide via e.g., Calvin-Benson cycle. A chloroplast has a double membrane envelope as boundary that separates it from its environment (Fig. 2.3). It is an open, non-equilibrium thermodynamic system with high order of self-organization. There is exchange of materials, namely, biomolecules, oxygen, and carbon dioxide across the envelope. Energy in form of heat and light also gets exchanged. The chloroplast has a life cycle that is well programmed and controlled by the plastid genome as well as the nuclear genome. Thus a chloroplast is a living system in miniature.

II. Chloroplast Development

A. Stages of Chloroplast Development

The changes in the structure and metabolism of the plastid with time are accompanied by changes in the thermodynamic parameters, e.g., free energy and entropy. Appearance of structural patterns, specific signaling systems, selective gene expression, and other biochemical events are associated with chloroplast development. The thermodynamic characteristics of proplastids, mature,

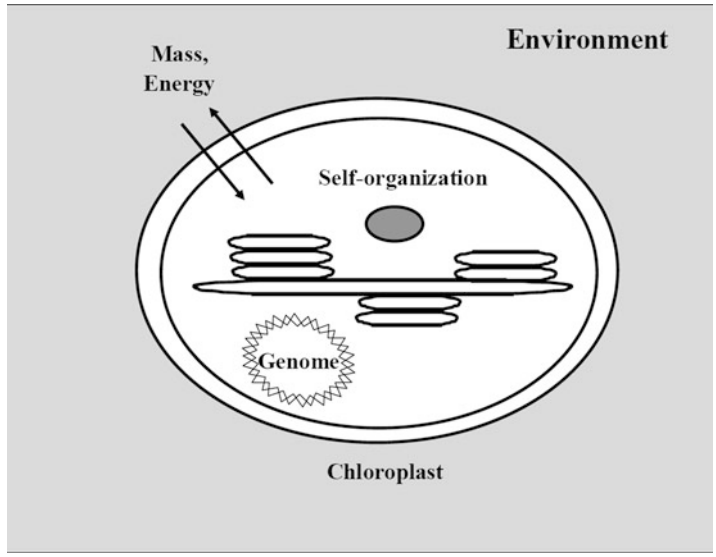

Fig. 2.3. Diagram depicting a chloroplast as an open thermodynamic system. The double membrane envelope acts as a boundary of the system. The system exchanges mass for example biomolecules, carbon dioxide, oxygen, and water with the environment. Energy exchange with the environment takes place in the form of light and heat predominantly. Metabolic network (self-organization) in the system derives order from disorder and genome is responsible for perpetuating order from order.

and aging chloroplasts, are discussed here. The structural changes are schematically shown in Figs. 2.4 and 2.5. The development of the proplastid to the mature chloroplast is a 'build up' process with biosynthesis of membrane structure, organization into grana and lumen, assembly of multimeric protein complexes and onset of well regulated electron transport and metabolic processes (Fig. 2.4) (Adam et al. 2011). On the other hand, transformation of mature chloroplasts to gerontoplasts is basically a dismantling process with disruption of membrane organization, disassembly of protein complexes, changes in the metabolism, and degradation of cellular molecules including pigments, proteins, and lipids.

B. Characteristics of Well Defined States of Chloroplasts

The development of the chloroplast involves a chain of transformations of the organelle from a proplastid to the photosynthetically active mature chloroplast, then to a gerontoplast, and finally to complete degradation of the plastid. These structurally different plastid forms also have distinct physiology. The above transformations are preprogrammed, well-regulated genetic and metabolic events. However, beyond the stage of the gerontoplast, there is complete degradation with point of no return that results in dissolution of the plastid.

1. Structure and Organization

Proplastids are located in embryonic tissues and meristems. They are small (0.5–1.0 μm in diameter) double membrane compartments, which enclose nucleic acids, proteins, lipids, precursors of internal membrane structures, a few starch grains and plastoglobuli in the stroma (Thomson and Whatley 1980). As noted above, proplastids differentiate into mature plastids.

A mature green plastid, the chloroplast, is structurally and functionally a well organized organelle (Catsky and Sestak 1997). A membrane system, composed of thylakoids, is dispersed in the chloroplast. A fully developed thylakoid membrane system organizes at places

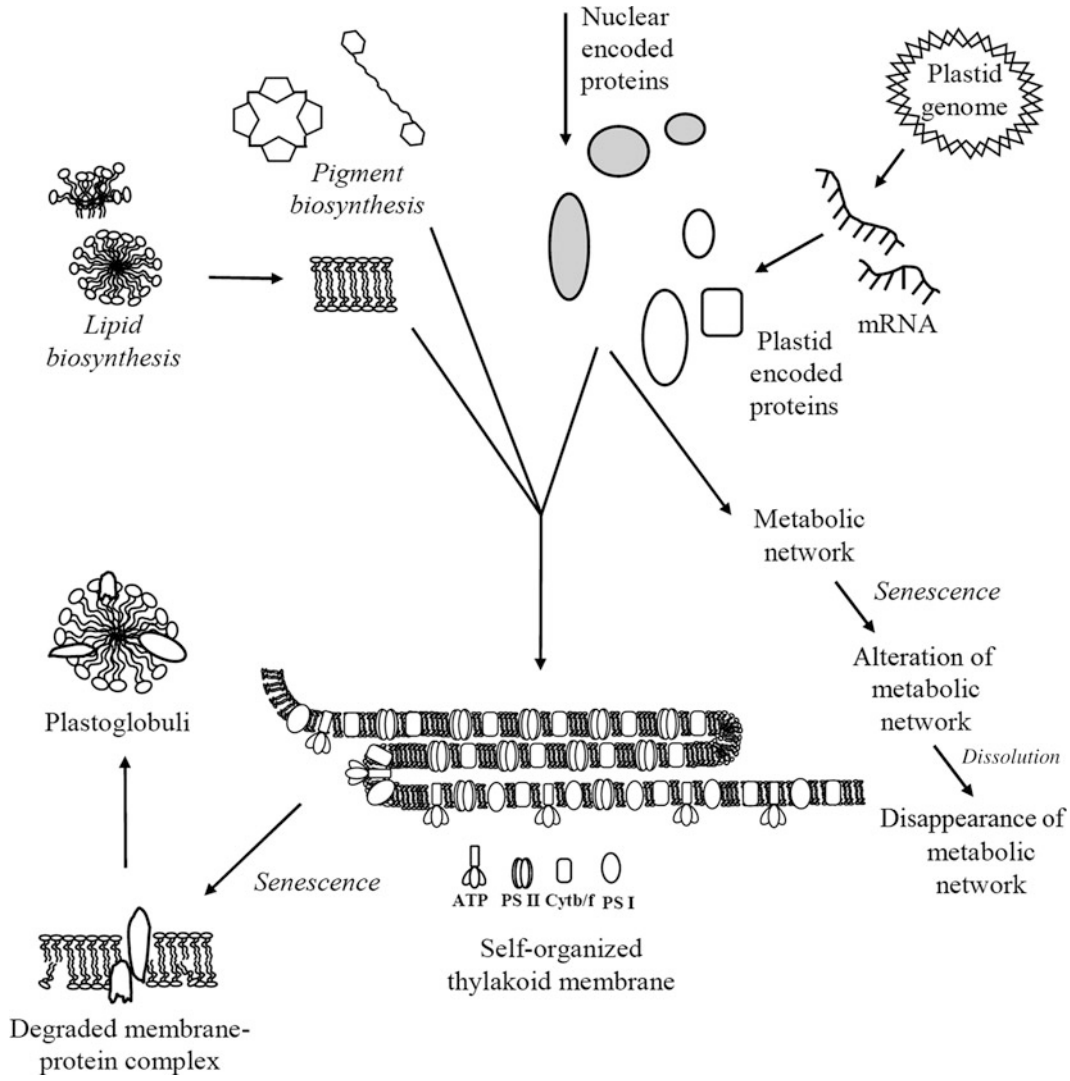

Fig. 2.4. Diagram of the structural changes during development of self-organized structures in proplastid. Biosynthesis of proteins, pigments, lipids and their organization to orderly structures, namely thylakoid membranes, and metabolic networks, are shown in the figure. The self-organization is perturbed during senescence and the metabolic network disappears upon dissolution.

into stacked membranous structures called grana. Unstacked thylakoid membranes are called stromal lamellae. There are four major protein complexes constituting the electron transport system located in the thylakoid lipid bilayer (Fig. 2.4). These complexes are PSI, PSII, Cyt b/f complex, and ATP synthase (CF_1 CF_0). Each complex consists of several protein subunits, some of which are encoded by the nuclear genome and the rest by the plastome (Goldschmidt-Clermont 1998). The carbon dioxide fixing enzyme system is located in the stroma.

A mature chloroplast undergoes structural and functional changes during senescence of green leaves. The changes result in

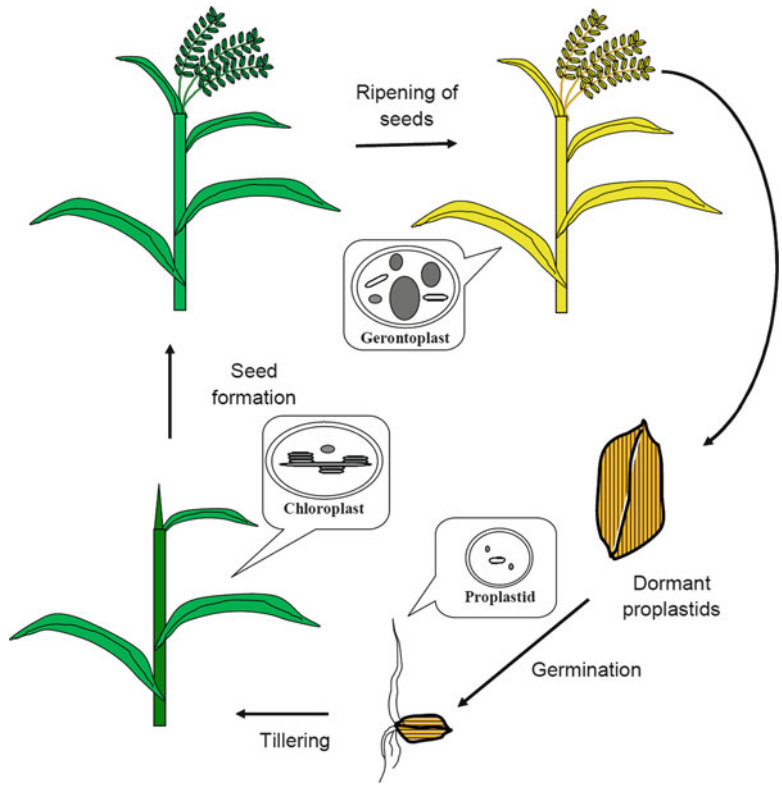

Fig. 2.5. Schematic representation of the movement of biomass in the form of nutrients from proplastid to chloroplasts. Then, the nutrients are mobilized to gerontoplasts and finally to the seeds. The seeds upon germination give rise to transformation of proplastid to chloroplasts in the seedlings. Seedlings grow into plants with mature chloroplast containing leaves. These leaves undergo senescence and from gerontoplasts nutrients are transported to seeds. Thus, indirectly gerontoplasts transfer mass to a new system, the proplastid, which builds up order from disorder while the gerontoplast itself heads toward complete disorder-death. The cycle of appearance-sustenance-disappearance continues.

differentiation of a chloroplast into a gerontoplast. Gerontoplasts are smaller in size compared to chloroplasts. Unstacked and degraded thylakoid membranes, loss of photosynthetic pigments, a large number of plastoglobuli with lipophilic materials, a few starch grains, and an intact double layer envelope membrane are structural features of the gerontoplast (Biswal and Biswal 1988; Biswal 2005). A gerontoplast develops from a mature chloroplast without any further growth and it undergoes gradual loss of biosynthetic potential (Thomas et al. 2001, 2003).

A gerontoplast can regenerate to form a chloroplast under certain conditions through a reverse pathway different from the forward one (Smart 1994; Thomas 1994; Zavaleta-Mancera et al. 1999a, b; Van Doorn and Yoshimoto 2010, see also Fulgosi et al., Chap. 26). During development of a gerontoplast the regeneration capability is limited to a threshold of degeneration, the so-called point of no return. The gerontoplast then loses the structural integrity and dissolution of the plastid occurs.

2. Metabolism

Metabolic networks in a biosystem are mathematically described by sets of catalytic elements connected with the substrate

fluxes and signals. Each catalytic element belongs to a self-organized set of enzymes (De la Fuente et al. 1999; De la Fuente 2010). These catalytic sets of enzymes are designated as metabolic subsystems. They may exhibit oscillatory and stationary activity patterns (De la Fuente et al. 1999). Global metabolic structures are able to self-organize spontaneously. They are characterized by a set of different active enzymes known as metabolic cores. Other metabolic subsystems exhibit structural plasticity and have conditional dynamic on-off switch states. The global metabolic structure could be present in all living cells (De la Fuente et al. 1999; Almaas et al. 2004, 2005; Almaas 2007; De la Fuente 2010). This may also exhibit emergence of chaotic behaviour within it (De la Fuente et al. 2008, 2009, 2010). Chaotic patterns are sensitive to the initial conditions. A small change in the initial state leads to large changes in the later state of the system. The deviations of the chaotic patterns are dependent on the quantum of perturbation of the initial conditions. The chaos, which has long-term correlations, may be advantageous to the biosystem due to fast and specific responses during the adaptation of the metabolic network to environmental changes (De la Fuente 2010). For example, calcium is associated with the regulation of cell metabolism (Berridge 1993). Cytosolic calcium may exhibit chaotic transitions in response to environmental perturbation (Dixon et al. 1995). It not only exhibits fast and specific metabolic responses during the adaptation to environmental perturbations, but it is also associated with long-term memory properties.

Proplastids import biomolecules including proteins. Synthesis of biomolecules also goes on inside the proplastid. Increase in volume, accumulation of mass and organization of structural components are major biological events that occur in proplastids (Biswal et al. 2003).

Photosynthesis is the major function of the chloroplast. Photosynthesis has evolved as a non-linear system where charge separation between special pair chlorophylls (primary electron donor) and another porphyrin molecule (primary electron acceptor) occurs through a branched pathway with very high efficiency (Juretić and Županović 2003). A special chlorophyll molecule is strategically located close to a primary electron acceptor and a primary electron donor. Similar to an electrical circuit, when the net electron current flows, dissipation occurs and steady state affinity or photocell voltage decreases. In the photosynthetic apparatus, the large majority of photons absorbed by reaction centers are utilized in photoelectron transfer. This implies that a photosynthetic system works far from the chemical equilibrium state for the absorbed and emitted photons (Juretić and Županović 2003, 2005). The MEP and the optimal photochemical yield of the system are estimated to be 19.78 kJ mol^{-1} s^{-1} K^{-1} and 0.946, respectively (Juretić and Županović 2003, 2005). Thus photosynthetic proton pumping and electron transport operate close to the MEP mode, which synthesizes ATP and reduces NADP to NADPH at optimal rates. The evolution of the photosynthetic apparatus is guided by the principle of MEP coupling with the thermodynamic evolution of its surrounding universe (Juretić and Županović 2003). It has become possible to measure thermodynamic parameters of the photosynthetic apparatus by photoacoustic devices (see a review by Hou 2011, and references therein). However, proper selection and preparation of samples for obtaining information of the desired system along with its environment for appropriate interpretation are required. Besides, sustenance of photosynthesis by overcoming variations in environmental parameters also remains an important function of the chloroplast. The fluctuations are overcome by maintaining the redox status through photostasis, the balance between the production of NADPH (and ATP) and carbon dioxide fixation. Mathematically the energy balance state of PSII can be expressed as follows (Falkowski and Chen 2003):

$$\sigma_{PSII} E_k = \tau^{-1}, \tag{2.5}$$

where, σ_{PSII} is the effective absorption cross-section of PSII; E_k, the irradiance at which

the maximum photosynthetic quantum yield balances the photosynthetic capacity; and τ^{-1}, the rate at which the photosynthetic electrons are utilized by the sink.

The variation is decoded basically by energy imbalance between the source (determined by the redox state of the chloroplast) and the sink (determined primarily by carbon and nitrogen metabolisms) (Ensminger et al. 2006; Wilson et al. 2006). The changes induced by environmental fluctuations create a signaling system for the regulation of gene expression causing modification in cellular metabolism for photosynthetic adaptation (Ensminger et al. 2006; see the review by Biswal et al. 2011). Maintenance of wear and tear, i.e., damage to proteins, pigments, and the membrane system by biosynthesis is a continuous process. Photosynthesis and maintenance of steady states are two important biological processes that occur in a mature chloroplast.

A decline of photosynthesis marks the transformation of chloroplasts to gerontoplasts. Loss of pigments, unstacking of thylakoid membranes, dissociation of protein complexes in thylakoids, loss of Rubisco and other enzymes involved in the fixation of carbon, imbalance in the redox status, and biosynthesis of senescence inducing proteins are the major metabolic phenomena in gerontoplasts. Import of some of the nuclear-encoded proteins into gerontoplasts suggests that during senescence the envelope membrane is capable of active transport (Kawakami and Watanabe 1993). The gerontoplast is an organelle, which exports nutrients, primarily nitrogen containing molecules, to other actively growing and reproductive parts of the plant (see the review by Biswal et al. 2012).

3. Genetics

Genes regulate the processes of a biosystem and its life-cycle including formation, growth, reproduction, aging, and death. Biosystems go through developmental cycles preserving information about the self-organization processes, which have a high probability of success. This is crucial for the continuation of life (Kay 1984). Biosystems are at the most sophisticated end of the complexity scale. When a new biosystem is generated before the death of an earlier one, the information from the latter to the former is transferred to preserve the self-organization processes with a potential to improve its efficiency. The genes are the databases of successful self-organization processes. Genes constrain the process of self-organization to options with high probability of success while self-organization is the mechanism of development (Schneider and Kay 1995). Formation, growth and steady state of a biosystem away from equilibrium can be described as *order from disorder* while the continuance of developmental cycles from generation to generation is conceived as *order from order*. The existence of a biosystem is dependent on both the processes *order from disorder* to generate life and *order from order* to ensure the continuance of life (Schneider and Kay 1995).

Development of plastids from chloroplasts to gerontoplasts is neither governed by external or internal physicochemical forces, nor it has option to choose the time of occurrence. Rather, physicochemical forces that facilitate the development are controlled by genes through a well programmed genetic expression along a definite time arrow.

The genetic basis of development of the photosynthetic organelle has been investigated in green algae and higher plants (Ryberg and Sundqvist 1991; Barkan et al. 1995; Mache et al. 1997; Leon et al. 1998; Pyke 1999; Rochaix 2011). These studies showed that at the level of gene expression chloroplast development is regulated similarly in all green organisms.

Plastids have semi-autonomous status. They are regulated by both plastid and nuclear genomes. Plastids possess their own genome with a transcriptional machinery (see Kanamaru and Sugita, Chap. 10 in this volume) and are capable of synthesizing some of their own proteins. However, a large number of different plastid proteins are encoded by the nuclear genome. Many multimeric thylakoid complexes and soluble complexes in the

stroma are composed of some components encoded by plastid genes and some by nuclear genes (Goldschmidt-Clermont 1998). Down-regulation of these photosynthesis-associated genes (*PAGs*) in mature chloroplasts parallels the up-regulation of senescence-associated genes (*SAGs*) (see reviews by Biswal 1999; Biswal et al. 2003).

III. Thermodynamics of Developmental States of the Chloroplast System

A. Thermodynamic Characteristics of Proplastids: A Simple System

The proplastid is an organelle with less ordered subsystems in stroma but has the facilities for active transport across the double membrane envelope. The proplastids also possess the plastid genome and ribosomes for protein synthesis. The metabolic subsystems are yet to be organized. Active transport and potentiality for biosynthesis of proteins, nucleic acids, fatty acids, and pigments suggest that proplastids have a certain level of order, which is much lower than that of the chloroplast (Fig. 2.4). It is a relatively unstable system, which is transformed to a stable chloroplast on receiving specific signals (fluctuations) (Fig. 2.6).

B. From Proplastids to Mature Chloroplasts: Toward Order

During transformation of proplastids to chloroplasts, the organelle becomes more

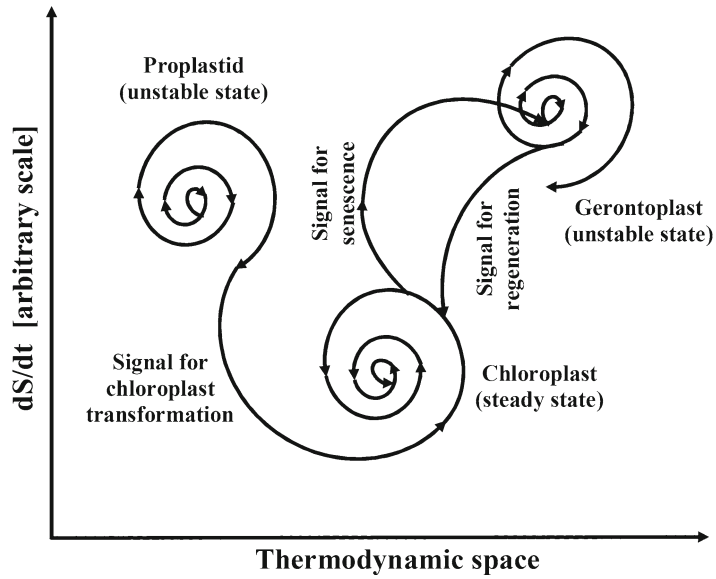

Fig. 2.6. Schematic representation of structure and fluctuation at different developmental states of the plastid system with arbitrary rate of entropy production (dS/dt) in thermodynamic space. The proplastid is at a relatively unstable state and fluctuations are amplified (signal for chloroplast transformation), which drive it to a steady state. Small fluctuations (stress) in steady state are damped by adaptive responses, and the system oscillates about the stable steady state. However, with aging, fluctuations are amplified (senescence signaling) and the system moves to an unstable state – the gerontoplast. A gerontoplast then degrades to a collapsing system, which is at complete equilibrium. Regeneration signals in gerontoplasts may regenerate the steady state of the chloroplast under special circumstances.

and more self-organized with the appearance of characteristic dissipative structures (Fig. 2.6). Biosynthesis of new molecules and their organization in complexes in both membrane and stroma occur in a precise manner, indicating that building up of chloroplasts is associated with a decrease in entropy of the system.

1. Signals for Plastid Differentiation

Proplastids differentiate in mesophyll tissue to chloroplasts in light. Light activates COP9 signalosome through phytochrome. This signaling system associated with gene expression during chloroplast development regulates expression of both nuclear and plastid genes in different signal transduction pathways. Plastid division, biosynthesis of proteins, import of nuclear proteins, intra-organelle protein targeting, pigment biosynthesis, and assembly of functional protein complexes are the major processes that take place during proplastid differentiation (Waters and Pyke 2005).

2. Self-organization of Subsystems and Biological Control

Proteins accumulated in proplastids interact specifically to form different complexes. Formation of functional protein complexes in the stroma or in the membranes does not occur spontaneously; it is rather governed by physico-chemical interactions. These are endergonic processes governed by biological control (genetic programming) leading to self-organization with MEP. A few specific examples of the biological control during chloroplast development are provided below: PSI assembly in thylakoid membrane starts with insertion of PsaB subunit (Rochaix 2011). It is designated as an anchor subunit. Unless the anchor subunit is inserted into the membrane, other subunits do not assemble. Assembly of other subunits occurs in a definite sequence. PsaA subunit binds to PsaB forming the CPI complex in the next step. Only after formation of the CPI complex, the PsaC subunit is assembled. Subsequently, other subunits assemble to form the PSI complex. Synthesis of PsaA is inhibited in the absence of PsaB subunit. PsaA, in the present case, is designated as "control by epistasy of synthesis" (CES) subunit. PsaC is also a CES subunit. Similarly, in PSII, D2 is an anchor subunit and D1 and CP47 are CES subunits (Kanervo et al. 2007). Protein targeting and events associated with the formation of grana structures are other examples of biological control of chloroplast development. Import of nuclear proteins from the cytosol to the stroma through the chloroplast envelope is regulated by Tic and Toc proteins (Vothknecht and Soll 2005; Andrès et al. 2010; Shi and Theg 2010; Strittmatter et al. 2010; Mulo 2011, see also Ling et al., Chap. 12). Targeting of proteins through the thylakoid membrane to the lumen is accomplished by Sec and SRP proteins (Robinson and Mant 2005). Lipid-protein interaction self-organizes to the complex thylakoid structures (Fig. 2.4). Thylakoid membranes stack at places to form more organized grana structures. Membrane appression at grana decreases the excluded volume. In other words, more space is available for the diffusion of proteins into the stroma. This boosts entropy production and acts as a driving force for grana formation (Chow et al. 2005; Kim et al. 2005). Grana formation has many important implications in photosynthesis, e.g., spatial separation of PSI and PSII, light harvesting, regulation of state transition, cyclic ATP synthesis (Chow et al. 2005). Grana formation results in a higher degree of internal compartmentalization. Hence, it may decrease entropy to a great extent (Marín et al. 2009). A proteolytic machinery also operates during the differentiation of plastids in order to degrade the unstable proteins or the assembly of proteins, where one or more subunits or prosthetic groups are missing (Adam 2005). Further, posttranslational modifications, hydrolysis of the peptide tag after targeting or transport, require proteolytic enzymes. The proteolytic

machinery is active in mature chloroplasts for regulating protein assembly (Adam 2005). The process of oxidative damage of D1, proteolysis and de novo replacement is a well known example. During senescence, proteolytic and other hydrolytic machineries are obviously active in dismantling of protein assemblies and conversion of macromolecules into smaller molecules. All these phenomena are under biological control and driven by the MEP principle. The metabolic network is also self-organized in the stroma of plastids (Fig. 2.4). Self-organization occurs in proplastids, which undergo transformation to a state of increased order associated with the MEP. The decrease in entropy of the system is achieved primarily at the expense of cytoplasmic energy sources while entropy of the universe is increased.

Biological control of chloroplast development has evolved through "learning" by the system of the most appropriate pathway and storing it into the "memory" subsystem of the genome. Transformation of *order from order*, evolving the process down to the generations, is a thermodynamic characteristic of a living system (Kauffman 2000).

3. Entropy of the System

The thermodynamic expression in terms of partial molar properties for this transformation, which occurs in an open system, away from the equilibrium, can be written as

$$\Delta G_i = \Delta H_i - T\Delta S_i \qquad (2.6)$$

where ΔG_i, ΔH_i and ΔS_i are the changes in partial molar free energy (chemical potential), enthalpy, and entropy, respectively; T is the temperature of the system in Kelvin. The free energy term, ΔG_i is not equal to zero because the process is away from the equilibrium. Entropy of the process increases as it is spontaneous. The change in entropy of the system is made up of two components: (a) d_eS, entropy exchange between the system and the surrounding and (b) d_iS, entropy change due to changes inside the system. The overall change in entropy (dS) of the system is expressed as

$$dS = d_eS + d_iS \qquad (2.7)$$

$d_iS = 0$ for reversible processes and $d_iS > 0$ for irreversible processes; however, d_iS is never less than zero.

In an open system, there is exchange of energy and mass between the system and the environment. The rate of change of entropy is expressed as

$$d_iS/dt = \Sigma J_k X_k > 0 \qquad (2.8)$$

where, J_k is the flux or rate of flow and X_k is the force responsible for such flux.

Entropy production is zero, at equilibrium; the flux (J_k) and the forces (X_k) are zero.

$$J_k = L_{kk} X_k \qquad (2.9)$$

where, $L_{kk} > 0$, and L_{kk} is called phenomenological coefficient for a system close to equilibrium. However, in the case of open systems far from equilibrium, where the thermodynamic forces are considerable, the flux is no longer a linear function of the force (Haase 1968; Yon-Kahn and Hervé 2010). During the developmental phase of the chloroplast from the proplastid, $dS/dt < 0$, as the system becomes more organized. The component d_iS/dt is always greater than zero, but the other component d_eS/dt can be less than zero. Hence, with magnitude of d_eS/dt ($d_eS/dt < 0$) being greater than that of d_iS/dt, the total rate of production of entropy is negative, i.e., entropy within the chloroplast decreases during the development of the chloroplast.

Transportation and targeting biomolecules to specific sites within chloroplasts during development is associated with positive ΔG_i quantity as it is an active, precise and directional process. Hence, these processes must be coupled to a process with large negative ΔG_i value. Weak interactions and reversible chemical bond formation can provide necessary negative free energy. Phosphorylation,

weak polar and hydrophobic interactions, steric compatibility and charge-charge interaction are a few such processes.

C. Mature Chloroplasts: The Order

A fully developed mature chloroplast is characterized by a steady state with respect to its structure and functions. No gross structural change is observed in a mature chloroplast even though the turnover of proteins and other biomolecules may be high. The biological processes of a chloroplast are in a dynamic steady state within the organelle and with the cytoplasmic environment. The major biological processes occurring in a chloroplast are (1) photoelectron transport; (2) synthesis of sugars through the Calvin-Benson cycle; (3) transport of sugars and other metabolites including phosphoglyceric acid (PGA) and dicarboxylic acids across the chloroplast membrane. The mature organelle maintains a stable steady state.

A chloroplast in a steady state exhibits a decrease in the entropy production and the rate of total entropy production is zero.

$$dS/dt = d_eS/dt + d_iS/dt = 0 \qquad (2.10)$$

$$\text{or } d_eS/dt = -d_iS/dt \qquad (2.11)$$

At steady state, d_iS/dt decreases to a minimum and $d_i^2S/dt^2 = 0$.

The steady state of the chloroplast is maintained and it itself does not come out of the state in spite of the perturbation occurring inside the system. Whenever there are changes in external forces, namely temperature, CO_2 concentration in the environment, osmotic pressure, and the intensity of light to perturb the system, the flux opposes these changes by the alterations in the redox state of the system, enzyme activity, and stress induced gene expression. These are known as the adaptational responses, which tend to restore the steady state as far as possible (Le Chatelier's principle). The flux caused by the perturbation may tend to dampen the perturbation so as to bring the system back to the steady state. Therefore, a chloroplast in non-equilibrium steady state, with $dS/dt = 0$, resists the stress condition by various stress adaptation mechanisms.

D. Aging of Chloroplasts: Breaking Down the Order

Aging chloroplasts exhibit the breaking down of structural organization and loss in photosynthetic function. Their adaptability weakens. Signaling systems induce the genomic information to perturb the steady state, and the chloroplast is moved to an unstable state – the gerontoplast (Fig. 2.6). The self-organized structures in the unstable gerontoplasts degrade. Degradation of macromolecules to small metabolites including amino acids, fatty acids, sugars and their transport to a new site of the growing cell in the plant are the major processes mediated by the gerontoplast (Lim et al. 2007; Biswal et al. 2012) (Fig. 2.5). Consequently, there is an increase in d_eS/dt, which becomes positive as the dissipative structures of the system collapse. The rate of production of the total entropy dS/dt becomes positive. However, the envelope is intact and active transport occurs in a gerontoplast. Some elements of order still persist with increasing disorder. There is a scope to recover the dissipative structures, i.e., by transformation of gerontoplast back to chloroplast. The gerontoplast may move to a steady state similar to the mature chloroplast (Fig. 2.6) (Smart 1994; Thomas 1994; Zavaleta-Mancera et al. 1999a, b; Van Doorn and Yoshimoto 2010; Parlitz et al. 2011, see also Fulgosi et al., Chap. 26).

E. Dissolution: The Disorder

The collapsing dissipative structures in gerontoplasts drift the system towards equilibrium. A point of no return is reached on the way to the equilibrium state. The process of lysis proceeds. Metabolic activity, active transport across envelope, and genetic

regulation disappear. The organelle reaches equilibrium. There is a complete collapse of dissipative structures. The complete disorder with maximum rate of production of total entropy persists.

Thus, the differentiating states of plastids during development appear to have different thermodynamic characteristics.

IV. Chloroplast Development: Recapitulating the Nature of the Living System

The nature of living systems is observed in chloroplast development. Hence, the observations on chloroplast development could be extrapolated to recapitule the nature of living systems or a self-organized dissipative structure in general.

A. Thermodynamics of Manifestation, Sustenance, and Dissolution

Manifestation of self-organized dissipative structures in proplastids, sustenance of self-organization in chloroplasts, and dissolution of self-organization in gerontoplasts constitute the states of development. Before lysis of plastids, the biomass is transported to newly generated proplastids developing into chloroplasts, and finally the biomass is stored in the seeds, which contain dormant proplastids. Germination of seeds gives rise to chloroplasts developing from proplastids in the cotyledons and leaves of seedlings. A seedling grows to a whole new plant which finally decays after producing seeds. Thus the life cycle of chloroplasts along with plant development continues. The life cycle of chloroplasts in a monocarpic plant is depicted in Fig. 2.5.

V. Conclusions

Chloroplast development is similar to the life cycle of any biosystem. It is an open thermodynamic system, far away from equilibrium and, hence, the principle of non-equilibrium thermodynamic systems could be applied to reveal the unknown aspects of the chloroplast system. However, very few reports are available on the study of the thermodynamic parameters of the chloroplast system. Thermodynamics may be useful in investigating metabolic subsystems – core, plastic and signaling networks (Almaas et al. 2005; De la Fuente et al. 2009; De la Fuente 2010). There are several grey areas in chloroplast development (Biswal et al. 2003, 2012). Many questions in this area still remain unanswered. Thermodynamic principles and mathematical approaches may be applied to answer these questions in the future.

The "state of the art" techniques, namely, photoacoustic devices, microarray, and tandem mass spectroscopy (MS-MS) may be applied for accumulation of experimental data on thermodynamic parameters at different states of development of chloroplasts along with changing proteomes and transcriptomes. The techniques are also useful to study self-organized dissipative structures in the chloroplast in the landscape of non-equilibrium thermodynamics.

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Part II

Chloroplast Biogenesis During Leaf Development

Chapter 3

Etioplasts and Their Significance in Chloroplast Biogenesis

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Summary

Etioplasts are considered as convenient but not completely adequate laboratory models of proplastid-to-chloroplast development. These plastids are formed in light-deprived tissues of angiosperm plants that would become chlorenchyma in the light. Etioplasts have a unique inner membrane consisting of highly regular, paracrystalline prolamellar bodies (PLBs) and of lamellar prothylakoids (PTs). First, we recall different situations where etioplasts or PLBs do appear and play an important role during normal leaf ontogenesis and chloroplast biogenesis under natural light conditions. These structures appear almost exclusively in

This chapter is dedicated to the late Professor Christer Sundqvist, a friend and major contributor to the etioplast-chloroplast transition art.

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young tissues with not completely differentiated chloroplasts and photosynthetic apparatus, and under conditions where light is either temporally and/or spatially limited during development. PLBs can be formed in young leaves during the dark phase of the light–dark cycles (LDC); or in young seedlings developing in the soil from seeds; in water plants or inside special structures, where a decreasing light gradient is naturally formed, e.g. buds, enveloping sheaths of outer leaves. Having discussed the relevance of etioplasts in chloroplast biogenesis, we then outline the structure, organization and assembly of etioplast inner membranes in etiolated seedlings. Furthermore, the different factors important for PLB formation, and in parallel, the molecular composition of the PLBs are reviewed in details. A special lipid composition, a high lipid per protein ratio, the presence of oligomers of NADPH:protochlorophyllide (Pchlide) oxidoreductase (LPOR) proteins binding Pchlide, NADPH and carotenoids may all be important for the stabilization and formation of the special cubic membrane of the PLBs. Therefore, the biosynthesis of pigments in etioplasts is also discussed. The last part focuses on the etioplast-to-chloroplast transition during greening of etiolated seedlings, and summarizes the ultrastructural, molecular and physiological changes observed during this process. Finally, the significance of PLBs in plant development and leaf ontogeny is outlined.

I. Introduction

Land plants and eucaryotic algae contain plastids, the intracellular compartments responsible – among others – for photosynthesis. Plastids derive from polyphyletic endosymbiotic events where ancestral photosynthetic procaryotes were engulfed and retained by heterotrophic, aerobic protoeucaryotes. In land plants (and algae) these two organisms became inextricably associated due to several processes that occurred during their co-evolution including the transfer of genetic information from the endosymbiont to the nuclear genome of the host. This way, although retaining (part of) its own DNA, nucleic acid and protein synthesis machinery, the plastid got fully integrated in the host cell, became semi-autonomic and therefore intensive co-regulation and communication occur between the host nucleus and the organelle (Waters and Pyke 2004).

The regulation is even more evident when considering the diversification of plastids within the plant body. Plastids got specialized in parallel with the increasing genetic, developmental and morphological complexity and diversity acquired by embryophytes. In non-photosynthetic organs, tissues and cells, the most ancient plastid type, the chloroplast, has partially or completely lost its photosynthetic activity, and distinct plastid types harboring other vital physiological functions and metabolites have differentiated. Therefore, plastid differentiation occurs concomitantly with cellular differentiation and is under the control of genetic and environmental factors. However, the functional and morphological differences between plastid types should be considered only as an acquired, but not definitive and irreversible specialization. Consistently with this, the term plastid (meaning 'formed, mouldable') reflects the high plasticity of these organelles that contributed to the fitness and successful adaptation of higher plants to the changing environment. Although plastid types are highly and dynamically interconvertible

Abbreviations: ALA – 5-aminolevulinic acid; Chl(s) – Chlorophyll(s); Chlide – Chlorophyllide; Coprogen III – Coproporphyrinogen III; DGDG – Digalactosyl diacylglycerol; DPOR – Dark-operative NADPH: Pchlide oxidoreductase; GG – Geranylgeraniol; GGPP – Geranylgeraniol diphosphate; GSA – Glutamate-1-semialdehyde; IPP – Isopentenyl diphosphate; LDC – Light–dark cycle; LPOR – Light-dependent NADPH: Pchlide oxidoreductase; LPOR-A, -B, -C – Isoforms of LPOR; MGDG – Monogalactosyl diacylglycerol; Pchlide – Protochlorophyllide; PGB – Porphobilinogen; PLB – Prolamellar body; Protogen IX – Porphyrinogen III; PSI – Photosystem I; PSII – Photosystem II; PT – Prothylakoid; Urogen III – Uroporphyrinogen III

(Waters and Pyke 2004; Solymosi and Schoefs 2008), their classification is based on their most obvious functions, generally reflected also in their morphological traits.

Proplastids defined as small, undifferentiated organelles with no or poorly developed internal membranes, have at least two basically different functional forms. Proplastids present in meristematic and embryonic tissues as well as in dedifferentiated cells are the precursors to all other plastid types, while proplastids found in root nodules in proximity of symbiotic intracellular nitrogen fixing bacteria play a major role in incorporating fixed nitrogen into a large number of biologically important nitrogenous compounds (Wise 2006).

Chromoplasts present in red-, orange- and yellow-colored plant parts (e.g. carrot roots, fruits, petals or tepals) accumulate carotenoids and are thought to play a role mostly in plant reproduction.

Amyloplasts are characteristic mostly for storage organs and tissues (present in e.g. roots, tubers, bulbs, rhizomes, seeds or fruits) or are associated with root gravisensing in the columella region of the calyptra. They are colorless and belong to leucoplasts, the plastid type which, in contrast to the chloroplast and the chromoplast, does not contain significant amounts of pigments. Leucoplasts are specialized for storage of e.g. starch (amyloplasts), oils and lipids (elaioplasts present e.g. in tapetum cells of anthers) or proteins (proteinoplasts e.g. in phloem cells) (Gunning and Steer 1996; Wise 2006).

The physiologically and biochemically best characterized and studied plastid type is the chloroplast, responsible for photosynthesis. Chloroplasts can be found in all green, photosynthetically active plant tissues (chlorenchyma) or cells (e.g. guard cells of stomata) present in various organs including green roots (e.g. epiphyte orchids), seeds (e.g. chloroembryophytes), fruits, flower parts (green petals, sepals, tepals, pistil), and most obviously green stems or leaves. During foliar senescence chloroplast thylakoids and pigments are dismantled and large plastoglobuli are formed in the so-called gerontoplasts or senescing chloroplasts (Mulisch and Krupinska, Chap. 14; Lichtenthaler, Chap. 15).

It is generally accepted that the differentiation of proplastids into mature chloroplasts is the obligatory pathway of chloroplast development, occurring in much of the above ground tissues in most plants (Pyke 2007). However, there are several special cases when this process occurs from other plastid types (e.g. chromoplasts – for instance during the greening of carrot roots, amyloplasts or even in rejuvenating gerontoplasts).

In spite of data and models describing the interconversion and development of the different plastids in distinct cell types, the exact molecular regulation of plastid biogenesis by the host cell is largely unknown. Besides internal factors (e.g. co-regulation with host cell differentiation, hormones), external factors, and in angiosperms especially the light conditions influence plastid differentiation. Hence, when putative photosynthetically active cells develop under light deprivation, chloroplast development is impaired, and etioplasts differentiate in them. Etioplasts are characterized by their unique inner membrane system consisting of the paracrystalline prolamellar body (PLB) with lipids in cubic phase and the interconnected lamellar prothylakoids (PTs) (Fig. 3.1a). On the physiological and biochemical level, the most important feature of etioplasts is that they lack chlorophyll (Chl) but instead accumulate low amounts of protochlorophyllide (Pchlide) and the photoenzyme, NADPH: Pchlide oxidoreductase (LPOR). LPOR drives the light-dependent step of Chl biosynthesis, i.e. the transformation of Pchlide into chlorophyllide (Chlide) (Solymosi and Schoefs 2008). Etiolated leaves are yellow due to carotenoids, which are not masked by the presence of high amounts of chlorophyllous pigments (Fig. 3.2). Etioplast differentiation and the transformation of etioplasts into chloroplasts upon light exposure have received considerable attention. In the next subchapters we discuss the significance of etioplasts in chloroplast differentiation, then give a detailed overview about the data on etioplast structure, differentiation

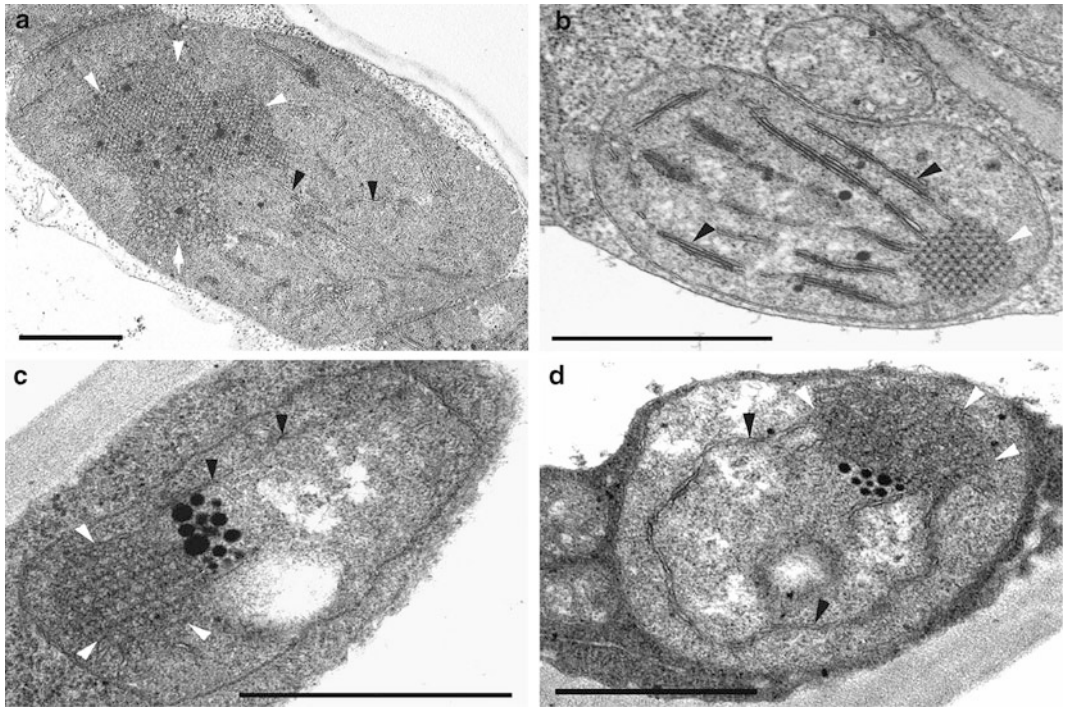

Fig. 3.1. Electron micrographs representing an etioplast of an etiolated 7-day-old wheat leaf grown under laboratory conditions (**a**), an etio-chloroplast characteristic for the inner leaf primordium of an opening horse chestnut bud collected in the nature (**b**), and etioplasts characteristic for the innermost leaves of Savoy cabbage heads (**c**, **d**). *White arrowheads* show the PLBs, the *white arrow* (on *panel A*) shows an "open" region of the PLB lattice with wider spacing between the membrane tubules. *Black arrowheads* indicate prothylakoids (**a**, **c**, **d**) or developing grana (**b**). *Bar* = 1 μm.

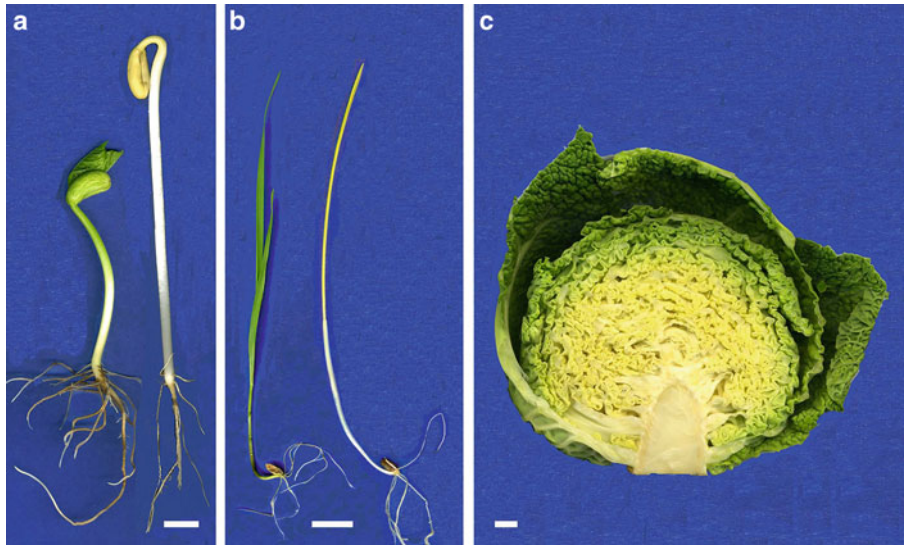

Fig. 3.2. 10-day-old light-grown and dark-grown (etiolated) bean (**a**), or wheat (**b**) seedlings, and a Savoy cabbage head collected from natural conditions with inner leaves developing under the shading of the outer, green leaves (**c**). Please note the pale (*yellow*) color of the etiolated or light-deprived tissues lacking chlorophylls. *Bar* = 1 cm.

and composition, pigment synthesis and etioplast-to-chloroplast transformation in etiolated leaves.

II. The Significance of Etioplasts in Chloroplast Differentiation

Dark-grown angiosperms represent a good model to investigate photomorphogenetic and light-dependent processes, since chloroplast differentiation, Chl biosynthesis and as a consequence photosynthesis, and other light-induced reactions (i.e. all processes related to photoreceptors) are inhibited in them, and can be induced and studied in a synchronized way. Thus, etiolated systems facilitate data interpretation thanks to the complete lack of chlorophylls and functional thylakoids, providing a good reference or starting point for further analyses and comparisons. Moreover, in contrast with light-grown tissues, cell and plastid divisions are often restricted or slower (Verbelen and de Greef 1979a), and after a certain period plastid development reaches a stationary phase in etiolated organs (Whatley 1977a). Thus, prolonged dark-growth results in a relatively homogenous population of plastids, which contain most thylakoid components that do not require light for synthesis (Smith 1978; Biswal et al. 2003; Solymosi and Schoefs 2008). Upon illumination, profound molecular alterations and rearrangements occur within the etioplasts, and their components are used to form functional chloroplasts rapidly (Gunning 2001; Blomqvist et al. 2006, 2008; Kleffmann et al. 2007).

Under natural conditions, chloroplast differentiation in general originates from proplastids without involving the etioplast stage. Therefore, studies investigating the etioplast-chloroplast transition as a model of organelle biogenesis have been debated both at the theoretical and the practical level. However, there are several data reporting prolonged darkness and the involvement of etioplasts in chloroplast biogenesis under natural conditions (Kutik 1998; Solymosi and Schoefs 2008, 2010). The role of PLBs in chloroplast differentiation of cotyledons germinating in the soil is often considered to be evident (Kirk and Tilney-Bassett 1967; Waters 2004), but in contrast its role during normal leaf development is often questioned. Here we review data regarding the role of PLBs in chloroplast biogenesis of true leaves under natural conditions.

With very few exceptions (Solymosi and Schoefs 2010), PLBs have been described in developmentally young (i.e. often meristematic) tissues containing differentiating chloroplasts, but have not been detected in chloroplasts of fully differentiated leaf cells having reached their final Chl content (Table 3.1, Ikeda 1970, 1971; Rascio et al. 1976, 1985; Bennett et al. 1987). Some authors suggest therefore to distinguish the 'young chloroplast' stage during proplastid-to-chloroplast transition, which stage may involve PLBs (Perner 1956). Plastids that contain both PLBs and developing or differentiated grana are termed etio-chloroplasts.

Furthermore, the occurrence of PLBs in chloroplasts is often associated with low light intensity (e.g. below the threshold at 10 μmol m^{-2} s^{-1} photon flux density in maize leaves) or with the complete absence of light (Bennett et al. 1987, Table 3.1). Darkness or decreasing light intensity gradients are conditions that occur in the nature where illumination is often limited. This limitation is spatial and/or temporal, i.e. sunlight is limited in space and thus the overall light intensity reaching the developing leaf is too low or zero, or illumination is limited in time and PLBs are reformed during the dark period of the LDC.

Low light intensity can occur in several situations. For instance below 2.2 or 5 mm soil, the incident light intensity decreases to 1% or 0.5%, respectively (Smith 1982, 1994). Day light (1900 μmol m^{-2} s^{-1}) can be strongly attenuated by canopies, for instance it decreases to 17.7 μmol m^{-2} s^{-1} under ivy canopy (Smith 1982). Depending on the transparency (turbidity) and depth of lakes, incident light intensity can dramatically decrease in them (Smith 1982). Plant structures (e.g. overlapping bud scales or outer leaves, fruit pericarps,

Table 3.1. Involvement of PLBs in plastid biogenesis in leaves under natural conditions. Examples are shown for different plant species and experimental conditions e.g. sample collection, light conditions, developmental age of the sample and chlorophyll biosynthesis (data are only included when available). Light intensity is converted (indicated by '~') to photon flux density for comparison.

Plant species	Sample collection, light conditions	PLB containing plant material, Chl biosynthesis	Reference
Appearance of PLBs during the dark phase of the light–dark cycles			
Barley (*Hordeum vulgare*)	"Outdoors", varying between ~19–222 μmol m^{-2} s^{-1} during the day, PLBs present only during the night	"Primary leaves", age not indicated, their Chl content has not reached the Chl content of fully green leaves	Ikeda 1971
Bean (*Phaseolus vulgaris*)	16:8 h LDC, 50 μmol m^{-2} s^{-1}	Primary leaves after 3 days and until 7 days of germination, PLBs appear only during the night, intensive Chl synthesis	Schoefs and Franck 2008
Maize (*Zea mays*)	The PLB is "perhaps a relic of membrane growth in darkness during the night before the early morning harvesting of the material"	In mesophyll cells of a young, still growing leaf with no fully mature chloroplasts, "as indicated by the presence of … PLB" in them	Gunning and Steer 1996
Appearance of PLBs in seeds germinating in the soil			
Bean (*Phaseolus vulgaris*)	"Growth cabinet", 12:12 h LDC (light intensity not indicated), seeds sown in silver sand (depth not indicated but based on a Figure ~ 1 cm depth)	PLBs appear in primary leaves after 3 days of germination (leaves still buried in the soil), but amorphous PLBs persist until full chloroplast differentiation	Whatley 1974
	Continuous light of ~74 μmol m^{-2} s^{-1}, seeds sown in vermiculite (1 cm depth)	PLBs appear in primary leaves 6 days after germination, but disappear on the next day	Verbelen and de Greef 1979b
Appearance of PLBs under natural, high light conditions			
Bean (*Phaseolus vulgaris*)	12:12 h LDC, light intensity "only about 1/5 of that outside at noon in midsummer" (~400 μmol m^{-2} s^{-1}), shading provided by the cotyledons	Young primary leaves at the beginning of their development (until day 5)	Whatley 1977a, b
Ceratozamia mexicana	"Greenhouse under natural photoperiod" during winter; from spring until end of summer "outside" at a "shadowy site"	"Very young dark red-brown leaflets", 2–5 cm long, curled leaves; but no PLBs in mature leaf chloroplasts	Bonzi et al. 1992
Durian *(Durio zibethinus)*	"Botanic gardens at Kew and in Oxford"	Young cells, "young chloroplast in a 20 mm pale green leaf", probably low Chl content	Whatley 1992
Ginkgo (*Ginkgo biloba*)	"Growth chamber", 12:12 h LDC, ~340 μmol m^{-2} s^{-1}	Young leaves, juvenile stages of proplastid-to-chloroplast development, absent from mature chloroplasts	Rascio et al. 1984a
Hart's-tongue fern (*Phyllitis scolopendrium*)	"Open-grown plants from the Botanic Garden of Padua University"	"Very juvenile stages", apical region of a tightly coiled and hairy crozier, low Chl content, intensive Chl synthesis	Mariani Colombo et al. 1983

Horse chestnut (*Aesculus hippocastanum*)	From parks and a small forest in Budapest during daytime, transient shading by bud scales and outer leaf primordia (~1% of incident natural light, enriched in red and far-red light)	Developing leaf primordia of opening buds, young, meristematic cells with etioplasts or etio-chloroplasts, low Chl content, intensive Chl synthesis	Fig. 3.1b, Solymosi et al. 2006a
Maize (*Zea mays*)	16:8 h LDC, ~340 μmol m^{-2} s^{-1}, shading provided by the enveloping sheaths of outer leaves	Young mesophyll and bundle sheath cells near the intercalary meristem of inner, enrolled young leaves, ~17% of their final Chl content	Leech et al. 1973
	12:12 h LDC, light intensity "only about 1/5 of that outside at noon in midsummer" (~400 μmol m^{-2} s^{-1})	"5 cm below tip of third leaf of 22 cm plant" – probably young and inner (enrolled) leaf	Whatley 1977b
Marine eelgrass (*Zostera capricornii*)	Plants still covered by water were collected during low tide, transient shading by water and sheathing leaf bases of surrounding older leaves	Young, still differentiating cells close to the intercalary meristem, low Chl content	Adamson et al. 1985
River spiderwort (*Tradescantia albiflora*)	"High light in a glass house"	6–8 week old plants, in "leaf two from the shoot tip" (young leaf), low Chl content	Adamson et al. 1980
Savoy cabbage (*Brassica oleracea* cv. sabauda)	From agricultural fields in Vecsés, in spring, during daytime, shading provided by outer leaves in the head, prolonged etiolation, no light reaches the inner leaves	Young, innermost leaf primordia, etioplasts, no Chls, but only its precursors	Fig. 3.1c, d
Sesame (*Sesamum indicum*)	"In a greenhouse"	Epidermal cells of very young (0.5 cm long) leaves	Platt-Aloia and Thomson 1977
Spinach (*Spinacia oleracea*)	"In a greenhouse"	"Yellow basal region" and "middle pale green region" of a 5 cm long young leaf, differentiating cells, probably low Chl content	Rascio et al. 1985
		In "young" leaves grown in autumn, low Chl content, PLBs absent from similar leaves in summer	Polettini et al. 1986
Tobacco (*Nicotiana tabacum*)	"Greenhouse"	1 cm long, young leaf primordia on the shoot tip	Stetler and Laetsch 1969
	"Open air-grown"	Basal and apical zones of leaves shorter than 12 cm	Casadoro and Rascio 1979
Wheat (*Triticum aestivum*)	"Glasshouse-grown"	Only in young leaves with fast cellular differentiation	Casadoro and Rascio 1979

(continued)

Table 3.1. (continued)

Plant species	Sample collection, light conditions	PLB containing plant material, Chl biosynthesis	Reference
White cabbage (*Brassica oleracea* cv. capitata)	As in Savoy cabbage (see above), but PLBs are less frequent, and occur only in the innermost leaves	Youngest, innermost leaf primordia, etioplasts, no Chls, but only its precursors	Solymosi et al. 2004
Appearance of PLBs under relatively low light in laboratory conditions (but using LDC illumination)			
Barley (*Hordeum vulgare*)	16:8 h LDC, 5 μmol m^{-2} s^{-1}	Young (7-d-old), light-grown leaves, amorphous PLBs present in all leaf segments	Wellburn et al. 1982
Maize (*Zea mays*)	Osram, fluorescent tube, 15:9 h LDC, ~6 μmol m^{-2} s^{-1} (PLBs absent at ~83 μmol m^{-2} s^{-1})	Young (10-d-old), light-grown leaves transferred to low light (~6 μmol m^{-2} s^{-1}) for 2–3 days	Wrischer 1966
	Fluorescent tube, 12:12 h LDC, ~76 μmol m^{-2} s^{-1}	PLBs present during both the dark and the light phase, but only in young leaves and close to the intercalary meristem	Rascio et al. 1976
Sugar cane (*Saccharum officinarum*)	"Growth chamber", 14:10 h LDC, ~30 μmol m^{-2} s^{-1}	In young mesophyll and bundle sheath cells, from leaf regions close to the basal meristem, and/or containing 50 or 75% of their final Chl content	Laetsch and Price 1969

seed coats) may also provide partial or complete shading to inner tissues resulting in PLB formation in them (Table 3.1, Vogelmann 1989; Solymosi and Schoefs 2008, 2010). At sunset, the light intensity (photon flux density) is around 26.5 μmol m^{-2} s^{-1} and this value further decreases during the night (moonlight equals appr. 0.005 μmol m^{-2} s^{-1}) (Smith 1982). At the same time, the red and far-red composition of the light is often altered in the situations mentioned above (Smith 1982, 1994), which may favour PLB formation (Solymosi and Schoefs 2008).

Taken together, spatial light limitation may occur in leaves developing (1) in the soil or under litter coverage, (2) under strong shade conditions in forests with thick canopies or in water, (3) under coverage by other, outer organs or tissue layers in special plant structures (Table 3.1, Solymosi and Schoefs 2008, 2010).

There are to our knowledge almost no literature about PLBs in plants grown under thick canopies or in water (Adamson et al. 1985), and only few studies about etiolated leaves developing from deeply buried seeds in the soil (Whatley 1974; Verbelen and de Greef 1979b).

During bud break (Fig. 3.1b, Solymosi et al. 2006a), and in the closed buds of some species (Solymosi and Böddi 2006), transient etiolation occurs in the inner leaf primordia. However, after bud break these etioplasts or etio-chloroplasts are converted to chloroplasts, suggesting a possible involvement of PLBs in chloroplast biogenesis under natural conditions. In cabbage heads (Fig. 3.2c) etioplasts differentiate in the innermost, young leaves (Fig. 3.1c, d, Solymosi et al. 2004), but these are not involved in normal chloroplast differentiation, unless the leaves get exposed to light.

Temporal light deprivation occurs during the night (Smith 1982, 1994). This situation may induce PLB formation during the early and very intensive period of plant development, i.e. in differentiating chloroplasts of young leaves (Ikeda 1970, 1971; Schoefs and Franck 2008) or cotyledons (Rebeiz and Rebeiz 1986).

In contrast to similar structures of etioplasts of dark-grown seedlings, the PLBs of etio-chloroplasts appear already 5 min after sunset under natural conditions, and disappear shortly at sunrise (within 15–20 min), when increasing light intensity reaches them (Ikeda 1970, 1971). They are in general smaller (0.5−1 μm, Adamson et al. 1985) and more numerous (Ikeda 1971; Whatley 1974) than the PLBs in etioplasts of dark-germinated seedlings. Similar structures have often been reported in plants grown under laboratory conditions in low light (Table 3.1). These PLBs have in general a highly regular structure, but in some cases they are ‘amorphous’ (Whatley 1974, 1977b; Wellburn et al. 1982). Due to the structural and possible functional differences between PLBs of etioplasts formed in dark-germinated seedlings under laboratory conditions, and the PLBs assembled in the nature under low light conditions and associated with developing chloroplast thylakoids, some authors prefer to distinguish these two by referring to the latter as “prothylakoid body” (Wellburn 1982) or “low-light PLB” (Wrischer 1966; Biswal et al. 2003). However, experimental knowledge about the physiology, molecular biology and regulation of PLBs occurring in the nature is rather scarce (Solymosi and Schoefs 2010). Normal PLB isolating methods were not successful to isolate them, which may further confirm differences in the stability of these membranes when compared with PLBs present in etioplasts (Wellburn 1982). The etio-chloroplast stage in this case seems to represent a highly dynamic system with PLBs formed between chloroplast thylakoids (especially grana) during the night and/or at low light conditions, and disappearing quickly upon illumination with high light intensity. It is tempting to speculate that this structure enables fast conversion and transfer of different molecules synthesized during the night (lipids, proteins) or at the very beginning of the illumination (Chls) to the developing thylakoids. In addition, it has been shown that PLBs, and the large oligomers of the ternary complexes of LPOR characteristic for them, have a crucial role in preventing pigment photooxidation

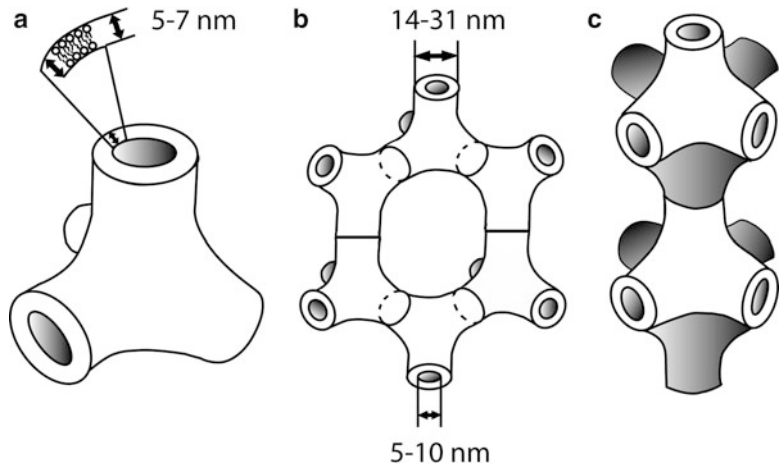

Fig. 3.3. Model representing the tetrapodel (**a**) and hexapodel units (**c**) of membrane tubules that build up the PLB structure. The internal proportions in the tetrapodel and hexapodel units are not shown correctly. (**b**), six tetrapodel units join each-other to form a hexagonal ring, the building unit of the PLBs. The units can join each other in several ways, here only one possibility is shown. Some dimensions of the PLBs are also indicated. (**c**), the hexapodel units join each other in a square lattice, which is very rarely found in PLBs.

and bleaching, as well as oxidative stress upon light exposure of dark-grown seedlings (Solymosi and Schoefs 2010).

The above data demonstrate that there are natural conditions under which PLBs can differentiate in leaves and may be involved in chloroplast biogenesis. However, in most of these situations cell and plastid differentiation are probably not synchronous in the nature, and great heterogeneity may be observed among different cells of the same leaf. Therefore, such systems are not so convenient for systematic scientific studies as etiolated plants grown under laboratory conditions.

III. Etioplast Structure and Differentiation

Since the first reports from the 1950s, the ultrastructure of etioplasts has been widely studied and characterized in dark-grown angiosperms. The etioplasts are lens-shaped or rather oval bodies around 1–5 μm in diameter. The cells of etiolated barley leaves contain 33–65 plastids, but this number increases with cell age (Robertson and Laetsch 1974). There are variations in this number between species and it also changes upon light exposure, e.g. it increases from 80 to 100 in oat (Ohnishi and Yamada 1980). Etioplasts often contain plastoglobuli, starch and may enclose phytoferritin. The etioplast inner membranes are arranged into PLBs and PTs that are interconnected (Fig. 3.1a, c, d). The ratio of PT and PLB membranes within the etioplasts can differ among species, organs and tissues, and changes during the ontogenesis of the plant and with the growth conditions. The PTs are flat, lamellar membranes resembling stroma thylakoids (Schnepf 1964), and they stretch out from PLBs into the stroma and can reach a length of 1–2 μm. There are often perforations in the PTs. Sometimes (depending on the age of the tissue and on the plant species studied) PTs are arranged in continuous PT-stacks (e.g. Rascio et al. 1986).

The PLB is a highly organized, semi-crystalline membrane structure, built up by cubic lattices of branched tubules of interconnected membranes (Figs. 3.3 and 3.4). The size of the PLBs varies usually between 0.5 and 2 μm. This membrane structure is unique because its lipids form a bicontinuous cubic phase instead of the usual lamellar structure of the lipid bilayers characteristic for most membranes

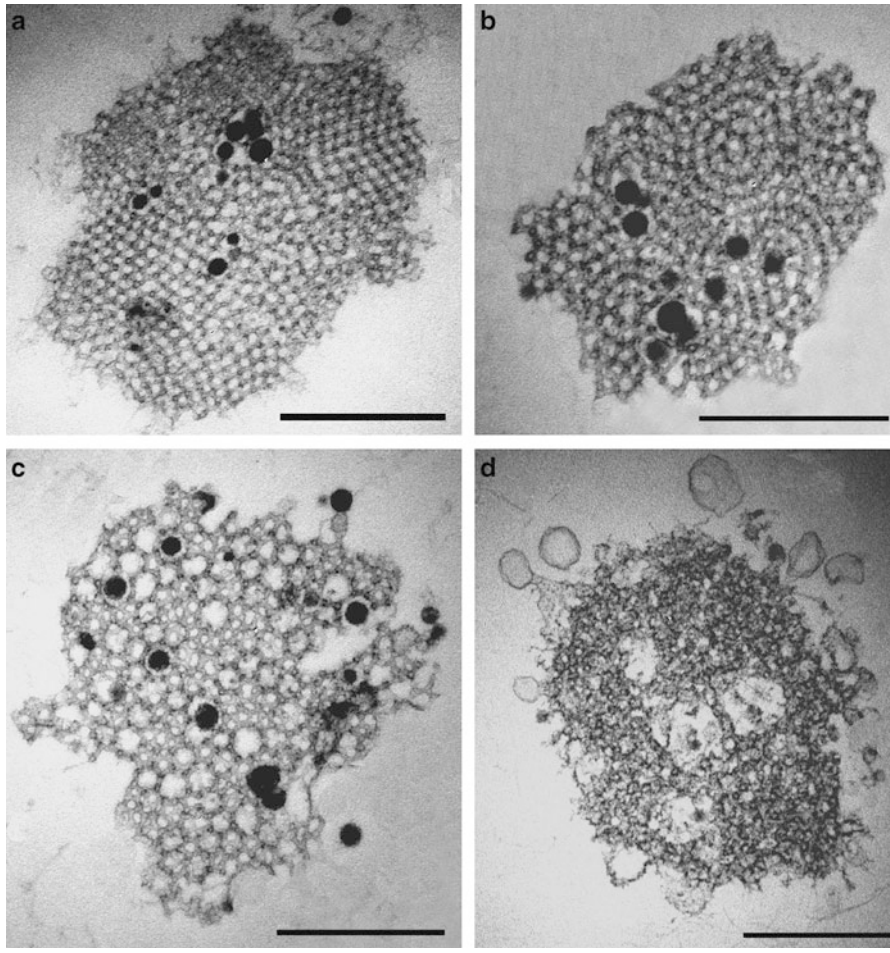

Fig. 3.4. Electron micrographs representing different characteristic features and appearances of PLBs. The PLBs were isolated from wheat leaves and studied as described in Myśliwa-Kurdziel et al. (1999) and Solymosi et al. (2006b). (**a–c**), samples kept in the dark; (**d**), sample illuminated for 40 min with 100 μmol m^{-2} s^{-1} at 20°C. Please note that different lattice types and/or structural appearances can be observed even within the same PLB (**a**). (**b**), centric or "spiral" PLB; (**c**), "open" PLB. Please note the disruption of the PLB lattice and vesicle formation on the PLB surface upon illumination (**d**). *Bar* = 0.5 μm.

(Selstam 1998). This membrane network has an enormous surface area and is considered to be the most compact store of unstacked cell membranes as its surface to volume ratio can amount to 50 (Gunning 2004). Its curved shape allows its fast transformation into thylakoids: rough estimations indicate that one PLB can transform directly into approximately 100 μm^2 of thylakoid membranes (Gunning 2004).

The membrane tubules of the PLBs are formed by membrane bilayers (5–7 nm thick), have an outer diameter of 14–31 nm and encircle a lumen with a diameter of 5–10 nm (Fig. 3.3, Gunning 1965; Selstam 1998). Thus, the bicontinuous membrane separates two compartments, the stroma of the etioplast and the PLB lumen. A plastid ribosome is commonly present at the center of each hexagonal unit in the prolamellar body lattice but it is not clear whether it has any structural or physiological role in PLB stabilization (Gunning 2004). Plastoglobuli also often occur in between the PLB lattice and disrupt its regularity (e.g. Fig. 3.4a–c).

The building units of the PLBs are usually 4-armed (tetrapodel or tetrahedral) or rarely 6-armed (Gunning 1965). The 4-armed or 6-armed units can be connected in different ways (Fig. 3.3), thus forming PLBs with different structural appearances and different (narrow or wide) spacing between the membranes (Fig. 3.4a–c, Gunning 1965, 2001, 2004). Besides the lattice type, the sectioning plane also influences the structural appearance of the PLB in ultrathin sections. The two most common PLB geometries are analogues of the arrangement of the atoms in crystal lattice of zinc sulphide, i.e., they correspond to wurtzite or zincblende crystal forms. In both cases six tetrahedrally branched units are joined in a hexagonal ring (e.g. Fig. 3.3b). Such rings can extend in all directions in the plane of the ring, and successive planes can also join in the third dimension, in two different ways. If the arms of the basic units are parallel to one another in the successive planes in the third dimension, then a crystal structure analogous to the mineral "wurtzite" is formed. In the zincblende (or diamond type) lattice the hexagons are held together in a structure with the same symmetry as the carbon atoms in the diamond crystal, i.e. the arms of the basic units are rotated 60° relative to one another in the successive planes, consequently the successive planes lie "out of register" with one another (Gunning 1965, 2004; Gunning and Steer 1996). Furthermore, the tetrahedral building units may form pentagonal rings and may be combined to form, very complex lattice structures. For instance, the spiral or so-called "centric" PLBs (Fig. 3.4b) have 20 large tetrahedral units radiating outwards, one from each of the 20 vertices of a central pentagonal dodecahedron (Gunning and Steer 1996). In 3D, this creates an icosahedral shape. PLBs with unusual, relatively wide-spacing structure were also termed noncrystalline PLB or "open" PLBs (Fig. 3.4c, Weier and Brown 1970; Gunning and Steer 1996; Gunning 2001, 2004). These have a complicated structure, the membrane surface is composed almost entirely of large polyhedra and it is difficult to discern elements of symmetry (Gunning 2001, 2004). Rarely, square lattice with six-armed tubes as building units interconnected at right angles to each other can be found in PLBs (e.g. Murakami et al. 1985, Fig. 3.3c). The physiological significance of the different types of PLBs is not known. They can occur in a single species, within the same etioplast and even in adjacent regions of a PLB (Figs. 3.1a and 3.4a, Henningsen and Boynton 1969; Henningsen et al. 1993; Gunning and Steer 1996), and their proportions may be affected by the plant age (Robertson and Laetsch 1974).

In contrast, much less is known about factors that determine and influence the assembly and differentiation of etioplast inner membranes. Because the intercalary meristem of monocots is located at the base of the leaf, a plastid developmental gradient is naturally organized along the leaf: the youngest cells being close to the basal meristem while the oldest ones being located at the leaf tip. Etioplast differentiation has been often studied along this spatial developmental gradient in monocots (Fig. 3.5, e.g. Robertson and Laetsch 1974; Mackender 1978; Boffey et al. 1980; Lütz 1981a, b) or during the life cycle of plastids in dicots (e.g., Weier and Brown 1970; Sperling et al. 1998). The inner membranes are assumed to be formed by vesicles budding off from the envelope of the proplastids (Fig. 3.5, Selstam 1998). According to Bradbeer et al. (1974a) the vesicles fuse and form a perforated membrane that develops into PLBs and PTs. At this early stage of plastid differentiation the plastids contain several, parallelly arranged perforated prothylakoid sheets (Fig. 3.5, Rascio et al. 1986) similar to the early phase of proplastid-to-chloroplast transformation. The plastids may contain large starch grains between the perforated sheets and at this developmental stage they resemble amyloplasts (Mackender 1978; Younis et al. 1995). In older leaf segments small and irregular PLBs appear between the parallel membranes, which are later transformed into "open" PLBs (Fig. 3.5, Rascio et al. 1986). The PLBs of the next developmental stage become highly regular and are often transformed into polycentric PLBs or PLBs with

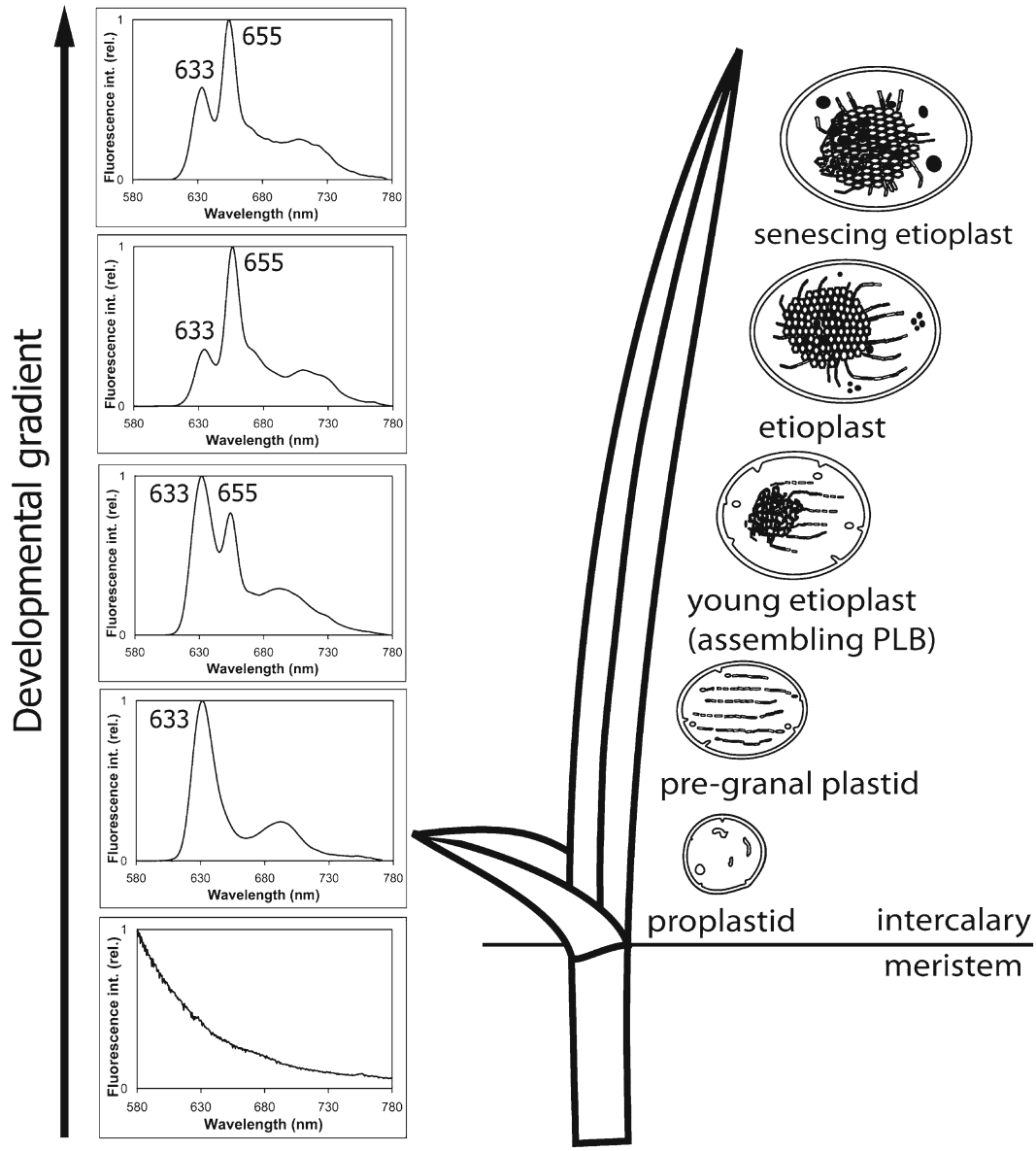

Fig. 3.5. Different stages of etioplast differentiation along a cereal leaf. The youngest cells close to the intercalary meristem contain proplastids, which differentiate into pre-granal plastids with perforated prothylakoids arranged parallelly. In some species amyloplasts with large starch grains and perforated prothylakoids are the characteristics at this stage (not shown). The two first stages can be also observed during proplastid-to-chloroplast differentiation in light-grown plants. Extensive vesicle budding from the envelope occurs until the young etioplast stage, in which the membranes fuse to form a small and irregular PLB. The next step is the formation of highly regular PLBs in mature etioplasts, which lose their regular structure during senescence, and the amount of prothylakoids also decreases in them. The size and number of plastoglobuli inside the PLBs also increases with age. For comparison, characteristic 77 K fluorescence emission spectra corresponding to different, sequential developmental stages have been also added to the figure. In this case, a baseline with no signal of chlorophyllous pigments is found only in the youngest meristematic cells or at the very beginning of germination. Then the fluorescence band with an emission maximum at around 633 nm, characteristic for non-photoactive protochlorophyllide, appears and is dominating in the spectra. In parallel with the appearance of PLBs, first a shoulder, then a dominating band appears at 655 nm, which corresponds to photoactive Pchlide. The lowest 633/655 ratio is characteristic for mature etioplasts with highly regular PLBs, and this ratio increases with senescence. Please note that the cereal leaf drawn in the middle is only for illustration of the sequence and tendencies of plastid differentiation and spectral changes, but the timing and exact "position" of the processes may vary among species and within the different cell types of the leaf.

building units arranged into hexagonal rings. Close to the leaf tip the senescence of the etioplasts can be observed (Fig. 3.5, Rascio et al. 1984b, 1986). During senescence PTs seem to be degraded or sometimes arranged in parallel stacks, while large, but not very regular PLBs with increased number of plastoglobuli within the lattice are characteristic. The timing of PLB formation varies between species, and in some cases proplastids and amyloplasts are characteristic at the early stages of differentiation (Younis et al. 1995). The frequency and size of the PLBs increase with ageing (Bradbeer et al. 1974a; Lütz 1981a, b; Rascio et al. 1988). Etioplast ultrastructure slightly varies in different cells or tissues of the leaves, like bundle sheath cells of C4 plants or in epidermal cells (Solymosi and Schoefs 2008).

IV. Molecular Composition of Prolamellar Bodies and Prothylakoids

The special cubic membrane structure of the PLBs is stabilized by several factors including a specific lipid composition, a high lipid per protein ratio, the presence of oligomers of LPOR proteins binding Pchlide, NADPH and carotenoids. In order to understand the factors contributing to the appearance of PLBs, the molecular composition of the etioplast and its isolated inner membrane subfractions have been studied in details by several authors.

The lipid composition of the PLBs and the PTs is basically similar to that of thylakoids (Selstam 1998), but the proportions of the distinct lipid fractions are slightly different, and the amount of lipids per plastid increases three times during the etioplast-chloroplast transformation in maize (Guillot-Salomon et al. 1973). Comparative studies on isolated PTs and PLBs have shown that the ratio of the cone-shaped monogalactosyl diacylglycerol (MGDG) to digalactosyl diacylglycerol (DGDG) is slightly higher (1.6–1.8) in PLBs than in PTs (1.1–1.4) (Bahl et al. 1976; Ryberg et al. 1983; Selstam and Sandelius 1984). Sulfolipids (i.e. sulfoquinovosyl diacylglycerol) and phospholipids are present in relatively low amounts in the membranes; their molar ratio varies around 6–7% (Ryberg et al. 1983; Selstam and Sandelius 1984). The fatty acid composition of the respective lipid fractions of the PLBs and PTs do not differ significantly (Ryberg et al. 1983; Selstam and Sandelius 1984), and are similar to those of chloroplasts in green leaves (Bahl et al. 1976). Therefore, probably other specific interactions (i.e. lipid-protein or protein-protein interactions, or the high lipid per protein ratio) must be responsible for PLB formation. In addition, mutants deficient in MGDG synthase 1–1, which contain 58% of MGDG relative to the wild-type plants, can form PLBs although these are smaller than in the wild-type plants (Jarvis et al. 2000).

There is 30% more lipid per protein in the PLB than in the PT (Ryberg et al. 1983; Selstam and Sandelius 1984; Protoschill-Krebs and Kesselmeier 1988). Thus, the PLB membrane is rich in lipids and in the H_{II} lipid MGDG, both factors that facilitate the formation of a cubic phase structure (Selstam 1998). However, the lipid composition of the inner membranes alone cannot account for the formation of the PLBs, because PLBs appear simultaneously and are interconnected with PTs in etioplasts (Fig. 3.1a, c, d) and with developing thylakoids and grana in etio-chloroplasts under natural conditions (Fig. 3.1b) or during plant greening, and may be transformed into each other rapidly (Ikeda 1970, 1971). Therefore, specific lipid-protein or protein-protein interactions or specific lateral heterogeneity of the plastid inner membranes should also be considered.

Studies on isolated etioplast inner membrane subfractions have shown that the membrane protein compositions of the PLBs and PTs differ significantly. Proteomic analyses (von Zychlinski et al. 2005; Blomqvist et al. 2006, 2008) confirmed that LPOR (MW 36 kDa) is the major membrane protein of the PLBs and accounts for 90–95% of its total proteins (Ikeuchi and Murakami 1983; Selstam and Sandelius 1984). Asymmetrically-placed proteins can induce saddle-shaped curvatures (Hyde et al. 1997), and

LPOR is a globular, membrane-associated protein found on the stromal side of the PLB (Aronsson et al. 2003a) in an oligomeric form (Wiktorsson et al. 1993; Ouazzani-Chahdi et al. 1998). The membrane anchoring of LPOR (Aronsson et al. 2001a), its specific interactions with MGDG (Klement et al. 1999; Engdahl et al. 2001) and its ability to form oligomers may all contribute to PLB formation. The oligomer formation of LPOR, its membrane association and the stability of PLB membranes are both enhanced by the presence of NADPH (Ryberg and Sundqvist 1988; Engdahl et al. 2001; Solymosi et al. 2006b) and ATP (Horton and Leech 1972; Engdahl et al. 2001), the latter suggesting the involvement of phosphorylation and dephosphorlyation to regulate oligomerization and monomer formation, respectively (Covello et al. 1987; Wiktorsson et al. 1996; Kovacheva et al. 2000).

There are three isoforms of LPOR, although LPOR-C is only found in Arabidopsis, and some species contain only one *LPOR* gene (Solymosi and Schoefs 2010). However, the two main isoforms of LPOR, LPOR-A and LPOR-B, are found in several species where LPOR-A reduces Pchlide when dark-grown material is illuminated, and LPOR-B is responsible for Pchlide photoreduction in green material (Aronsson et al. 2003b). Interestingly, LPOR-A import in Arabidopsis seems to be Pchlide-dependent in cotyledons (Kim and Apel 2004) but not in leaves (Jarvis et al. 1998; Aronsson et al. 2000, 2001b; Dahlin et al. 2000). Thus, the suggested organ-specific LPOR-A import is closely linked to the light-dependent transformation of a storage organ into a fully photosynthesizing leaf. This step would then only occur in the presence of LPOR-A's substrate, Pchlide, which is found in large amounts in etioplasts but in minor amounts in chloroplasts. This implies a novel import pathway being substrate dependent for which several specific components have been suggested (e.g. Toc33, OEP16, PTC52, Reinbothe et al. 2004a). However, the importance of these putative components for LPOR-A import has not been confirmed by other laboratories (Kim et al. 2005; Philippar et al. 2007; Boij et al. 2009; Pudelski et al. 2009), and in addition, data regarding OEP16 have been questioned by the authors themselves (Bonneville and Tichtinsky 2010). Thus, the significance of these components remains to be convincingly demonstrated.

The different light responses of the LPOR isoforms, as well as their significance in PLB formation are discussed elsewhere (Solymosi and Schoefs 2010). Data indicate that both LPOR-A and LPOR-B can accumulate and be present in the PLBs of dark-grown plants, and the size of PLBs correlates with the total amount of LPOR proteins rather than with the ratio of the different isoforms.

Besides LPOR, isolated PLBs have been also shown to contain 64 unique proteins, representing pigment biosynthesis, photosynthetic light reaction, Calvin cycle proteins, chaperones and protein synthesis (Blomqvist et al. 2008). Proteins involved in photosynthetic light reactions are the most numerous in the PLBs which strengthens the assumption that the PLB membranes are precursors to the thylakoids and are used for the formation of the photosynthetic membranes during greening. Chl synthase, the enzyme catalyzing the transformation of Chlide into Chl (Fig. 3.6, Rüdiger et al. 1980) is a highly hydrophobic protein with several transmembrane helices, and is localized to the PLBs (Blomqvist et al. 2008), but its activity can be measured also in PT-enriched fractions (Lindsten et al. 1990).

In the PTs, LPOR is present only in minor amounts and many other proteins, however, are dominating (Ikeuchi and Murakami 1983; Selstam and Sandelius 1984; Dehesh and Ryberg 1985; Ryberg and Dehesh 1986; Lindsten et al. 1988). Other proteins localized to the PTs are for example the chloroplast coupling factor (CF_1) (Wellburn 1977), and some of the proteins associated with the oxygen evolving complex (OEC) of photosystem II (PSII) (Blomqvist et al. 2006, 2008).

Etioplast inner membranes contain different pigments, including the pigment precursors of Chl (for instance Pchlide) or their derivatives (Fig. 3.6). HPLC studies have

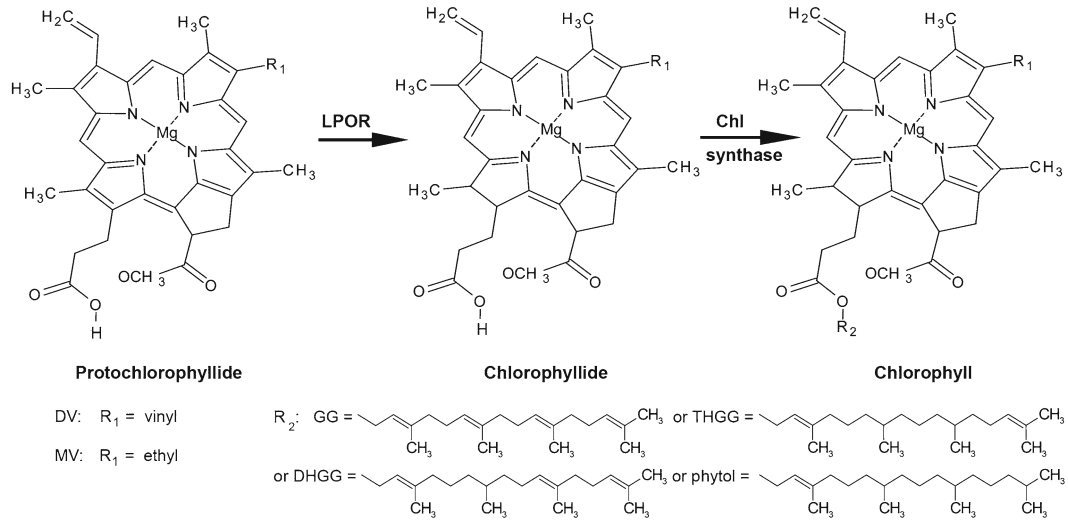

Fig. 3.6. Molecular alterations of the porphyrin ring during the last steps of chlorophyll biosynthesis. The different side chains of the porphyrin (or chlorin) ring are presented. The different esters involved in the esterification of Chlide and in the formation of Chlide-phytol, i.e. Chl are also shown. *DV* divinyl, *MV* monovinyl, *GG* geranylgeraniol, *DHGG* dihydrogeranylgeraniol, *THGG* tetrahydrogeranylgeraniol.

demonstrated that Pchlide esters are present in relatively high amounts in the PTs, while in the PLBs Pchlide dominates (Böddi et al. 1989). In etiolated seedlings where phytol is present only in low amount, Pchlide is esterified mostly with geranylgeraniol (GG), dihydro- or tetrahydro-GG (Schoch et al. 1977; Schoefs et al. 1998). Data about mutants impaired in Chl biosynthesis indicate that the accumulation of Pchlide is crucial for proper PLB assembly (Mascia and Robertson 1978; Solymosi and Schoefs 2010). Besides being localized to etioplast inner membranes (Laflèche et al. 1972; Ryberg and Sundqvist 1982), Pchlide and its esters are present also in the envelope (Bräutigam et al. 2008), the thylakoids of chloroplasts or developing chloroplasts (Dehesh et al. 1986a; Barthélemy et al. 2000). Thus, the results show that the presence of Pchlide pigments is an indispensable condition for PLB formation, however, several data indicate that only the Pchlide pigments bound to the active site of LPOR oligomers appear concomitantly with PLBs (Klein and Schiff 1972; Sperling et al. 1998; Masuda et al. 2003). Thus, Pchlide is needed for the formation of photoactive oligomers of LPOR associated to the PLB membranes, and this way the presence of the pigment, and NADPH, only indirectly regulates PLB formation. Upon irradiation, when the newly formed Chlide is dissociated from LPOR, the enzyme undergoes disaggregation, is often prone to proteolytic degradation and in parallel, the regular PLB structure is also disrupted. This also outlines the importance of photoactive LPOR oligomers binding NADPH and Pchlide in the stabilization of PLB membranes.

Most etiolated gymnosperms or lower plants contain etio-chloroplasts with relatively low amounts of Chls and accumulate to some extent Pchlide as well. In addition to LPOR these plants possess another, light-independent mechanism for Pchlide photoreduction involving a dark-operative POR (DPOR) and can synthesize Chls in the dark (e.g. Laudi and Manzini 1975; Walles and Hudák 1975; Selstam et al. 1987; Schoefs and Franck 2003). Due to the presence of Chls that may activate gene expression and stabilize the structure of some polypeptides, the etio-chloroplasts of dark-grown gymnosperms accumulate and contain several proteins of the photosynthetic light reactions in their grana, however, these proteins are often not fully functional or properly organized (Canovas et al. 1993). At the same time their

PLBs contain LPOR in oligomeric forms (Selstam and Widell 1986; Selstam et al. 1987; Forreiter and Apel 1993; Schoefs and Franck 1998). This outlines that the lack of functional thylakoids and grana formation in etioplasts of angiosperms is due to the complete inhibition of Chl synthesis in these plants, but also shows that the formation of PLBs is more related to the presence and accumulation of LPOR oligomers and/or to a specific lipid ratio with relatively high MGDG content (Selstam and Widell 1986) than solely to the presence or the absence of Chls.

Etioplasts contain a set of carotenoids similar to that of chloroplasts (Selstam and Sandelius 1984; Schoefs et al. 1998). The carotenoid composition and the ratio of the different pigments of isolated PLB and PT fractions are similar (Böddi et al. 1989; Sundqvist and Dahlin 1997). Pigment analyses performed on isolated photoactive dimers and large oligomers of LPOR-Chlide-NADPH ternary complexes have demonstrated the presence of carotenoids such as zeaxanthin and violaxanthin molecules (Ouazzani-Chahdi et al. 1998). Several data indicate that carotenoids are important in membrane association of LPOR (Denev et al. 2005) and that the correct and complete carotenogenesis pathway leading to the synthesis of polar carotenoids (xanthophylls) is required for the maintenance of stable LPOR oligomers and PLB membranes (Moro et al. 2004). The inhibition of the transformation of poly-cis-carotenoids to the trans-forms disrupts PLB formation, which may suggest that trans-carotenoids play a specific role in PLB assembly, for instance in regulating the membrane fluidity and/or stabilizing the curved membranes (Park et al. 2002).

The data above show that there are specific differences in the composition of PLB and PT membranes, especially (1) in the ratio of the different galactolipids, (2) in the lipid to protein ratio, (3) in the protein composition, and (4) in the photosynthetic pigments and their precursors. At the same time they outline the importance of etioplast inner membranes, especially the PLBs as precursors to chloroplast membranes upon light exposure of dark-grown tissues. The very compact membrane structure of PLBs furnishes lipids for the rapid formation of thylakoids upon illumination, the etioplast inner membranes contain proteins needed for the rapid synthesis of Chls and for the rapid onset of photosynthetic activity upon illumination. The oligomers of the ternary complexes of LPOR, NADPH and Pchlide, together with carotenoids and enzymes of the antioxidant defense system prevent photooxidation of the pigments upon light exposure (Sperling et al. 1997; Kanervo et al. 2008).

V. Pigment Biosynthesis in Etioplasts

During the etioplast-to-chloroplast transition there is a dramatic increase of chlorophyllous pigments, and the carotenoid pigment composition is also significantly altered. Thus, the biosynthesis of carotenoids, and the biosynthesis of Chls with emphasis on the light-dependent conversion in etiolated leaves are highlighted here.

A. Carotenoid Biosynthesis

Carotenoid pigments are yellow (xanthophylls), pale yellow (ζ-carotene), orange (ß-carotene), red (lycopene) or colorless (phytoene) and present in all plant tissues. The carotenoid biosynthesis pathway, starting with phytoene, involves desaturations, cyclizations, hydroxylations, and epoxidations. The enzymes involved in carotenoid biosynthesis are nuclear encoded (Kirk and Tilney-Bassett 1978), and the majority are located in the plastids where the synthesis occurs (Kreuz et al. 1982; Lütke-Brinkhaus et al. 1982; Lütke-Brinkhaus and Kleinig 1987; Linden et al. 1993). The carotenoid biosynthesis in green tissue is somewhat different from the one in fruits and flowers, which have additional control and regulatory mechanisms not reviewed here (Thelander et al. 1986). The regulation of the carotenoid biosynthesis in green tissues is not fully understood yet,

i.e. despite expression of genes related to carotenoid synthesis in etiolated plants, the carotenoid biosynthesis is stimulated and activated upon transfer to light (Albrecht and Sandmann 1994; von Lintig et al. 1997; Welsch et al. 2000).

In plastids, pyruvate and glyceraldehyde-3-phosphate produce isopentenyl diphosphate (IPP) through several enzymatic steps (Lichtenthaler et al. 1997; Hirschberg 2001). Thus, this 5-carbon compound is the first building block of carotenoids just as of all other isoprenoids. Three molecules of IPP are added to dimethylallyl diphosphate catalyzed by GG diphosphate (GGPP) synthase, which produces a 20-carbon molecule GGPP. Two molecules of GGPP are then the starting material for the first carotenoid phytoene, synthesized by phytoene synthase. Phytoene is converted into ζ-carotene by phytoene desaturase, which is followed by a conversion of ζ-carotene into lycopene by the enzyme ζ-carotene desaturase (Mayer et al. 1992; Norris et al. 1995).

Next come two cyclization reactions of lycopene, which marks a branching point. The reactions are catalyzed by lycopene β-cyclase and lycopene ε-cyclase, giving the products α-carotene (two β rings) in one branch, and β-carotene (one β and one ε ring) in another branch (Cunningham et al. 1996; Pogson et al. 1996). These cyclic carotene products are further modified ending up as xantophylls through specific hydroxylation enzymes for the β-ring and ε-ring (Bouvier et al. 1998). Thus, the xantophylls are either derived from α-carotene (i.e. lutein) or from β-carotene (i.e. zeaxanthin, violaxanthin, and neoxanthin) (Rissler and Pogson 2001). The xanthophyll zeaxanthin produced after the hydroxylation can be converted into violaxanthin via antheraxanthin by the enzyme zeaxanthin epoxidase (Bouvier et al. 1998). However, violaxanthin can be converted back to zeaxanthin by violaxanthin deepoxidase thus, creating the "xanthophyll cycle" that plays an important protective role for the photosystems through thermal dissipation of excess excitation energy within the antenna complexes.

B. Chlorophyll Biosynthesis

The tetrapyrrole biosynthetic pathway leading to Chl (see also Tripathy and Dalal, Chap. 27) starts with the production of 5-aminolevulinic acid (ALA). In higher plants, ALA is produced through a three-step C5 pathway from a five-carbon skeleton of glutamate (Friedmann et al. 1987; von Wettstein et al. 1995). The glutamate is ligated to $tRNA^{Glu}$ by Glutamyl-tRNA synthetase. Glutamate-tRNA is then reduced by Glutamyl-tRNA reductase to glutamate-1-semialdehyde (GSA), which is further transaminated by GSA aminotransferase to produce ALA.

Six enzymatic steps later, the ALA has been turned into protoporphyrin IX, which also marks a branching point in the tetrapyrrole pathway: one branch leading to heme synthesis and the other Mg-branch leading to Chl production (Fig. 3.7). The synthesis starts with condensation of two molecules of ALA into porphobilinogen (PGB, a pyrrole molecule) by ALA dehydratase, then four molecules of PGB are polymerized into hydroxymethylbilane by PGB deaminase. This linear tetrapyrrole is converted into a closed ring structure through isomerization by uroporphyrinogen III (urogen III) synthase to produce urogen III, which is then decarboxylated into coproporphyrinogen III (coprogen III) by urogen III decarboxylase. This molecule is then oxidized in two steps with protoporhyrinogen IX (protogen IX) as an intermediate to produce protoporphyrin IX. These steps are catalyzed by coprogen III oxidase and protogen IX oxidase, respectively. The former converts the propionic acid side chains into vinyl groups, the latter is responsible for the formation of the highly conjugated macrocycle via dehydrogenation.

In the Mg-branch leading to Chl formation, the first step is the insertion of Mg^{2+} by the enzyme magnesium-protoporphyrin chelatase (Mg chelatase). The product Mg-protoporphyrin IX is methylated by Mg-protoporphyrin IX methyltransferase to form Mg protoporphyrin IX monomethyl ester, and next Mg-protoporphyrin IX monomethyl ester cyclase catalyzes the incorpora-

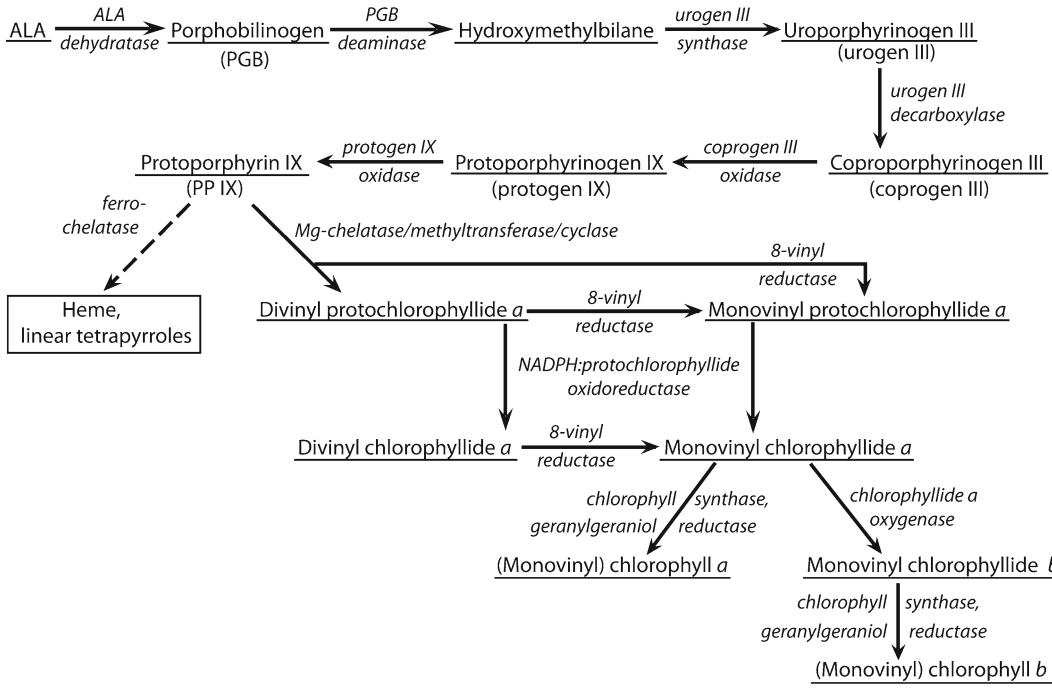

Fig. 3.7. Chl biosynthetic pathway starting from ALA. *Dashed arrow* and the *boxed text* indicate the branching point of Chl and heme (and linear tetrapyrrole) biosynthetic routes. The last steps of Chl biosynthesis are in this chart simplified (not all monovinyl and divinyl transformation pathways are indicated) and represent the biosynthesis of Chl *b* in dark-grown angiosperms upon irradiation (Schoefs et al. 1998).

tion of oxygen to form 3,8-divinyl Pchlide *a*, having vinyl groups in ring A and B of the molecule (Fig. 3.6). The next step is the strictly light-dependent reaction in angiosperms catalyzed by LPOR, which reduces the D-ring of the divinyl Pchlide thereby forming 3,8-divinyl Chlide *a*. In addition, a light-independent Pchlide reductase (DPOR) exists in algae, cyanobacteria and gymnosperms (Fujita and Bauer 2003; Schoefs and Franck 2003). The 8-vinyl group in ring B is reduced by 8-vinyl reductase to form 3-monovinyl Chlide *a*. Interestingly, 8-vinyl reductase can also convert both 3,8-divinyl Pchlide and Mg protoporphyrin IX monomethyl ester to 3-monovinyl Pchlide *a* although with less efficiency than using 3,8-divinyl Chlide as substrate (Tanaka and Tanaka 2007). LPOR can also use 3-monovinyl Pchlide *a* as substrate to produce 3-monovinyl Chlide *a* (Fig. 3.7). The final step to produce Chl is the esterification of the 17-propionate on the D-ring by Chl synthase (Rüdiger et al. 1980) resulting in addition of a side chain to the porphyrin ring and this way in Chl *a* formation (Figs. 3.6 and 3.7). Chlide can be esterified either directly with phytol or first with GG and then the added GG moiety can be reduced stepwise to phytol by GG reductase (Fig. 3.6, Benz et al. 1980; Rüdiger et al. 1980; Schoefs and Bertrand 2000).

The presence of Chl *b* among organisms is restricted to land plants, green algae, euglenophytes and chlorarachniophytes and some cyanobacterial taxa. Chl *a* can be converted into Chl *b* through oxygenation either directly or indirectly, that is, via Chlide *a* oxygenation into Chlide *b* and the subsequent esterification of Chlide *b* to Chl *b* by Chl synthase similarly to Chlide *a* esterification (Schoefs et al. 1998; Oster et al. 2000). The conversion of Chlide *a* to Chlide *b* is catalyzed by Chlide *a* oxygenase (CAO). The reaction occurs as a two-step reaction with 7-hydroxymethyl Chlide *a* as an intermediate (Oster et al. 2000).

C. Spectroscopy as a Tool to Study Chlorophyll Biosynthesis in Etiolated Plants

Pchlide is suitable for spectroscopic studies and has different spectral properties depending on its molecular environment, i.e. on its interactions with other Pchlide molecules, with proteins (e.g. with LPOR) and/or other molecules like NADPH. Both absorption and fluorescence spectra have been analyzed in detail (see Sundqvist and Dahlin 1997; Schoefs 1999, 2001, 2005; Schoefs and Franck 2003). Four distinct molecular populations of Pchlide – having distinct spectral properties and thus being denoted as spectral forms – have been identified in the 77 K fluorescence emission spectra of etiolated leaves (Böddi et al. 1992). For simplicity we will use the fluorescence emission maxima to denote the different spectral forms, and only the data strongly related to etioplast development in leaves will be discussed. The PTs contain predominantly a Pchlide spectral form with emission maximum at 633 nm, representing non-photoactive, probably not LPOR-bound pigments. Photoactive dimers of Pchlide-LPOR-NADPH ternary complexes localized to the surface of the PLBs have a fluorescence emission maximum at 644 nm. The major photoactive Pchlide form (with emission maximum at 655 nm) corresponding to large Pchlide-LPOR-NADPH oligomers is characteristic for the PLBs and always accumulates concomitantly with development of PLBs (Solymosi and Schoefs 2008, 2010).

The absorption and the fluorescence emission spectra reflect the changes in the pigment organization during leaf ontogenesis and plastid differentiation, i.e. the ratio of the different spectral forms of Pchlide is altered during ageing (Fig. 3.5). This process can be studied either by recording the spectra of seedlings with different age or by studying the cereal leaf gradient from the youngest cells close to the basal meristem towards the leaf tip, these two approaches giving basically similar results (Fig. 3.5). In the beginning of plastid development in proplastids no pigments can be detected and baseline is characteristic for the spectra (Fig. 3.5, He et al. 1994; Younis et al. 1995). Later, i.e. in young etiolated leaves or in the basal, youngest cells of cereal leaves, the short-wavelength, non-photoactive Pchlide form with emission maximum at 633 nm is dominating and no or only very few and small PLBs are found in the plastids (Klein and Schiff 1972; Younis et al. 1995; Reinbothe et al. 2004b). In parallel with the appearance of small PLBs, and with the increase in their size and frequency in the plastids, the fluorescence band at 655 nm appears first only as a shoulder (e.g. 30 h of germination in wheat leaves) and then becomes dominating in the spectrum (48 h of germination in wheat) (Fig. 3.5, Younis et al. 1995). The ratio of photoactive to non-photoactive Pchlide varies among species and is the highest in the spectra of plant species with highly regular paracrystalline PLBs like maize (Böddi et al. 1992; Amirjani and Sundqvist 2004; Amirjani et al. 2006), and in the cells with the most regular and/or largest PLBs in fully mature etioplasts. During senescence of the etioplasts, the relative contributions of the photoactive Pchlide forms with emission maxima at 644 and 655 nm decrease (Fig. 3.5). Similar tendencies were found in absorption spectra (Klein and Schiff 1972). Although the ratio of the different spectral forms may be related to some extent to the changes in LPOR content of the plastids (Davies et al. 1989; He et al. 1994; Reinbothe et al. 2004b; Amirjani et al. 2006), the fluorescence ratio of the different spectral forms alone cannot be correlated directly with the amount of the different molecular populations of Pchlide, due to energy migration from the short-wavelength to the long-wavelength Pchlide (Kahn et al. 1970; Kis-Petik et al. 1999; Amirjani and Sundqvist 2004). It should be noted that the spectra reflect the total Pchlide fluorescence of all plastids present in a leaf or leaf segment, although some variability of plastid differentiation can be found even within the different regions of the leaf.

Similar data have been obtained during studies about plastids containing PLBs under natural conditions (Cohen and Rebeiz 1978; Schoefs and Franck 2008). In the beginning

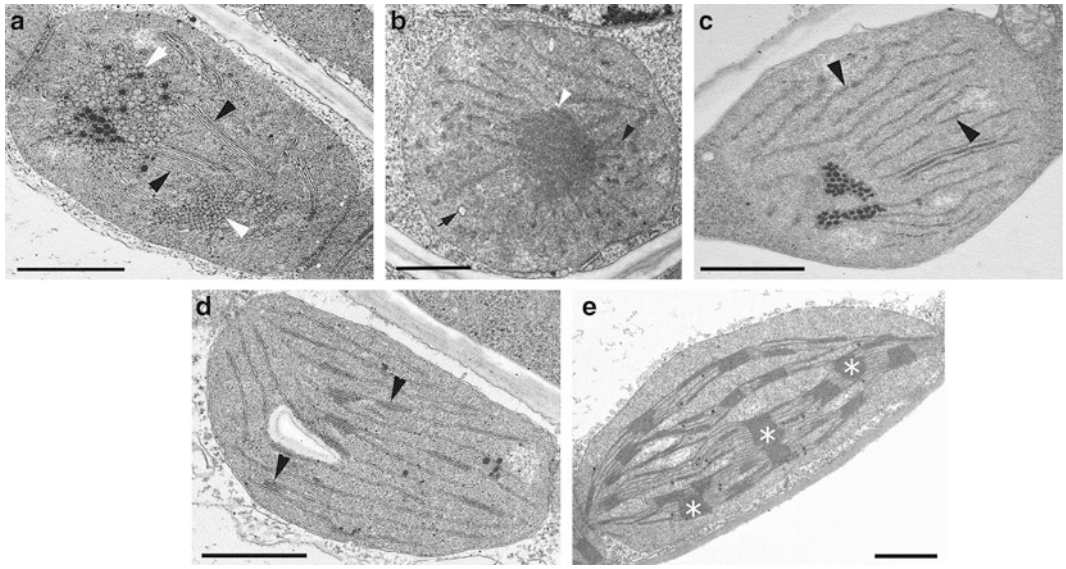

Fig. 3.8. Different steps of etioplast-chloroplast transformation in 7-day-old etiolated wheat leaves illuminated for 8 h with continuous white light of 50 μmol s^{-1} m^{-2}. (**a**), Etioplast before irradiation with two PLBs (*white arrow* indicating "open" PLB region). (**b**), Etio-chloroplast containing developing thylakoids with remnants of the PLB after 3 h illumination. (**c**), Developing thylakoids, incipient grana stacks in a plastid after 3 h illumination. No PLB membranes are observed, but clusters of plastoglobuli may indicate previous presence of a PLB. (**d**), Young chloroplast with developing grana after 8 h illumination. (**e**), Young chloroplast from a 7-day-old, light-grown wheat leaf. *White arrowheads*, PLBs or their remnants; *black arrowheads*, (pro)thylakoids or developing grana; *black arrow*, membrane vesicle; *asterisk*, fully differentiated grana.

of development only the short-wavelength Pchlide form accumulates, while later the photoactive Pchlide with emission maximum at 655 nm appears and accumulates during the dark phase of the LDCs in PLBs, until the young chloroplasts have reached their final size and maturation (Cohen and Rebeiz 1978; Schoefs and Franck 2008). Several authors have shown that these spectral forms and photoactive Pchlide with fluorescence emission maximum at 653–655 nm appear also in light-grown seedlings during the early stages of plastid development (Cohen and Rebeiz 1978; Franck et al. 1993; Schoefs et al. 2000). Photoactive Pchlide represents in such cases an intermediate in Chl biosynthesis, so there is always a steady-state amount of it that can be detected during greening even in the light and may be even in the absence of PLBs.

VI. The Etioplast-to-Chloroplast Transition upon Irradiation of Etiolated Leaves

A. Structural Alterations During Greening

Since the pioneering light microscopic observations from the 1950s, the transformation of etioplasts into chloroplasts, and especially the loss of the regular structure of the PLBs upon irradiation of etiolated leaves have been studied in detail in many laboratories and several schemes have been presented (Figs. 3.8 and 3.9). In these works, the term etio-chloroplast describes fully mature etioplasts of relatively old dark-grown tissues that have been irradiated and in which PLBs are disappearing while thylakoid and grana formation has started. The process starts with

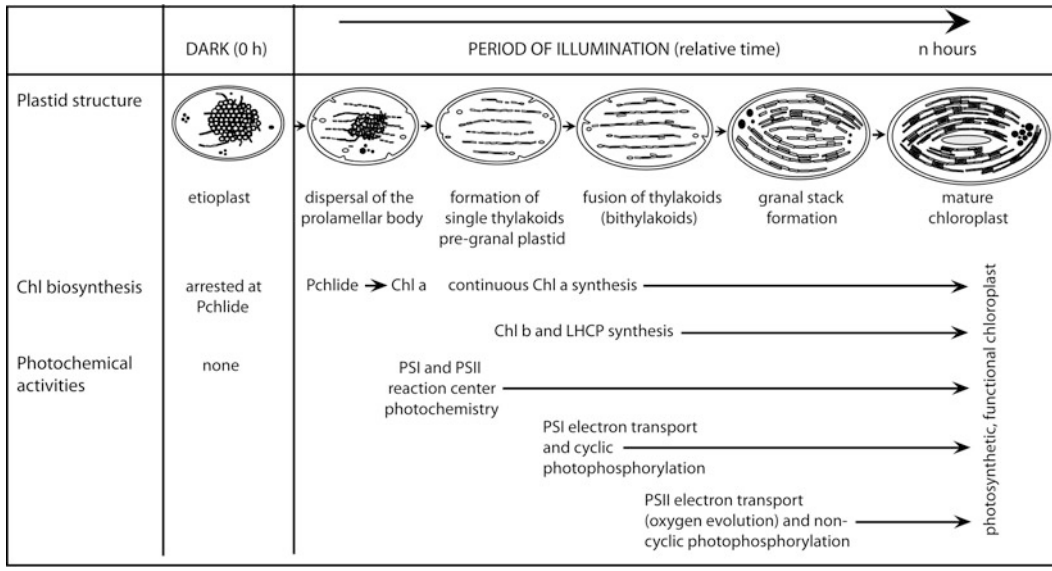

Fig. 3.9. Different subsequent steps of etioplast-to-chloroplast transformation in illuminated etiolated leaves (modified after Leech and Baker 1983). Plastid structure, Chl biosynthesis and photosynthetic activity are summarized and compared. The period of illumination needed for the processes, or the different steps may vary according to the species studied and the light intensity used. *LHCP* light-harvesting complex.

the loosening of PLB tubules, i.e. the semi-crystalline regularity of PLBs is lost, the PLBs slightly shrink and (pro)thylakoid membranes emanating from the PLBs appear (Fig. 3.8b) and become prominent already within 2–5 min after illumination, in parallel with the rapid esterification (15–30 s) of about 15% of the newly formed Chlide by Chl synthase (Gunning 1965, 2004; Domanskii et al. 2003). During this early reorganization of the etioplast inner membranes, the cubic-to-lamellar phase transition occurs on the surface of the PLBs, where the formation of vesicles has been observed under certain experimental conditions (Figs. 3.3d and 3.8; Klein et al. 1964; Kohn and Klein 1976; Ryberg and Sundqvist 1988; Solymosi et al. 2006b). This stage might be referred to as the transformation of PLBs into PTs, or to so-called 'primary thylakoids'. The dispersal and reorganization of the PLBs leads to the formation of evenly spaced, planar membranes. These membranes retain many perforations derived from the PLBs, and – similarly to proplastid-to-chloroplast differentiation – at a later stage of development these perforations may play an important role in initiating the development of overlapping regions of the thylakoids, and in grana formation (Fig. 3.8c–e; Gunning 2004). After the rearrangement and insertion of specific compounds (lipids, pigments, proteins), and their special assembly, the PTs develop, and can be termed thylakoids that later start to aggregate to form grana (von Wettstein 1959; Leech and Baker 1983; Selstam 1998; Gunning 2004). At these stages, many polyribosome configurations are lying on and close to the developing thylakoids indicating active protein synthesis in the plastids (Gunning 2004).

B. Molecular Alterations During Greening

The etioplast-to-chloroplast transition is a very complex process that requires the strict coordination between the synthesis of pigments (Chls and carotenoids), membranes (i.e. lipids), and proteins (encoded both in the nucleus or in the chloroplast), and is under the control of several internal and external factors. First of all, the disruption of the PLB

membrane is strongly associated with Pchlide photoreduction and with the subsequent Chl formation (Schoefs 1999, 2001, 2005; Schoefs and Franck 2003). Pchlide phototransformation is the first event after illumination, occurring on a μs-ms time scale, and it triggers further processes leading to the etioplast-to-chloroplast transformation. The mechanism of Pchlide photoreduction is independent from the developmental stage of the leaf, but the fate of the different photoproducts formed during the reaction is age-related. In young leaves Chlide is quickly released from the LPOR active site and probably gets rapidly esterified to yield Chl with a fluorescence emission maximum at 675 nm (Schoefs and Franck 1993). At the same time, the photoactive Pchlide complexes are rapidly regenerated from the non-photoactive Pchlide pool.

In old leaves, where Pchlide gets accumulated to higher extent, only about 15% of the newly formed Chlide undergoes the rapid shift and esterification (observed 15–30 s after illumination in young leaves), while the majority of Chlide remains attached to LPOR active site after phototransformation, and undergoes several molecular modifications that are reflected by different spectral shifts (Domanskii and Rüdiger 2001; Domanskii et al. 2003; Rassadina et al. 2004). First of all, in plastids with PLBs, LPOR oligomers containing attached Chlide and $NADP^+$ are formed with characteristic fluorescence emission at 690 nm. Within 5–60 s $NADP^+$ is reduced and oligomers of Chlide-LPOR-NADPH ternary complexes are formed with emission maximum at 696 nm. These two latter forms are interconvertible and function in the Chlide microcycle, that is involved in photoprotection of the newly formed pigment from photooxidation (Franck and Inoue 1984), and the formation of which is also indispensable for the development of active PSII in greening etiolated leaves (Franck et al. 1997). The Chlide-LPOR-NADPH ternary complexes later undergo a progressive spectral blue shift (Shibata shift) towards 680 nm during 30–60 min after the onset of illumination (Shibata 1957). Several factors that inhibit the Shibata shift (e.g. NADPH – Ryberg and Sundqvist 1988; low temperatures – e.g. Treffry 1970; Henningsen and Boynton 1974; specific chemical cross-linkers: Wiktorsson et al. 1993) also inhibit the dissociation of the PLB structure and/or the breakdown of the oligomers and/or Chlide esterification. Although the molecular background of Shibata shift is still not completely understood, several cellular and molecular events occur simultaneously and are strongly associated with it such as the disruption of the PLB structure (Domanskii et al. 2003), the conformational changes and disaggregation of the LPOR oligomers (Böddi et al. 1990), dephosphorylation (Wiktorsson et al. 1996; Kovacheva et al. 2000), the translocation of LPOR and Chl synthase to the PTs (Ryberg and Dehesh 1986; Lindsten et al. 1993), the release of Chlide from LPOR oligomers and the regeneration of photoactive Pchlide (Rassadina et al. 2004). All these processes are prerequisites for the esterification of the majority of the newly formed Chlide (Domanskii and Rüdiger 2001; Domanskii et al. 2003). This conclusion is further supported by the fact that Chl synthase is present in the PLBs, but is latent as long as the regular structure of the PLB is preserved (Lindsten et al. 1990).

Thus, Chlide esterification is kinetically limited in old leaves and/or in situations when the PLB structure and/or the structure of the LPOR oligomers is retained after illumination. This limitation is either due to the slow or inhibited diffusion of GG (and/or phytol) to Chl synthase, or to the relatively low amount of Chl synthase enzyme present in the membranes, or rather to the limited release of Chlide from LPOR oligomers, or to a combination of these processes. However, after the disruption and reorganization of the PLB membranes, the newly formed Chlide can get esterified. In agreement with these results, it is not surprising, that there is a short lag phase (1–2 h in old leaves, and 8 h in young leaves) before the major phase of rapid Chl biosynthesis begins (Schoefs et al. 1998). Initially, the Chl *a/b* ratio is high, but ratios characteristic of mature green tissue (e.g. 4.5–3.5) are established after a few

hours (Boffey et al. 1980) or 1 day (Kanervo et al. 2008).

Etioplasts are characterized by a highly reduced level of carotenoids and a lower proportion of β-carotene than that found in chloroplasts (Bahl 1977). The yellowish color of etiolated seedlings is due to the high content of carotenoids and the lack of chlorophylls. The carotenoid/chlorophyll ratio decreases in parallel with greening, together with the Chl *a/b* ratio and the total xanthophyll/β-carotene ratio (Barry et al. 1991). However, the rate of carotenoid synthesis (especially that of β-carotene) is also accelerated in the presence of light, but the different carotenoids are differently affected, and their synthesis also depends on the age of the studied tissue, the species studied, and the light intensity applied during greening and can therefore not be generalized (e.g. Bahl 1977; Barry et al. 1991; Schoefs et al. 1998).

The lipid content (especially that of galactolipids) of the plastids increases at least three-fold during the 24 h illumination of etiolated leaves (Guillot-Salomon et al. 1973), but this increase has a lag phase of 6–10 h from the beginning of the treatment and is longer than the lag phase for Chl biosynthesis (Selldén and Selstam 1976). During the lag phase, thylakoid formation results mainly from the dispersion and rearrangement of the membrane lipids of the PLBs and PTs, while *de novo* thylakoid formation starts only 10–15 h after the onset of irradiation (Bradbeer et al. 1974b). The lipid biosynthetic activity depends also on the age of the tissue under study. In old, metabolically less active cells the PLBs persist for a longer period, lipid biosynthesis and consequently thylakoid development and grana formation is slower than in young tissues and is more related to the reutilization of the etioplast inner membranes (Rascio et al. 1984b). The lipid and fatty acid composition of the membranes does not change dramatically during the greening process (Guillot-Salomon et al. 1973; Selldén and Selstam 1976; Kesselmeier et al. 1987). However, the molar ratio of MGDG to DGDG decreases from 1.367 to 1.101 in isolated oat etioplasts during 60 min of irradiation (Kesselmeier et al. 1987). In mutants with special lipid composition, e.g. having low amounts of DGDG, both PLB disruption and grana formation are inhibited in the light (Henningsen et al. 1993). In addition, dark-grown mutants defective in MGDG synthesis have an increased ratio of photoactive to photoinactive Pchlide, that may represent a photoprotective mechanism to deal with high light intensities during etioplast-to-chloroplast transformation (Aronsson et al. 2008). The only new lipid molecule appearing during etioplast-to-chloroplast transition and proplastid-to-chloroplast transition is phosphatidyl glycerol containing the trienoic acid, Δ3trans hexadecenoic acid (Selldén and Selstam 1976).

During de-etiolation, the biogenesis of chloroplasts involves biosynthesis of pigments, development of thylakoids, as well as expression of genes for assembly of the photosynthetic apparatus. The synthesis of most proteins of the photosynthetic apparatus is activated by light or depends on its presence, and grana formation is considerably inhibited in the presence of protein synthesis inhibitors (Wrischer 1967). During greening of old etiolated plants, the translation of mRNA encoding D1, CP43 and CP47 seems to depend on the presence of Chl, indicating that Chl synthesis is the bottleneck of the regulation of the etioplast-to-chloroplast transformation (Paulsen 2001). Recent proteomic analyses of the PLB have outlined the importance of the PLB in the development of the photosynthetic activity during greening, as several proteins of the photosynthetic light and dark reactions (proteins of the ATP synthase complex, oxygen evolving complex, cytochrome b_6f, plastocyanin, one subunit of PSII, Rubisco, etc.) are present already in these membranes (Blomqvist et al. 2008; Kanervo et al. 2008).

The LPOR-A gene expression is downregulated upon irradiation of etiolated seedlings and the proteolytic degradation of LPOR-A was also observed in parallel with the dispersion of the PLB membranes during

greening (Apel 1981; Dehesh et al. 1986a, b). In etioplasts, LPOR is found in the envelope and the inner membranes; later it is associated mostly with appressed regions of thylakoids, while in mature chloroplasts it was found in the envelope (Barthélemy et al. 2000). Unfortunately, these studies did not involve analyses of the different LPOR isoforms. Similar to LPOR-A, enzymes involved in photoprotection and oxidative stress responses (e.g. gluthathione reductase, and stromal and thylakoid forms of ascorbate peroxidase) are all highly expressed in etiolated plants, but are down-regulated upon light exposure of the plants (Kanervo et al. 2008), indicating the role of PLBs and etioplasts in preventing photooxidation during greening.

C. Physiological Alterations During Greening, the Onset of Photosynthesis

During chloroplast differentiation, the efficiency of the photochemical processes increases due to the coordinated accumulation of PSI, PSII and the light-harvesting complexes. Consequently, the light energy required to saturate the photochemical reactions decreases, and the coupling of electron transport to phosphorylation, and this way ATP synthesis, also becomes more efficient (Leech and Baker 1983). Despite the fact that the assembly of the pigment-protein complexes and their insertion into developing thylakoids are of critical importance for the development of the photosynthetic activity, the details of these processes are not yet completely elucidated. For instance, the correct folding of the main apo protein of light-harvesting complexes requires the presence of Chl *a*, Chl *b*, violaxanthin, neoxanthin and lutein (Paulsen et al. 1990). Therefore, light-induced Chl accumulation in plastids is a prerequisite for, and tightly coupled with, the stable assembly of the pigment–protein complexes in the thylakoid membrane (Paulsen 2001). At the same time, the rapid onset of photosynthesis is crucial for the survival of the seedling, and many components (lipids, carotenoids and the phytol moiety of Chl) are synthesized from simultaneously fixed CO_2 during greening (Heinze et al. 1990).

During the lag phase of Chl and carotenoid synthesis, the formation of a minimal but functional photosynthetic apparatus is observed (Schoefs and Franck 1991; Franck 1993). Greening studies with etiolated plants using short (flash or seconds) illumination have clearly demonstrated that the Chl molecules formed during the photoreduction of Pchlide are (at least partially) used for the formation and assembly of small amounts of the photochemically active reaction centers of PSI and PSII, and this way a fully functional electron transport chain is rapidly formed (Fig. 3.9, Leech and Baker 1983; Franck 1993).

PSI activity can be detected very early (within 10 min) after the beginning of illumination (Bertrand et al. 1988), while the assembly of active PSII requires the formation of Chlide-LPOR-NADPH complexes and is usually observed on a slightly longer time scale (Franck et al. 1997). PSI driven cyclic photophosphorylation can be detected within 30 min after the onset of illumination, and appears before the non-cyclic process and oxygen evolution, and provides ATP for the synthesis of chloroplast components (Fig. 3.9, Leech and Baker 1983; Franck 1993).

During continuous greening of both young and old dark-grown leaves, the antenna size of the photosystems increases through the accumulation of light-harvesting complexes together with Chl *b*. The time needed for "free" Chl to be fully integrated, and therefore for reaching maximal photosynthetic efficiency, varies very much with plant age and experimental methods (works on intact leaves or on isolated etiochloroplasts), but it takes several hours (Franck 1993). For instance, complete PSI and PSII core complex assembly and 40% of the maximal photochemical efficiency appear already after 6 h of greening in etiolated pea, but fully active and stable photosystems have been detected only after 24 h illumination (Kanervo et al. 2008).

VII. Concluding Remarks

We have presented data indicating that etioplasts or etio-chloroplasts may appear and are involved in chloroplast biogenesis in young and differentiating leaves. However, data about etioplast and etio-chloroplast differentiation under natural conditions are still rather scarce, and their molecular composition and role in grana development still need to be elucidated. Extensive studies on etioplasts of dark-grown angiosperms and on their greening have highlighted the importance of the PLB membranes in chloroplast development. They have two vital functions. First, they host the oligomers of LPOR that bind NADPH, Pchlide and carotenoids, and represent a unique photoprotective mechanism against pigment photooxidation upon light exposure. Second, PLBs enable a fast onset of photosynthetic activity upon light exposure, because (1) the organization of their pigment-protein complexes enables relatively rapid Chl synthesis when compared with proplastids, (2) they serve as precursors to developing thylakoids as they contain several proteins of the photosynthetic light and dark reactions, and (3) their extremely compactly packed membranes act as a lipid reservoir for the assembly of the thylakoid membranes.

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Chapter 4

The Biogenesis of the Thylakoid Membrane: Photosystem II, a Case Study

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Summary

The thylakoid membrane of cyanobacteria and higher plants is a highly organized system of internal membranes that enclose the protein complexes of the photosynthetic apparatus. Photosystem II (PSII) is a central component of this machinery and it is responsible for catalyzing the light induced electron transfer from water to plastoquinone, generating almost all of the oxygen in the atmosphere. PSII is a multimeric chlorophyll-protein complex of dual genetic origin, whose biogenesis is a complicated and highly regulated process.

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Nuclear-encoded auxiliary factors coordinate the expression of plastid- and nuclear-encoded PSII subunits in response to environmental and endogenous cues. Studies performed with photosynthesis mutants of green algae and higher plants have revealed that these factors control the expression of the plastid-encoded genes at the posttranscriptional level. Many of the regulatory proteins are able to modify the efficiency of translation of specific PSII subunit transcripts, and redox regulation plays an important role in the control of these processes. The assembly of PSII proteins into a functional complex also requires the activity of nuclear-encoded factors, several of which are conserved throughout the plant kingdom, but some appear only in higher plants and green algae, indicating that new requirements for PSII assembly have evolved during evolution. Recent studies suggest that the initial steps of PSII biogenesis are localized to a specific region of the interior membrane system of chloroplasts and cyanobacteria.

I. Introduction

Although the chloroplasts of plants and algae were first described 160 years ago, a detailed analysis of their basic structures, in particular of the thylakoids, followed only 100 years later in 1955 (Hodge et al. 1955; von Wettstein 1959). The thylakoids are the dominating structures of mature chloroplasts, and in higher plants they are differentiated into two types called the grana and the stroma lamellae. The grana consist of regular stacks of appressed membranes that form disc-shaped structures interconnected by the unstacked stromal lamellae, forming a continuous intertwined membrane system enclosing an internal aqueous space, the thylakoid lumen. However, complex grana structures are not essential for photosynthesis, and cyanobacteria, as well as many algae, contain numerous single layers of thylakoid membranes inside their cells or chloroplasts, respectively. The typical lipid bilayer of thylakoids is composed mainly of galactosyl diglycerides, which are special lipids exclusively found in chloroplasts (Shimojima et al. 2010; see also Block et al. Chap. 7). The large protein complexes of the photosynthetic machinery are embedded in these membranes: PSII and its light harvesting system II (LHCII), the cytochrome *b6f* complex, PSI and the ATP synthase complex. While PSII is located in appressed grana domains, PSI and the ATP synthase complex are most abundant in stroma lamellae and grana margins. The cytochrome *b6f* complex is distributed quite evenly in the thylakoid membrane. This organization is characteristic of the chloroplast of higher land plants.

Approximately 1,500 million years ago, chloroplasts evolved by endosymbiosis, a process in which a eukaryotic, mitochondriate cell engulfed and established an endosymbiotic relationship with a cyanobacterium (Dyall et al. 2004). Evolution into the chloroplasts of red and green algae, and eventually that of land plants, required the integration of the biology of the endosymbiont into the system of the host cell. This

Abbreviations: ADP – Adenosine-diphosphate; ATAB – Arabidopsis translation of *psaB* mRNA; ATP – Adenosine-triphosphate; CES – Control by epistasy of synthesis; CRP – Chloroplast RNA processing; EMS – Ethyl-methanesulfonate; Hcf – High chlorophyll fluorescence; LHCII – Light harvesting complex II; Mbb – Accumulation/maturation of *psbB* mRNA; OEC – Oxygen evolving complex; PABP – Poly(A)-binding protein; PDI – Protein disulfide isomerase; PPIase – Peptidyl-prolyl isomerase; PSI – Photosystem I; PSII – Photosystem II; RB – RNA binding; RBP – RNA binding protein; RBS – Ribosome binding site; RC – PSII reaction center-like complex containing D1, D2 and PsbE/F; RC47 – PSII core complex lacking CP43; RCC1 – Monomeric PSII core complex; RCC2 – Dimeric PSII core complex; cpSRP – Signal recognition particle; SDR – Short chain dehydrogenase reductase; Tab – Translation of *psaB* mRNA; Tba – Translation of *psbA* mRNA; Tbc – Translation of *psbC* mRNA; TPR – Tetratrico peptide repeat; UTR – Untranslated region; UV – Ultraviolet; Ycf – Hypothetical chloroplast open-reading frame

adaptation process was accompanied by a massive transfer of cyanobacterial genes into the nucleus of the host, resulting in the loss of genetic autonomy. However, the biochemistry of the endosymbiont was retained in the chloroplast and was even enlarged during evolution (Timmis et al. 2004; Lunn 2007). The expression of nearly every protein complex or pathway inside the organelle is therefore the product of the concerted teamwork of two genetic compartments, the nucleus and the plastome. The synthesis of plastid-encoded proteins depends on numerous nuclear-encoded factors that are involved in virtually every step of chloroplast gene expression, starting from transcription up to final complex assembly. These factors coordinate the spatiotemporal pathway of thylakoid membrane complex biogenesis. During recent years many of these regulators have been identified using forward and reverse genetic approaches. However, the functions and regulatory mechanisms that govern membrane biogenesis are mostly unknown to date. The present review summarizes and discusses the current knowledge concerning thylakoid membrane biogenesis. PSII was used as a model system based on the enormous reservoir of available data on the structure, composition and regulation of gene expression of this complex. A brief comment on the components of PSII is followed by a description of the different aspects of complex biogenesis and its regulation.

II. Photosystem II (PSII), a Multisubunit-Cofactor Assembly of Dual Genetic Origin

PSII is a multisubunit protein-cofactor assembly with the capacity to function as a water-plastoquinone oxidoreductase. Over the last decade, X-ray crystallography complemented by spectroscopic techniques provided several 3-D models of the PSII core complex with increasing resolution, recently refined to 1.9 Å (Zouni et al. 2001; Ferreira et al. 2004; Guskov et al. 2009; Umena et al. 2011). These structures were all based on PSII from thermophylic cyanobacteria, mostly *Thermosynechococcus elongatus*. Crystallographic studies of the PSII core complex from algae and higher plants are at an early stage; however, it is reasonable to believe that the basic PSII structures are conserved amongst all types of oxygenic organisms except for some minor differences in subunit content.

The heart of PSII is the reaction center, composed of the subunits D1 (PsbA) and D2 (PsbD). These subunits bind the pigment cofactors chlorophyll, pheophytin and plastoquinone, which perform light-induced charge separation and oxidation of water (Fig. 4.1). The reaction center is flanked by the two large chlorophyll binding subunits CP47 (PsbB) and CP43 (PsbC), which constitute the inner antenna system of the PSII core complex. Depending on the organism studied, this core complex is surrounded by 13–16 low molecular mass intrinsic protein subunits. The exact role of these single trans-membrane-spanning proteins is still elusive. Recent findings show that the majority of them are involved in stabilization, assembly or dimerization of the PSII complex (Komenda 2005; Takasaka et al. 2010). On the luminal side of the thylakoid membrane, extrinsic polypeptides form the oxygen-evolving complex (OEC). In higher plants, these proteins designated PsbO, PsbP, PsbQ participate in the stabilization of the $CaMn_4$ cluster bound to the PsbA and PsbC proteins and are required for efficient oxygen evolution (Roose et al. 2007). A detailed summary of biochemical, structural and functional characteristics of the PSII photosynthetic complex is reviewed elsewhere (Wydrzynski et al. 2005).

The PSII core complexes associate with the peripheral antenna system, which, in green plants, is composed of the heterotrimeric LHCII and the minor antenna proteins CP24, CP26 and CP29. Two to four copies of LHCII trimers associate with a PSII core dimer, resulting in the so called supercomplexes, or even megacomplexes (Dekker and Boekema 2005; Kovacs et al. 2006). This highly developed antenna system of green

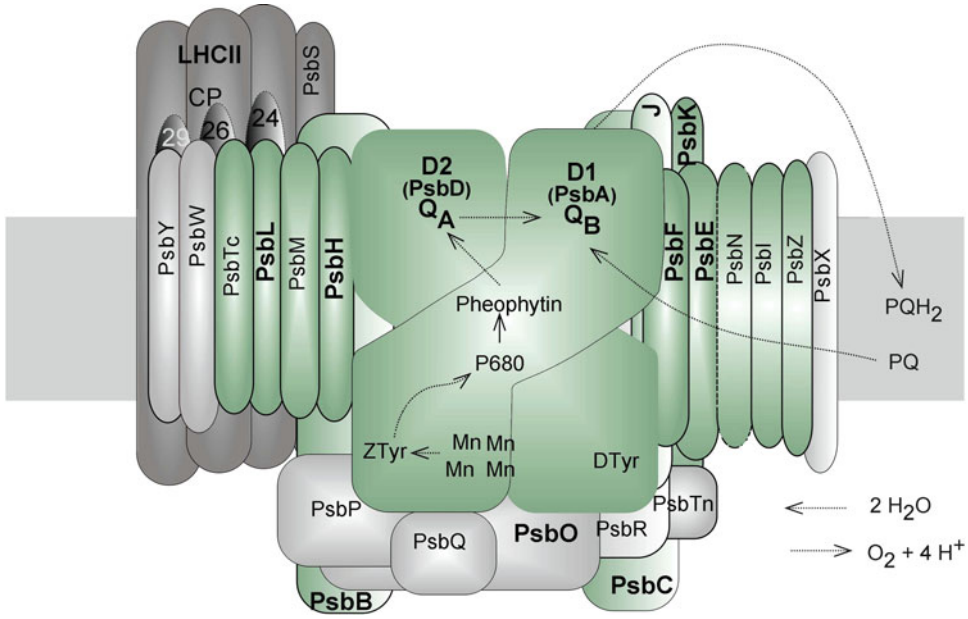

Fig. 4.1. Subunit organization of the monomeric PSII-LHCII supercomplex. *Green* colored polypeptides represent subunits encoded by the plastome, subunits depicted in *grey* are contributed by the nucleus. The letters in *bold* represent proteins essential for assembly and stable accumulation of the whole complex. PsbN might not be a bona fide PSII component and is therefore marked with *dashed line.* (Modified according to Hankamer et al. (1997).)

plants ensures efficient light capture and transfer of excitation energy to the reaction center of PSII. To cope with imbalances in excitation energy between PSII and PSI the so-called state transition process as a short-term acclimation leads to redistribution of a mobile LHCII fraction between the photosystems resulting in their equal stimulation. This reversible fine-tuning is triggered by the redox-state of the plastoquinone pool, which is sensed and transduced to the LHCII by the kinase/phosphatase pair STN7/PPH1 (Kanervo et al. 2005; Kargul and Barber 2008; Rochaix 2011).

The PSII membrane complex is of dual genetic origin: the intrinsic subunits of the PSII core complex are almost completely encoded by the plastome, whereas components of the extrinsic oxygen evolving complex (PsbO, PsbP, PsbQ), and PsbR, certain low molecular mass subunits (PsbS, PsbTn, PsbW, PsbX, PsbY) and the LHCII complex are encoded by nuclear genes (Fig. 4.1). The concerted synthesis and assembly of such an elaborate protein complex requires the contribution of many auxiliary factors. Moreover, the expression of two genetically different compartments has to be coordinated to ensure the precise stoichiometry of PSII components. In addition, the synthesis and accumulation of the constituent subunits is regulated by external signals and by the developmental program of the plant organ or cell. Nuclear-encoded factors integrate these processes during PSII biogenesis by coordinating specific steps of PSII gene expression and by fine-tuning the synthesis of PSII components during acclimation to specific conditions.

III. Model Organisms for Studying the Biogenesis of PSII

Both biochemical and genetic approaches have been used to dissect the complex regulatory network that governs plastid biogenesis.

The genetic approach was based on the use of model organisms such as *Arabidopsis thaliana* (Arabidopsis), *Zea mays* (maize) and *Chlamydomonas reinhardtii* (Chlamydomonas), each of which offered specific advantages. Arabidopsis rapidly evolved as a powerful genetic model for the study of higher plants because of its short life cycle and small genome. High throughput techniques including forward and reverse genetic approaches, microarrays, protein separation and identification and bioinformatics have enabled researchers to characterize gene products involved in chloroplast biogenesis and specify their functions (Chi et al. 2012). However, biochemical studies using wild-type Arabidopsis and its photosynthesis mutants are difficult due to small plant size. Although maize mutants are larger and more suitable for this kind of analysis (Stern et al. 2004), the long generation time and large nuclear genome of maize has hampered gene identification in isolated mutant lines. Chlamydomonas combines several advantages for genetic, molecular and biochemical analysis of photosynthesis mutants (Dent et al. 2001). An advantage of the green alga is its ability to grow heterotrophically even in darkness in an acetate-containing medium. Under these conditions, chlorophyll is synthesized and a complete photosynthetic apparatus can be generated. Thus it is possible to study biochemical or biophysical characteristics of light sensitive mutants in dark-grown algae cells avoiding secondary effects induced by photo-oxidative damage often found in higher plant photosynthetic mutants. Moreover, the plastome of Chlamydomonas can be modified by transformation, which occurs by homologous recombination, thus simplifying gene knockouts and site directed mutagenesis in the chloroplast. The use of this technique has resulted in significant contributions towards the understanding of the regulatory principles of chloroplast gene expression (Sect. IV.A.3). The recent completion of the Chlamydomonas nuclear genome sequence has enabled comparative, phylogenomic studies, identifying uncharacterized genes encoding proteins that are likely associated with the function and biogenesis of chloroplasts (Merchant et al. 2007; Grossman et al. 2010). In addition to these eukaryotic organisms, the cyanobacterium Synechocystis sp. PCC6803 (Synechocystis) is also used as a model organism to analyze PSII biogenesis; however, basic aspects of regulated expression of photosynthetic genes differ between cyanobacteria and chloroplasts, and this is paralleled by different sets of required auxiliary factors.

Genetic screens for photosynthetic mutants of land plants are generally based on the detection of increased PSII chlorophyll fluorescence (*h*igh *c*hlorophyll *f*luorescence (hcf) phenotype), which primarily indicates a block in the photosynthetic electron transport chain. The hcf phenotype is mostly accompanied by chlorophyll deficiency and is visible in the dark under UV-light (Miles 1980, 1994).

IV. Biogenesis of PSII: Nuclear-Encoded Auxiliary Factors are Involved in Many Different Steps of Plastid Gene Expression

Previous work based on the analysis of photosynthetic mutants of higher plants and Chlamydomonas shows that chloroplast gene expression is predominantly controlled at the post-transcriptional level. Nuclear-encoded auxiliary factors have diverse functions at all levels of plastid gene expression including RNA processing, transcript stabilization, translation, protein turnover and all aspects of complex assembly (Fig. 4.2) (Wollman et al. 1999; Rochaix 2007). Likewise in cyanobacteria, the majority of the genes encoded by higher plant chloroplasts are organized into polycistronic transcription units (Herrmann et al. 1992; Sugiura 1992). These gene clusters are often heterogeneous, such as the *psbB* transcription unit, which consists of genes encoding components of PSII and the cytochrome *b6f* complex (Barkan 1988; Westhoff et al. 1988). In contrast to cyanobacteria, the primary products

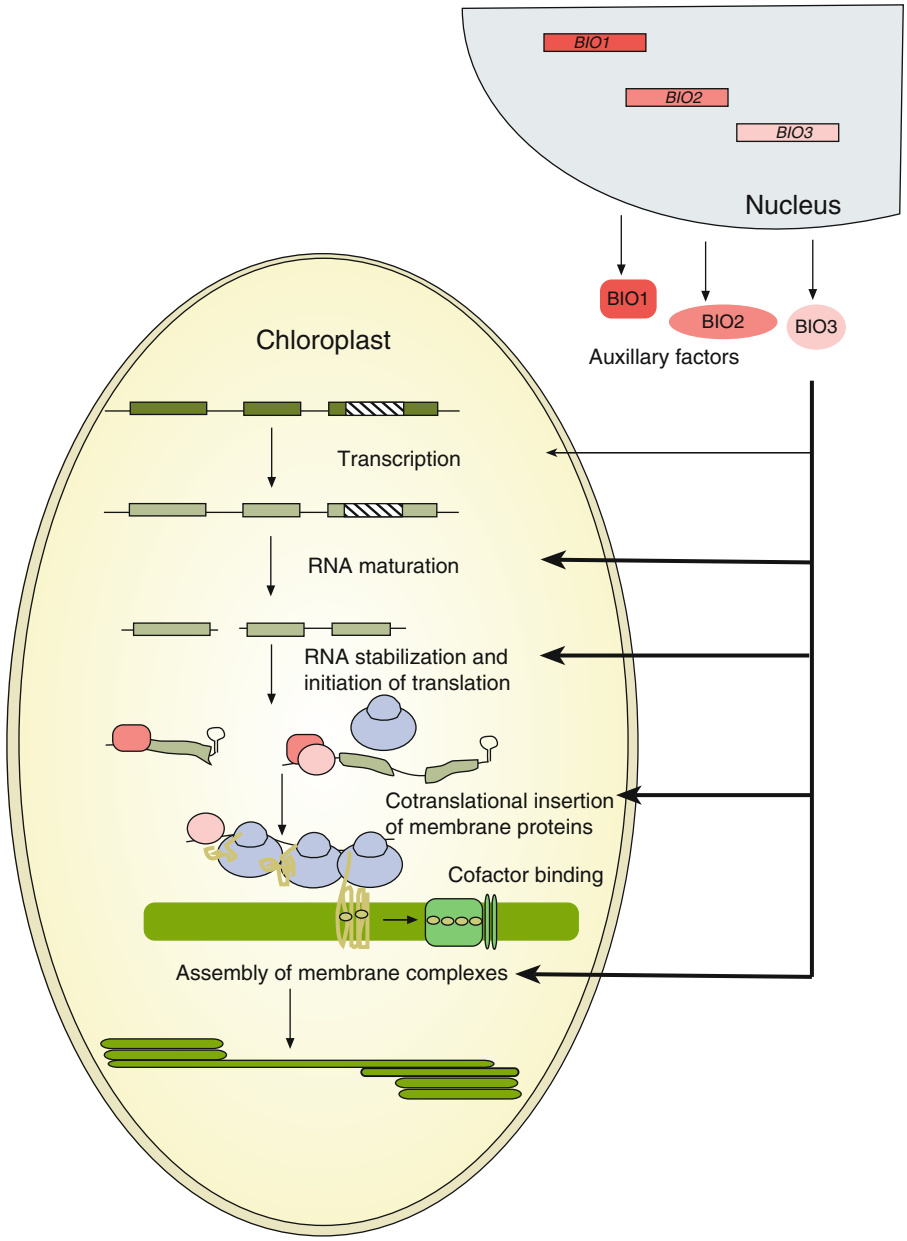

Fig. 4.2. Schematic representation of the mechanisms involved in the control of chloroplast gene expression in higher plants. Nuclear-encoded auxiliary factors accomplish diverse functions at all levels of plastid gene expression. These regulatory factors (symbolized by Bio1–3) coordinate plastid and nuclear gene expression during biogenesis of the thylakoid membrane in response to endogenous and environmental cues. The *thin arrow* pointing to transcription indicates that this process is a minor target of nuclear-encoded regulatory factors. (The figure is based on a schematic presented by Rochaix (1996).)

of these transcription units need further modifications to produce translatable RNAs. These posttranscriptional modifications include RNA cleavage, RNA stabilization, intron splicing and in many cases, RNA editing (Monde et al. 2000a; Stern et al. 2010). Chloroplast mRNA translation also differs considerably from the original prokaryotic process, since it became mostly uncoupled from transcription, which converted the translation machinery of the chloroplast into a highly regulated system. Moreover, the process of translation in chloroplasts is accompanied by cofactor binding, protein folding, membrane insertion of proteins and complex assembly, which provide further possible steps for the intervention of nuclear-encoded factors in plastid gene expression (Fig. 4.2).

A. Posttranscriptional Control: The Biogenesis of the PSII Core Complex Is Regulated at the Translational Level

As summarized below, the stability and translation of chloroplast messages depends on the concerted action of RNA secondary structures and sequence motifs in the 5′ and 3′ untranslated regions (UTR) and of nuclear-encoded transacting factors (Marin-Navarro et al. 2007; del Campo 2009, Stern 2010; Zhelyazkova et al. 2012). These processes of post-transcriptional regulation are mainly mediated by proteins that act in a gene-specific manner. Table 4.1 summarizes the complement of factors involved in RNA processing, stabilization and translation of RNAs encoding PSII subunits.

Table 4.1. Nuclear-encoded factors involved in processing, stabilization and translation of plastid-encoded transcripts of PSII subunits

Protein factor	Target	Motifs	Function	Reference
CrRB47	*psbA* 5′ UTR	Poly(A) binding protein	RNA binding protein, activates initiation of translation of *psbA* mRNA	(Yohn et al. 1996; 1998b)
CrRB60	*psbA* 5′ UTR	Disulfide isomerase	Reacts with disulfide of RB47, and determines its redox state	(Trebitsh et al. 2001; Alergand et al. 2006)
CrTba1	*psbA* mRNA	Oxido-reductase	Involved in redox regulation of RB47	(Somanchi et al. 2005)
AtHCF173	*psbA* mRNA	SDR	Involved in initiation of D1 synthesis	(Schult et al. 2007)
CrAc115	*psbD*		Stabilization of D2 translation intermediates	(Rattanachaikunsopon et al. 1999)
CrNac2	*psbD* 5′ UTR	TPR	Stabilization of *psbD* mRNA	(Boudreau et al. 2000)
CrRBP40	*psbD* 5′ UTR	RRM-like motif	RNA binding factor, affects initiation of D2 synthesis	(Schwarz et al. 2007)
AtTAB2	*psbD*/C (*psaA/B*)		RNA binding protein, involved in translation of *psbD*/C and psaA/B	(Barneche et al. 2006)
AtHCF107	*psbB*, *psbH*	R-TPRs	Processing/stabilization of *psbH* containing transcripts, synthesis/accumulation of CP47	(Felder et al. 2001; Sane et al. 2005)
CrMbb1[a]	*psbB*, *psbH*	TPR	Maturation/stabilization and translation of *psbB* and psbH RNAs	(Vaistij et al. 2000)
CrTbc2	*psbC*	PPPEW repeats	Translation of *psbC* mRNA	(Auchincloss et al. 2002)

Cr Chlamydomonas reinhardtii, *At Arabidopsis thaliana*

[a]Mbb1 is an ortholog of HCF107, but due to slight differences in their functions both factors are listed separately

1. Redox-Regulation Is Involved in the Expression of Reaction Center Protein D1

a. A Sophisticated Regulatory Network Identified in Chlamydomonas

Synthesis and assembly of the D1/D2 heterodimer and bound cofactors requires coupling to external stimuli and fine-tuning in response to expression of its assembly partners. Light plays an important role in regulated D1 expression and considerably increases D1 synthesis rate in higher plants and algal cells (Fromm et al. 1985; Klein et al. 1988; Malnoe et al. 1988; Krupinska and Apel 1989). Genetic and biochemical studies revealed the existence of a sophisticated regulatory network involved in translation initiation of the *psbA* mRNA in the green alga Chlamydomonas. The *psbA* 5′ UTR of Chlamydomonas, which contains a secondary structure combined with a Shine-Dalgarno-like sequence, binds a complex of four proteins: RB47, RB38, RB60 and RB55 (Danon and Mayfield 1991). Binding of the complex is mediated by the RB47 protein, which is essential for D1 translation and belongs to the family of poly(A)-binding proteins (PABP). RB47 contains four conserved RNA recognition motifs (Yohn et al. 1996, 1998a) and binds in its reduced form to the 5′ UTR of *psbA* mRNA to facilitate translation initiation (Fong et al. 2000). Light modulates the binding of the RB47 protein complex, which is paralleled by alterations in polyribosome association of the *psbA* mRNA reflecting different synthesis rates of the D1 protein. This process is regulated by RB60, a disulfide isomerase homolog (Trebitsh et al. 2001) able to modify the redox state of RB47 (Kim and Mayfield 2002). RB60 selectively reacts with the disulfide of RB47 through the formation of a disulfide intermediate, suggesting that the redox states of these two proteins are coupled (Alergand et al. 2006). Because the pool of RB60-thiols in the chloroplast becomes proportionally reduced with increasing light intensity, probably transduced from PSI by the ferredoxin-thioredoxin system, it was suggested that the purpose of this regulatory mechanism is to modulate *psbA* mRNA translation in response to incident light (Trebitsh et al. 2000). Moreover, the redox active cysteines of RB47 react in a pH-sensitive manner, suggesting that stromal pH changes resulting from illumination of the chloroplast may contribute to the regulation of *psbA* mRNA translation mediated by RB47 (Alergand et al. 2006). In a second possible mechanism, ADP-dependent phosphorylation of RB60 inactivates the binding of the complex to *psbA* mRNA. The inactivation of binding by phosphorylation of RB60 requires high ADP concentrations, which are normally only attained in chloroplasts in the dark, indicating that this mechanism is aimed at diminishing *psbA* mRNA translation in the night (Danon and Mayfield 1994). Another player in the regulation of D1 synthesis is the factor Tba1, a protein with homology to oxidoreductases (Somanchi et al. 2005). Tba1 is suggested to be involved in the redox activation of RB47 via RB60 to activate translation initiation of the *psbA* mRNA, although its exact role has not been determined.

b. Translation of *psbA* mRNA in Higher Plants: Functions of Auxiliary Factors Are Still Unclear

A comparable model for the light activated translation of the *psbA* mRNA has not been outlined for higher plants, but light is a pivotal stimulus for *psbA* gene expression. Direct evidence for the control of *psbA* translation initiation by light through 5′ UTR sequences came from gene fusion constructs in transformed tobacco plastids (Staub and Maliga 1994). Using an in vitro translation system, three cis-acting regulatory elements of the *psbA* 5′UTR of tobacco relevant for translational control have been identified: RBS1, RBS2 and between them an AU-Box. It is suggested that RBS1 and -2

bind to the 3′ end of the 16S rRNA, which causes the looping out of the AU-Box, possibly facilitating the interaction of trans-acting factors (Hirose and Sugiura 1996). The existence of a protein factor that binds to the AU-Box has been confirmed by gel retardation experiments. Further candidate factors have been proposed to be involved in the binding of these 5′-regulatory element, thereby controlling *psbA* translation. Recently, the Arabidopsis protein HCF173 was found to be essential for *psbA* translation (Schult et al. 2007). Mutants lacking HCF173 do not grow under photoautotrophic conditions and are characterized by impaired accumulation of *psbA* mRNA and a significant reduction of the ribosomal loading of this transcript, indicating that HCF173 is involved in stabilization and translation initiation of the *psbA* mRNA. HCF173 is weakly related to the short-chain dehydrogenase/reductase (SDR) superfamily, a class of enzymes that uses NAD(P)(H) as coenzymes and functions as dehydrogenases, dehydratases, epimerases, and isomerases of a variety of substrates. The protein is conserved in higher plants and Chlamydomonas and is mainly attached to chloroplast membranes, where it acts as a part of a high molecular mass complex that contains *psbA* mRNA. In plants and algae, formation of a translational initiation complex triggers the targeting of the *psbA* mRNA protein complex to the thylakoids, a process that is necessary for cotranslational membrane insertion of D1 (Zhang et al. 2000). Bearing this in mind, the authors speculate that the observed membrane binding of HCF173 might be necessary for membrane targeting of *psbA* mRNA translation. A similar tethering function was proposed for the RNA binding protein RBP63 from Chlamydomonas that binds to *psbA* mRNA and is localized to stroma thylakoid membranes (Ossenbühl et al. 2002). Proteins of 43- and 30 kDa that bind to the *psbA* 5′UTR were identified in Arabidopsis (Shen et al. 2001). Oxidizing conditions abolished the interactions of the proteins with the 5′UTR, while reducing conditions recovered their RNA binding activity. Thus, it can be hypothesized that redox-dependent interactions also participate in posttranscriptional control of *psbA* gene expression in higher plants. However, elucidation of the underlying regulatory mechanisms requires further investigation into the interacting protein partners, their enzymatic activities and the conditions of binding.

c. Translation of *psbD* mRNA: Identification of Interesting Regulatory Factors

Cis-acting RNA leader elements, termed PRB1 and PRB2, are also required for the stability and translation initiation of the Chlamydomonas *psbD* mRNA encoding the D2 protein (Nickelsen et al. 1999). The nuclear-encoded trans-factors required for this process are Nac2 and RBP40. Nac2 is a tetratricopeptide repeat (TPR) protein that is necessary for stable accumulation of the *psbD* mRNA (Kuchka et al. 1989; Boudreau et al. 2000). It is part of a high molecular mass complex that binds to the mature *psbD* transcript, very likely via the cis-element PRB2 (Nickelsen et al. 1999). This binding guides RBP40 to a U-rich site immediately downstream of PRB2 (Schwarz et al. 2007), probably inducing conformational changes within the region encompassing the start codon, and rendering this sequence accessible to components of the translational machinery. RBP40 binds the *psbD* 5′UTR directly. This interaction is very likely mediated by a motif that resembles a well-known RNA binding domain, the RNA recognition motif domain (Clery et al. 2008). Translation of the *psbD* mRNA in higher plants is possibly affected by the nuclear-encoded, evolutionarily conserved RNA binding protein ATAB2. This protein was identified in Arabidopsis as an ortholog of the Chlamydomonas Tab2 protein (Barneche et al. 2006). Tab2 is specifically required for the translation of *psaB*, which encodes one of the subunits of the PSI reaction center (Stampacchia et al. 1997; Dauvillee et al. 2003).

ATAB2 knockout mutants of Arabidopsis showed impaired accumulation of PSI and PSII, interestingly, not only plastome-encoded but also nuclear-encoded subunits are affected. Polysome association studies showed that *psaA/B* and *psbD*/C translation is decreased in the *atab2* mutant (Barneche et al. 2006). It is speculated that ATAB2 synchronizes the synthesis of PSI and PSII components affecting the anchoring subunits D2 and PsaB of both photosystems. Blue light photoreceptors regulate the expression of ATAB2, and therefore this factor might be part of a photoreceptor-mediated signaling pathway directly transducing light signals from the environment into the chloroplast (Barneche et al. 2006).

d. Translation Elongation: Close Association with Cofactor Binding and Membrane Insertion of D1 and D2

Molecular genetic studies show that translation initiation of the proteins D1 and D2 in Chlamydomonas and higher plants is under the rigorous control of the nucleus. However, important processes like cofactor binding, membrane insertion and assembly occur during the translation elongation of D1 and D2. To facilitate the proper association of chlorophyll to the D1 polypeptide chain, translation elongation is disrupted by ribosomal pausing in chloroplasts from barley (Kim et al. 1991). Specific pausing sites were identified by the occurrence of translation intermediates with sizes of 15–25 kDa. Although the barley mutant *vir-115* (viridis-115) lacks these pausing intermediates, the *psbA* mRNA accumulates and is associated with polysomes (Gamble and Mullet 1989; Kim et al. 1994). Thus, the nuclear locus VIR-115 was suggested to be involved in the pausing of ribosomes or the binding of cofactors to the nascent D1 chain. Similarly, translation of the D2 protein is regulated at the level of elongation by the nuclear gene *Ac115* in Chlamydomonas (Wu and Kuchka 1995). *Ac115* encodes a small protein of 113 amino acids that possesses a hydrophobic domain at the C-terminus, which may be a transmembrane domain or a region involved in protein-protein interaction (Rattanachaikunsopon et al. 1999). Ac115 has been suggested to be necessary for stabilization of D2 translation intermediates or it may serve to localize the *psbD* message and/or the nascent D2 protein to the thylakoids.

2. The Inner Antenna, CP47 and CP43, and the Small Subunit PsbH

a. Synthesis and Accumulation of CP47 Are Tightly Coupled with Expression of PsbH

Synthesis and/or accumulation of the PSII proteins CP47 and PsbH are specifically impaired in the Arabidopsis mutant *hcf107*. Rigorous examination of the hcf mutant phenotype identified altered processing and translation of mRNAs originating from the polycistronic *psbB-psbT-psbH-petB-petD* transcript (Felder et al. 2001). In Arabidopsis, *psbH*-containing transcripts occur with a long (−75) and a short (−45) 5′ UTR. It was suggested that the longer 5′ UTR forms a thermodynamically stable hairpin structure that conceals the translation initiation site and start codon. Processing into the short form enables ribosome binding and subsequent translation initiation. HCF107 is involved in the correct processing, stabilization and possibly even translation of this short *psbH* mRNA. Moreover, this factor might be involved in translation of *psbB*, because despite normal accumulation of the mature *psbB* transcript, the protein is not synthesized in the *hcf107* mutant. The mapped nuclear gene *HCF107* encodes a product that belongs to the family of TPR proteins (Sane et al. 2005). Due to modifications in the TPR consensus sequence, HCF107 was assigned to a separate group consisting of TPR proteins from yeast that are specifically involved in RNA processing events and are hence designated as RNA(R) TPR proteins (Ben-Yehuda et al. 2000). Immunolocalization studies showed that

HCF107 is primarily localized to plastid membranes. An ortholog of HCF107, the Chlamydomonas Mbb1 protein, has comparable but not similar functions. Mbb1 also acts separately on the *psbB* and *psbH* transcripts, but in contrast to Arabidopsis, it is necessary for maturation and/or accumulation of both *psbB* and *psbH* mRNAs (Vaistij et al. 2000). Moreover, in line with the data from Arabidopsis, chloroplast transformation with *psbB* 5′ UTR reporter constructs further indicates a role for this factor in translation. Both HCF107 and Mbb1 are part of a high molecular weight complex in chloroplasts. Concerning the function of TPR domain proteins, HCF107/MBB1 might be necessary to ensure the interaction of distinct components of the RNA-processing and translation machinery. Indeed, the association of the MBB1 complex with RNA was shown by size fractionation and subsequent RNAse treatment.

b. Regulators of *psbC* mRNA Translation

Translation of the chloroplast *psbC* mRNA in Chlamydomonas is controlled by its 5′ UTR and the products of the nuclear loci *TBC1*, *TBC2*, and possibly *TBC3* (Rochaix et al. 1989). Reporter gene expression studies revealed that the *TBC1* locus controls translation initiation of *psbC* mRNA via a region near the start codon and central parts of the 5′UTR sequence, enclosing a stem-loop structure that is essential for *psbC* translation (Zerges et al. 1997). The TBC1 gene has not been identified to date. The *Tbc2* gene was cloned and the coding sequence predicts a protein of 1,115 residues with nine copies of a novel degenerate 38-amino-acid octatricopeptide repeat (OPR) motif (Stern et al. 2010) near its C-terminal end (Auchincloss et al. 2002). The central region of the protein displays partial amino acid sequence identity with CRP1, a protein in maize that is implicated in the processing and translation of the chloroplast *petA* and *petD* mRNAs. The Tbc2 protein co-fractionates with the chloroplast stroma and is associated with a 400 kDa-protein complex. The function of Tbc2 is also mediated by specific *psbC* 5′ UTR sequences; however, the Tbc2 complex is not associated with RNA, indicating only transient or indirect interaction with the target sequence. The role of Tbc3 is unclear so far because the nuclear locus is represented by a suppressor mutation that reverses two different translational blocks of *psbC* mRNA (Zerges et al. 1997). A non-specific or newly generated RNA-binding affinity that compensates for the mutational defects in *psbC* mRNA translation can therefore not be ruled out.

3. Assembly-Controlled Steps That Regulate Translation of PSII Core Subunits

Studies with photosynthesis mutants indicate that unassembled components, particularly the ones forming the catalytic core, are post-translationally degraded (Rochaix 1995; Wollman et al. 1999). This active degradation prevents unspecific aggregation of non-assembled proteins, which possibly disturb membrane integrity, and limits the accumulation of partially matured core subunits that could contribute to the formation of deleterious redox intermediates. In Chlamydomonas, stoichiometric accumulation of the major chloroplast-encoded PSII subunits is regulated by a cascade of assembly-controlled processes (Minai et al. 2006). In brief, the synthesis of specific subunits is regulated by the availability of their direct assembly partners. This is a common mechanism that governs the biogenesis of all photosynthetic membrane complexes and the ATP synthase and has been termed ‘control by epistasy of synthesis’ (CES) (Choquet et al. 1998, 2003; Wostrikoff et al. 2004; Drapier et al. 2007). PSII assembly occurs as a sequential process initiated by the D2-PsbE/F subcomplex (Minagawa and Takahashi 2004). D1 and certain small subunits are then attached to establish the reaction center and subsequently the inner antenna proteins CP47 and CP43 are bound (cf. Sect. IV.B). In a mutant strain in which the *psbD*-gene is deleted and

therefore lacks the D2 protein, *psbA* mRNA translation is down-regulated by the accumulation of unassembled D1 (Minai et al. 2006). Reporter gene constructs demonstrate that this regulation is mediated by the *psbA* 5′UTR. Because this sequence is sufficient to confer the CES behavior to reporter gene expression, it is suggested that translation initiation is affected. The same negative feedback loop was observed for D1-dependent synthesis of CP47. Unassembled CP47 represses its translation until it is attached to the D1/D2 reaction center complex. The synthesis of the major subunits of PSII is therefore adjusted to the available amount of the assembly competent precomplex, which is in principle defined by the actual level of D2. The mechanisms underlying this autoregulation of translation are unclear, but it is tempting to speculate that at least some of the above-mentioned trans-acting factors are involved in these processes.

A mechanism comparable to assembly-controlled synthesis of protein complex subunits has not been identified in higher plants. Mutants of Arabidopsis with specific defects in single subunits of photosynthetic membrane complexes do not show a CES-like control of their assembly partners (Schult et al. 2007). In tobacco chloroplast deletion mutants (ΔpetB, ΔpetD, ΔpetA), the observed effects on translational control are marginal and no clear cut statement about a CES mechanism in this plant could be made (Monde et al. 2000b).

B. PSII Complex Assembly

Much emphasis has been placed on the detailed study of PSII assembly in cyanobacteria and in plants (Baena-Gonzalez and Aro 2002; Rokka et al. 2005; Nixon et al. 2010). Although these studies show parallels in the sequential order in which subunits associate into a mature PSII complex, knockout mutants have revealed differences in the complement of accessory proteins and the functional relevance of conserved PSII assembly factors between the two groups of organisms. In addition to the many auxiliary factors, structural PSII components including the OEC proteins PsbO, PsbP and PsbQ, and some low molecular weight subunits are thought to be required for assembly and regulation of the structural integrity of PSII supercomplexes and granal stacking in higher plants (Minagawa and Takahashi 2004; Shi and Schroder 2004; Suorsa and Aro 2007). Furthermore, as a consequence of harmful photosynthetic side reactions PSII continually undergoes a cycle of damage and repair. The D1 protein as the main target of photoinhibition is irreversibly damaged and must be replaced with a newly synthesized copy (Baena-Gonzalez and Aro 2002). Assembly factors as well as kinases, phosphatases and proteases exclusively involved in this repair cycle have been analyzed in great detail in photosynthetic organisms (Adam et al. 2006; Mulo et al. 2008; Nixon et al. 2010). The following paragraphs summarize the current knowledge about auxiliary proteins that are necessary for the biogenesis and maintenance of PSII.

Assembly processes have been studied by radioactive labeling of leaf proteins and subsequent separation of solubilized thylakoid membranes by native two-dimensional gel electrophoresis (Reisinger and Eichacker 2007), coupled with sensitive and specific identification of proteins by mass spectrometry (Ploscher et al. 2009). Moreover, the development of methods like yeast-two-hybrid split ubiquitin analyses, which is used to examine protein-protein interactions between intrinsic membrane proteins (Pasch et al. 2005), considerably improved the identification of candidate assembly factors. PSII assembly can be described as a sequential addition of subunits and cofactors to an initial acceptor complex consisting of D2, PsbE/F and PsbI (Rokka et al. 2005). First, the precursor of the D1 subunit (pD1) is assembled into the receptor complex to form the PSII reaction center-like assembly complex, RC. Subsequently, CP47, possibly together with PsbH, attaches to RC to generate

the so-called CP47-RC complex. In two further steps, the PsbM, PsbTc, and PsbR subunits are added to form CP43-free PSII monomers. The attachment of CP43, together with PsbK, allows the formation of the monomeric PSII core complex RCC1, which in turn binds the $CaMn_4$ cluster and the lumenal extrinsic subunits PsbO, PsbP and PsbQ. A prerequisite for the assembly of the oxygen-evolving complex, including the manganese cluster, is the C-terminal processing of the D1 precursor (Nixon et al. 1992; Roose and Pakrasi 2004). During this process, nine residues in higher plants and 16 in cyanobacteria are removed by the endoprotease CtpA (*c*arboxyl-*t*erminal *p*rocessing protease) (Diner et al. 1988; Fujita et al. 1989; Anbudurai et al. 1994). Processing of the D1 precursor accompanies assembly of the RC and can be visualized based on the differences in the migration of mature and immature D1 in SDS gel electrophoresis (Müller and Eichacker 1999). The assembled oxygen-evolving PSII complex forms a dimer (RCC2) and higher order supercomplexes in vivo, which are relevant for efficient light capture and photosynthetic performance. The PSII dimers are surrounded by up to four LHCII trimers and several minor LHCII proteins (CP29, CP26, and CP24), which serve as linkers for the antenna complexes in this supramolecular assembly (Dekker and Boekema 2005; Nelson and Yocum 2006).

The described assembly processes are assisted by many nuclear-encoded assembly factors that are involved in stabilization of assembly intermediates, processing of proteins, membrane insertion and cofactor binding, and possibly in many other unknown processes. Table 4.2 summarizes the data about known assembly factors of PSII and Fig. 4.3 represents a schematic drawing of the PSII assembly process. The specific functions of assembly factors are described in the following section.

Table 4.2. Nuclear-encoded factors involved in PSII assembly and maintenance

Factor[a]	Gene code[b]	Mutant[c]	Possible function	Reference
AtHCF136 (L)	At5g23120	No photoautotrophic growth, PSII subunits hardly detectable	Assembly of initial PSII intermediates	(Meurer et al. 1998; Plücken et al. 2002)
Ycf48 [HCF136]	slr2034	Slow rate of autotrophic growth, PSII assembly impaired	Assembly of initial PSII intermediates, involved in PSII repair cycle	(Komenda et al. 2008)
AtPAM68 (M)	At4g19100	Slow photoautotrophic growth, reduced PSII accumulation	Assistance in different PSII assembly steps	(Armbruster et al. 2010)
Sll0933 [PAM68]	sll0933	Photoautotrophic growth, normal PSII accumulation	–	(Armbruster et al. 2010)
AtLPA1 (M)	At2g02910	Impaired photoautotrophic growth, PSII 25–50%	Synthesis and assembly of D1 and D2	(Peng et al. 2006)
CrREP27 [LPA1]	–	Photoautotrophic growth	Synthesis of D1 during PSII repair	(Park et al. 2007; Dewez et al. 2009)
AtLPA2 (M)	At5g51545	Impaired photoautotrophic growth, PSII 30%	Synthesis of CP43, assembly of PSII Interaction with LPA3	(Ma et al. 2007)
AtLPA3 (M)	At1g73060	Impaired photoautotrophic growth, PSII 30%	Synthesis of CP43, assembly of PSII Interaction with LPA2	(Cai et al. 2010)

(continued)

Table 4.2. (continued)

Factor[a]	Gene code[b]	Mutant[c]	Possible function	Reference
AtCYP38 (L)	At1g01480	Impaired photoautotrophic growth, PSII reduced	Proper folding or assembly of D1	(Fu et al. 2007; Sirpio et al. 2008)
SoTLP40 [CYP38]	–	–	Regulation of dephosporylation of thylakoid membrane proteins	(Fulgosi et al. 1998; Vener et al. 1999; Rokka et al. 2000)
AtFKBP20-2 (L)	At3g60370	Impaired photoautotrophic growth	Assembly of PSII supercomplexes	(Lima et al. 2006)
AtTLP18.3 (L)	At1g54780	Photoautotrophic growth, light sensitive seedlings	Turnover of damaged D1 and dimerization of PSII	(Sirpio et al. 2007)
AtPPL1 (L)	At3g55330	Photoautotrophic growth, light sensitive seedlings	Efficient repair of photodamaged PSII, involved in donor side assembly	(Ishihara et al. 2007)
Sll1418 [PPL1]	Sll1418	Photoautotrophic growth	Stabilizing function at the donor side of PSII	(Sveshnikov et al. 2007)
AtPSB27 (L) [Slr1645 (L)]	At1g03600	Photoautotrophic growth	Efficient repair of photodamaged PSII	(Chen et al. 2006; Nowaczyk et al. 2006)
Slr0286 (L)	Slr0286	Photoautotrophic growth	Assembly of the OEC proteins	(Kufryk and Vermaas 2001)
PratA (P)	Slr2048	Slow photoautotrophic growth, reduced PSII accumulation	Facilitates C-terminal processing of D1	(Klinkert et al. 2004; Schottkowski et al. 2009)
AtLPA19 (L)	At1g05385	Slow photoautotrophic growth, PSII 25%	Facilitates C-terminal processing of D1	(Wei et al. 2010)

M membrane protein, *L* lumenal protein, *P* periplasmic protein, *Cr Chlamydomonas reinhardtii*, *At Arabidopsis thaliana*

[a]Orthologous proteins are indicated in parenthesis

[b]Gene codes are listed for Arabidopsis and Synechocystis only

[c]Percent values given refer to the levels of the major PSII core subunits in the mutants

1. Assembly of the PSII Reaction Center Complex and CP43

a. HCF136 Is Essential for the Initial Steps of Reaction Center Complex Assembly

Several assembly factors have been shown to be involved in the early steps of the assembly of the D1/D2 heterodimer, including the low molecular mass subunits psbE/F and psbI (Fig. 4.3). HCF136 (*h*igh *c*hlorophyll *f* luorescence 136) of Arabidopsis was one of the first PSII assembly factors characterized (Meurer et al. 1998). The protein is targeted to the lumenal side of stroma thylakoids and is absolutely required for accumulation of PSII proteins. The severe mutational defect leads to a pale green appearance of the seedlings, which are non-viable under photoautotrophic growth conditions. In vivo radiolabeling experiments showed that although all PSII subunits were synthesized at normal rates, the formation of the PSII RC complex was specifically blocked in the absence of HCF136 (Plücken et al. 2002). Two-dimensional gel electrophoretic separation of membrane proteins showed that HCF136 co-migrates with RC-like assembly complexes. Furthermore, affinity chromatography using immobilized HCF136 revealed its interaction with a PSII assembly complex containing at least D2 and PsbE/F. The HCF136 protein accumulates in etiolated seedlings, suggesting its presence during or

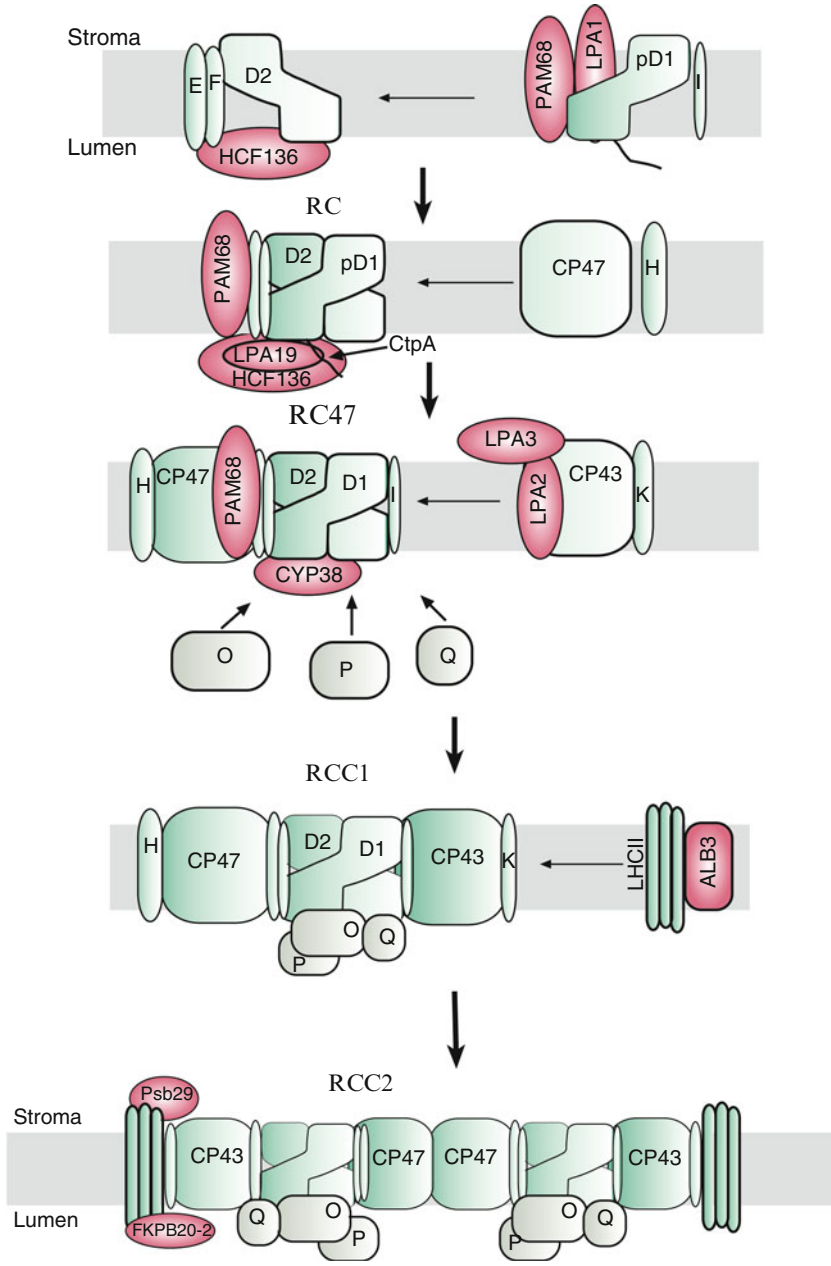

Fig. 4.3. Schematic drawing of PSII assembly in thylakoids of higher plants. The generation of different PSII assembly intermediates and the possible involvement of auxiliary factors are depicted. The formation of a pD1/psbI subcomplex is very likely during PSII assembly in Synechocystis, its occurrence in higher plants remains to be analyzed. For clarity, only some of the low molecular mass subunits are shown. The types of assembly intermediates are: RC, PSII reaction center-like complex containing D1 (pD1), D2 and PsbE/F; RC47, PSII core complex lacking CP43; RCC1, monomeric PSII core complex; RCC2, dimeric PSII core complex. *Grey* shaded subunits with the capital letters O, P, Q represent the OEC. CtpA is the D1 processing protease.

even before light-induced synthesis of PSII core proteins to facilitate the initial PSII assembly steps (Meurer et al. 1998). Knockout experiments in Synechocystis showed that the HCF136 ortholog Ycf48 is not strictly required for PSII assembly; however, the factor participated in optimizing PSII assembly through the formation of reaction center assembly intermediates (Komenda et al. 2008). Yeast two hybrid analysis using the split ubiquitin system revealed that Ycf48 interacts with unprocessed D1 (pD1). This interaction might be necessary to prevent premature processing and degradation of pD1 before its association with D2. In addition to its role in the assembly of PSII complexes, Ycf48 was suggested to be involved in the PSII repair cycle (Komenda et al. 2008).

b. Non-mandatory Factors Involved in Reaction Center Complex Assembly

The assembly factor LPA1 (*l*ow *P*SII *a*ssembly 1) is involved in the synthesis and/or stabilization of D1 and D2 in Arabidopsis (Peng et al. 2006). PSII subunits are significantly reduced in *lpa1* mutants, but homozygous plants are able to grow photoautotrophically, although with reduced growth rates. A reduction in the efficiency of PSII assembly was detected in *lpa1* mutants, as indicated by the accumulation of free PSII proteins and lower levels of PSII dimers in solubilized membrane complex preparations. LPA1 contains two transmembrane domains and four tetratricopeptide repeats, and it is an intrinsic component of the Arabidopsis thylakoid membrane. Yeast two-hybrid studies revealed that LPA1 interacts with D1 but not with D2. This factor might therefore be an integral membrane chaperone that is required for efficient assembly of the PSII reaction centers (Peng et al. 2006). CrRep27 (*re*pair-aberrant mutant of *P*SII) is the ortholog of LPA1 in Chlamydomonas (Park et al. 2007). Similar to LPA1, Rep27 is suggested to be involved in D1 synthesis, but as indicated by its name, Rep27 participates in D1 synthesis during the PSII repair cycle (Dewez et al. 2009).

A similar phenotype to that of *lpa1* was found in the Arabidopsis photosynthesis mutant *pam68* (*p*hotosynthesis *a*ffected *m*utant 68), which shows low levels of PSII core proteins and both, decreased stability and maturation of the D1 protein (Armbruster et al. 2010). In addition, this mutant shows an impairment in PSII complex assembly, as seen in the accumulation of RC precomplexes at the expense of PSII dimer and supercomplexes. These observations support a direct or indirect role of PAM68 in the integration of D1 into early PSII intermediates, promoting further transition of the RC precomplex to larger assembly complexes. PAM68 is an integral membrane protein characterized by two transmembrane domains and an acidic domain at the N-terminus. Orthologs of PAM68 exist in all sequenced photosynthetic eukaryotes and in cyanobacteria. In cyanobacteria, the factor appears to be less important for photosynthesis than in higher plants, as the disruption of the *PAM68* ortholog in Synechocystis caused no alterations in photoautotrophic growth, oxygen evolution or the levels of PSII proteins. Intriguingly, PAM68 interacts in split-ubiquitin assays with all major subunits of the core complex as well as with the assembly factors LPA1, HCF136, ALB3 and LPA2. The interaction with PSII proteins and assembly factors that appear at later stages of PSII assembly, and the inefficient accumulation of PSII dimers and supercomplexes might be an indication that the factor is necessary for the later steps of PSII assembly (Armbruster et al. 2010).

The factor ALB3 (Albino 3), defined as one interactor of PAM68, has important functions in the insertion of thylakoid membrane proteins. ALB3 is proposed to play a role as a translocase/insertase by catalyzing the lateral integration of proteins into the lipid bilayer, and it might be involved in folding and assembly of proteins into membrane protein complexes (Yi and Dalbey 2005). Inactivation of the *ALB3* gene leads to severe phenotypes in all photosynthetic organisms characterized by disturbed thylakoid integrity and strongly impaired

viability, indicating the importance of this integral membrane protein (Sundberg et al. 1997). The ALB3 factor is required for cpSRP dependent posttranslational insertion of light harvesting chl a/b binding proteins (Bellafiore et al. 2002; Moore et al. 2003). In addition, it interacts with D1, D2 and CP43 and other integral proteins of the thylakoid membrane (Ossenbühl et al. 2004; Pasch et al. 2005). These observations point to an involvement of ALB3 in co-translational membrane insertion of thylakoid proteins. A modification of the posttranslational cpSRP pathway was suggested to be associated with these cotranslational processes (Schunemann 2004). ALB3 is clearly a general component of the thylakoid protein targeting and integration machinery and its function has been described in recent studies (Richter et al. 2010; Wang and Dalbey 2011).

Recently, evidence was provided that CP43 assembly is organized in a concerted manner by the auxiliary proteins LPA2 and LPA3 (Ma et al. 2007; Cai et al. 2010). The Arabidopsis mutants *lpa2* and *lpa3* are both characterized by reduced CP43 protein synthesis rates and stability, which is paralleled by inefficient assembly of PSII intermediates and supercomplexes. Sequence analysis and biochemical studies showed that LPA2 is an integral thylakoid membrane protein that contains two transmembrane domains. By contrast, LPA3 lacks hydrophobic stretches and its membrane-binding characteristics resemble those of extrinsic proteins associated with the thylakoid membranes. Sequence comparisons revealed that both factors are restricted to green algae and higher plants, indicating that their functional relevance is coupled with the evolution of new requirements in chloroplasts. Yeast two hybrid assays and bimolecular fluorescence complementation indicated that LPA2 and LPA3 interact independently with the PSII core protein CP43 and with each other (Cai et al. 2010). The double mutation of LPA2 and LPA3 is more deleterious for the assembly of PSII than either single mutation. Moreover, the direct interaction of both factors with ALB3 was shown. Altogether, these data suggest the cooperation between LPA2 and LPA3 in assisting CP43 assembly possibly before, during, or after processing by the ALB3 translocase.

2. Assembly of Lumenal Exposed PSII Components: Proteomics Meets PSII Biogenesis

a. Immunophilins Assist in PSII Protein Folding

Analysis of the Arabidopsis thylakoid lumen proteome revealed the localization of several functionally distinct protein groups (Peltier et al. 2002; Schubert et al. 2002). The presence of a large group of isomerases suggested continuous folding and unfolding activity inside this compartment. The majority of these isomerases belong to the group of peptidyl-prolyl isomerases (PPIase), which have the ability to catalyze the cis-trans isomerization of proline-imidic peptide bonds and to accelerate protein folding (Golbik et al. 2005). One of these PPIase families is represented by the so-called immunophilins, originally identified as the cellular targets of immunosuppressant drugs. The immunophilins encompass two ubiquitous protein families: the FK-506 binding proteins or FKBPs, and the cyclosporin-binding proteins or cyclophilins (Romano et al. 2005). Arabidopsis cyclophylin 38 (CYP38) is required for the early steps of thylakoid biogenesis, as reflected by its detection in etiolated seedlings (Kanervo et al. 2008). The protein was originally discovered in spinach and designated TLP40 (*t*hylakoid *l*umen *P*PIase) (Fulgosi et al. 1998). TLP40, which is located primarily in non-appressed membranes, has PPIase activity and regulates the dephosphorylation of thylakoid membrane proteins by binding to a thylakoid protein phosphatase (Vener et al. 1999; Rokka et al. 2000). Arabidopsis *cyp38* mutant plants showed reduced growth and decreased accumulation of PSII, which was shown to originate from disorganized assembly at the donor-side of the complex (Fu et al. 2007; Sirpio et al. 2008). It is hypothesized that AtCYP38 is involved in

proper folding of D1, and possibly also CP43, into the growing PSII complex, thereby enabling the correct assembly of the oxygen evolving complex during PSII biogenesis and maintenance (Sirpio et al. 2008). Interestingly, the C-terminus and the luminal loops of D1, as well as the crucial E-loop of CP43, which are all involved in the ligation of the $CaMn_4$ cluster, contain several highly conserved proline residues (Anderson et al. 2002; Burnap 2004) as putative targets of AtCYP38 (Sirpio et al. 2008).

Knock-out of another immunophilin, FKBP20-2, induced the characteristics of a photosynthesis mutant in Arabidopsis (Lima et al. 2006). Spectroscopic and biochemical studies indicate that FKBP20-2 appears to be required during the association of PSII supercomplexes, although the function of this factor remains unclear.

Recently, it was reported that only two of the 16 luminal immunophilins of Arabidopsis possess peptidyl-prolyl-isomerase activity (Ingelsson et al. 2009). It is therefore suggested that the cellular functions of immunophilins in the thylakoid lumen are not related to their PPIase capacity and should be investigated beyond this enzymatic activity. Further research is required to clarify these uncertainties about the different members of this interesting protein family.

b. Luminal Proteins Required for Efficient Repair of Photodamaged PSII

Two other proteins of the lumen proteome have been identified as PSII repair proteins. The membrane attached AtTLP18.3 is involved in the turnover of damaged D1 proteins and possibly also in PSII complex dimerization (Sirpio et al. 2007). The even distribution of TLP18.3 between stroma and grana thylakoids is correlated with these functions, which are also restricted either to stroma (repair) or grana (dimerization) membranes. The second candidate repair factor belongs to the group of luminal proteins that comprise homologs of PsbP, the structural protein of the water splitting complex in algae and higher plants (Peltier et al. 2002; Schubert et al. 2002). One of these PsbP-like (PPL) proteins is encoded by the nuclear gene *AtPPL1* (Ishihara et al. 2007). AtPPL1 displays only limited sequence identity with PsbP (25%) while it shows higher sequence similarity (33%) to a cyanobacterial PsbP homolog (cyanoP), indicating a closer functional relationship with the latter. Analysis of a *ppl1* T-DNA insertion mutant shows that the PSII of the mutant is more susceptible to light-induced damage than that of the wild type, and the recovery of photoinhibited PSII activity was delayed, suggesting that PPL1 is required for efficient repair of photodamaged PSII (Ishihara et al. 2007). The cyanobacterial homolog of AtPPL1, Sll1418, is reported to have a stabilizing function at the donor side of PSII and to be involved in calcium and chloride association (Sveshnikov et al. 2007). Plants lacking AtPPL1 or AtTLP18.3 are not significantly affected by the deficiency, suggesting a redundancy of proteins that assist the described repair steps.

The factors Psb27, Psb28 and Psb29 were originally identified as substoichiometric components of CP47-His tagged preparations isolated from Synechocystis (Kashino et al. 2002), indicating an auxiliary function of these components. All three proteins are conserved throughout the plant kingdom. The most comprehensive data have been published for Psb27, which is a luminal protein found in a non-oxygen evolving PSII monomer that lacks extrinsic proteins PsbO, PsbU, PsbV (subunit of OEC in cyanobacteria) and the $CaMn_4$ cluster (Mamedov et al. 2007). An Arabidopsis PSB27 loss of function mutant shows that this factor is not essential for oxygenic photosynthesis and PSII formation (Chen et al. 2006). Recent data provide evidence that Psb27 is involved in the assembly of the water-splitting site of PSII during the recovery process after light-induced PSII inactivation (Nowaczyk et al. 2006; Roose and Pakrasi 2008).

Less is known about Psb28, which might bind to CP43-less PSII intermediates in Synechocystis. Knock-out of the gene indicated an involvement in chlorophyll synthesis and/or binding during PSII and also PSI assembly (Dobakova et al. 2009).

The data available on the *psb29* mutants are contradictory. Initial studies of Arabidopsis mutants described the accumulation of membrane vesicles in chloroplasts and altered thylakoid membrane organization (Wang et al. 2004). Later evidence was provided that PSII biogenesis is selectively disturbed in *Psb29* knockout mutants of Synechocystis and Arabidopsis (Keren et al. 2005). Further analysis is necessary to clarify the functional role of this protein.

3. A Periplasmic Factor Restricted to the Cyanobacterial PSII Assembly Machinery

The protein PratA of Synechocystis is a periplasmic TPR protein, necessary for adequate C-terminal processing of the D1 subunit of PSII (Klinkert et al. 2004). A null mutant of PratA that reveals considerably impaired growth rate, also shows residual photosynthetic activity, indicating that the factor is important but not indispensable. Yeast-two-hybrid experiments show that PratA interacts with the C-terminus of mature and immature D1, and residues 314–328 were defined as the binding-site residues (Schottkowski et al. 2009). The PratA protein was detected in two different complexes. Soluble PratA is part of a high molecular mass complex of approximately 200 kDa that is independent from the D1 protein. By contrast, membrane associated PratA was found in smaller complexes (70 kDa) that depend on the presence of D1, and these assemblies may represent early PSII assembly intermediates consisting at least of D1 and PratA (Schottkowski et al. 2009). Whether the C-terminal processing protease of D1, CtpA, is supported by this complex or PratA alone is unclear. The localization of the PSII assembly factor PratA to the periplasm supports the assumption that the initial steps of PSII assembly proceed in the cytoplasmic membrane or at least in a specific connected membrane compartment. Sucrose density gradient centrifugation showed that the membrane associated 70 kDa PratA complex is localized to a specific subfraction of membranes, which might represent a transfer and/or connecting region between plasma and thylakoid membrane (Schottkowski et al. 2009). The substantial accumulation of pD1 in this membrane fraction in the absence of PratA supports the current model that the initial steps of PSII biogenesis take place in specific membrane compartments in cyanobacteria (cf. Sect. V).

Although a PratA homologous protein is missing in algae and higher plants, the existence of a functional relative is possible. Recently, evidence was provided that a Psb27 homolog in Arabidopsis termed LPA19 facilitates D1 precursor processing during biogenesis of PSII (Wei et al. 2010). This factor is localized to the lumenal compartment and interacts with the C-terminus of D1. Mutants lacking the LPA19 protein grow poorly, especially under high light conditions, and show inefficient D1 maturation. Further precise analysis of the LPA19 protein and the corresponding mutant phenotype may give more information about the exact functional role of this factor in PSII assembly of higher plants.

V. A Spatiotemporal Pathway Is Involved in PSII Biogenesis

A widely accepted model suggests that chloroplast-encoded photosynthetic membrane proteins are synthesized by membrane bound polysomes (Chua et al. 1973; Margulies 1983; Klein et al. 1988; Zhang et al. 1999). Membrane fractionation experiments suggested that stroma thylakoids are the primary site for these processes in higher plants (Yamamoto et al. 1981; van Wijk et al. 1995). More recent studies in Chlamydomonas proposed that thylakoid membrane biogenesis

is initiated in the inner envelope membrane of the chloroplast (Zerges and Rochaix 1998; Zerges et al. 2002) but clear-cut statements were complicated by the impurity of membrane preparations. Recent data obtained using fluorescence confocal microscopy show that synthesis of PSII components and complex assembly processes are highly compartmentalized in Chlamydomonas (Uniacke and Zerges 2007). Under conditions that induce de novo synthesis of PSII, mRNAs encoding PSII core proteins (*psbA*, *psbC*) and the translation factor RB38 colocalize around the pyrenoid. This region contains specific thylakoid membranes and is termed the T Zone (T for translation). In two PSII assembly mutants, unassembled D1 and incompletely assembled PSII complexes colocalized inside the T zone. These observations indicate that the early steps of PSII assembly occur in these regions and that a quality control system enables only assembled PSII complexes to leave the T zone and enter photosynthetically active thylakoid membranes (Uniacke and Zerges 2007). To investigate the mechanism targeting mRNA to the T zone, Chlamydomonas cells were treated with the translational inhibitor lincomycin, which removes ribosomes and nascent polypeptide chains from the mRNA. The results of this study suggested that an mRNA-based targeting mechanism is involved in T zone association of the *psbA* transcript and subsequent de novo synthesis of the D1 protein (Uniacke and Zerges 2009). The auxiliary factors RB60 and RBP63 may be involved in this membrane tethering of the *psbA* mRNA based on the partitioning of these factors between the soluble stroma and a chloroplast membrane fraction, which is consistent with cycling between these two locations (Trebitsh et al. 2001; Ossenbühl et al. 2002).

Emphasis has also been placed on the investigation of initial steps of thylakoid membrane assembly in cyanobacteria. The original studies proposed that the plasma membrane, and not the thylakoid membrane, is the site for early steps of biogenesis of the photosynthetic reaction center complexes in cyanobacterial cells (Zak et al. 2001). However, the current model assumes that protein synthesis/assembly and chlorophyll synthesis/insertion are localized to a specialized membrane subfraction, representing a biosynthetic center associated with both plasma and thylakoid membranes (Nickelsen et al. 2011). These membrane compartments are termed PDMs (*P*rat A *d*efined *m*embranes) and they are marked by the location of the PSII biogenesis factor Prat A (Schottkowski et al. 2009). PDMs might be identical to the so-called convergence sites visualized by electron micrography (van de Meene et al. 2006). PSII biogenesis is suggested to begin at PDMs and progress during trafficking into the thylakoids. This localized biogenesis requires an efficient membrane flow from regions of initial assembly into the thylakoids. Contact sites between the PDMs, periplasmic and thylakoid membranes are suggested to ensure an internal exchange between these membrane systems of cyanobacteria.

The formation of thylakoid membranes during the development of proplastids into mature chloroplasts of higher plants is still highly elusive. The site of de novo protein synthesis of PSII and the other photosynthetic membrane complexes in proplastids and chloroplasts is not known. Moreover, several lines of evidence suggest that a vesicular transport system is involved in membrane thylakoid biogenesis (White and Hoober 1994; Kroll et al. 2001). Interestingly, proteins involved in vesicle formation or membrane fusion of vesicles, such as Vipp1 (*v*esicle *i*nducing *p*rotein in *p*lastids) and the dynamin-like GTPase FZL (*f*uzzy onions-*l*ike) are detected in two suborganellar locations (envelope and thylakoids), which is consistent with a trafficking function for these proteins (Kroll et al. 2001). However, which specific cargo is transported through this pathway is unclear. It is widely accepted that vesicles are necessary for the transport of lipids, whose synthesis proceeds at the inner envelope membrane (Douce 1974; Dormann and Benning 2002). However, the possible presence of non-lipid components such as photosynthetic protein complexes as part of the cargo is not clear.

An alternative to this vesicle transport system is the lateral migration of newly assembled PSII complexes from the site of origin to thylakoids with photosynthetic activity. These lateral movements are typically associated with the PSII repair cycle in mature thylakoids (Goral et al. 2010).

VI. Conclusions and Perspectives

Biogenesis of PSII in the chloroplast compartment depends on numerous auxiliary protein factors encoded by the nuclear genome of plants. The members of this highly diverse set of protein families impinge on all aspects of the biogenesis process. PSII biogenesis is integrated into the dynamic network between chloroplasts and their surrounding cytoplasm and is influenced by the developmental program and external growth conditions. The expression of plastid-encoded PSII subunits is predominantly controlled at the posttranscriptional level. Many factors regulate the fate and translational efficiency of specific transcripts by direct or indirect interactions with RNA 5′UTRs. Redox-regulation plays an important role during these processes. The availability and assembly of specific PSII core complex subunits regulates the formation and accumulation of the protein complex. Assembly factors are surprisingly different and are involved in protein processing, cofactor binding, and membrane insertion of proteins, as well as in the coordination of sequential or simultaneous steps of the assembly process. The function of some assembly factors seems to be essential, but many of them function as modulators or optimizers of certain assembly steps and obviously the assembly machinery can compensate for their loss of function.

Although significant progress has been made in understanding the complex processes involved in the biogenesis of PSII, the mechanistic and regulatory aspects of individual steps are largely unknown. Most, if not all, of the auxiliary factors identified so far are probably organized into high molecular weight complexes and these units are able to respond to environmental and developmental signals. The discovery of the individual proteins comprising such a complex is expected to shed light on the regulatory mechanisms and enzymatic activities involved. The improvement and application of sensitive techniques in molecular biology, biochemistry and biophysics will permit the purification of known factors and identification of their associated partners. A genetic approach to the analysis of allelic series might contribute to the identification and functional analysis of unknown protein domains involved in the diverse activities taking place during the biogenesis of PSII.

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Chapter 5

Organization and Assembly of Photosystem I

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Summary

Photosystem I (PSI), one of the two photosystems that drive electron transfer in oxygenic photosynthesis, is a very large pigment-protein complex with more than 100 cofactors. Crystal structure at 3.3 Å in higher plants, and that at 2.5 Å in cyanobacteria, gives us a detailed image of proteins and binding cofactors. However, most of the accumulated information is regarded as a snapshot of PSI. In spite of its general static image, components of PSI must be newly synthesized and assembled during initial synthesis, acclimation

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processes, and recovery from photoinhibition. Some factors required for such processes are common between higher plants, algae and cyanobacteria, but others are not. For the true comprehension of PSI, it is important to understand the dynamic nature of PSI including assembly or degradation. In this chapter, "four Ws and one H" of the PSI assembly are summarized.

I. Introduction

One of the definitions of living organisms is the ability to undergo metabolic changes. Proteins in the organisms are continuously synthesized, assembled to complexes in some cases, and degraded in the end. The photosynthetic machinery is no exception (Wollman et al. 1999; Kanervo et al. 2007). One of the well-known examples of proteins with high turnover rate is the D1 protein of photosystem II (PSII) in photosynthetic organisms. The dynamics of PSII has been extensively studied since the 1980s (Aro et al. 1993). Compared to PSII, the turnover rate of the other photosystem, photosystem I (PSI), is much slower, at least in mature leaves of higher plants, and the physiological significance of PSI turnover has been slighted in the past. Together with the technical difficulty to detect the turnover of PSI, dynamic aspects of PSI could not be regarded as a topic that should be enthusiastically studied.

The turnover of PSI is, however, very important in many steps during the life cycle of photosynthetic organisms. First, PSI should be actively synthesized and assembled in the process of greening, even if the turnover rate of PSI is very low in mature leaves of higher plants. In the case of microalgae including cyanobacteria, PSI should be constantly synthesized for their growth, since the growth rate of microalgae is solely determined by the rate of cell proliferation. Thus, several studies on PSI assembly have been carried out in a green alga *Chlamydomonas reinhardtii* with great success (Ozawa et al. 2010). Secondly, in spite of its static image, PSI is a regulatory target during acclimation to environmental changes, and its content could drastically increase or decrease upon the change of, for example, photon flux density or light quality (Hihara and Sonoike 2001; Muramatsu and Hihara 2012). Finally, PSI could be the site of damage under photoinhibitory conditions (Sonoike 2011). Photoinhibition of PSI is largely irreversible, and most of the inactivated PSI would be selectively degraded. Some of the PSI would be re-synthesized and newly assembled. Thus, synthesis and assembling are carried out even in mature leaves under such conditions. The PSI reaction center complex is a very large pigment-protein complex with many subunits and with more than 100 cofactors (Fromme and Grotjohann 2006; Nelson and Ben-Shem 2006). To place these proteins and factors into proper position, each component may assemble into the final complex one by one or may assemble into sub-complexes, which were finally integrated into mature complexes. In this review, assembly of PSI is discussed from the view points of "what", "where", "how" and "why".

Abbreviations: A_0 A_1 – Primary and secondary electron acceptors in PSI; CTAB – Cetyltrimethylammonium bromide; DPOR – Light-independent chlorophyll reductase; F_X F_A, and F_B – The terminal electron acceptors in PSI; LHCI – Light harvesting complex of photosystem I; LHCII – Light harvesting complex of photosystem II; LPOR – Light-dependent protochlorophyllide reductase; P700 – Primary electron donor of PSI; PSI – Photosystem I; PSII – Photosystem II; SDS – Sodium dodecyl sulfate

II. What Is Assembled into Photosystem I (PSI) Complex?

A. General Outline of PSI Structure

PSI core complexes in plants exist as monomers in vivo with the peripheral light-harvesting complex (LHCI) around one side of PSI (Nelson and Ben-Shem 2006). LHCI is

composed of four subunits, Lhca1–Lhca4, belonging to the chlorophyll *a*/*b* type of antenna protein family (Croce et al. 2006). Stoichiometry of LHCI and core complex is usually constant in higher plants but the stoichiometry was reported to change under different light conditions in red algae, which contain both LHCI and phycobilisomes as antenna systems (Tan et al. 1995). LHCI and PSI sub-complexes are first formed independently, and then assembled with each other at least in the green alga *Chlamydomonas reinhardtii*, in which LHCI could be assembled even in the mutant lacking the PSI core (Wollman and Bennoun 1982; Takahashi et al. 2004). In the case of *Arabidopsis thaliana*, the mutants lacking each of the four LHCI subunits showed assembly of remaining LHCI subunits to the PSI complex (Wientjes et al. 2009).

Although LHCI is the antenna chlorophyll protein complex specific to PSI, LHCII, the antenna complex serving for PSII, could also serve for PSI as antenna under particular condition. In the process called state transition, LHCII moves from PSII to PSI or from PSI to PSII upon changes in environmental light intensity and/or light quality (Minagawa 2011). Since PSI and LHCII form a complex under state-II (i.e. under the light preferentially exciting PSII) condition (Takahashi et al. 2006), LHCII could also be regarded as a component of PSI in a sense. In the case of red algae and cyanobacteria, phycobilisomes, the large extrinsic antenna complex of phycobiliproteins serving as PSII antenna, could also serve for PSI under state-II conditions (Murata 1969; Mullineaux and Emlyn-Jones 2005). Interestingly, two types of phycobilisomes were identified in *Synechocystis* sp. PCC 6803 (Kondo et al. 2005), and one of them is reported as specific antenna complex for PSI (Kondo et al. 2007). This specific antenna, CpcG2-phycobilisome, could be also regarded as a component of PSI. Along those similar lines, it was reported that PSI forms supercomplexes with the NADH dehydrogenase (NDH) complex (Peng et al. 2008) or with the cytochrome b_6f complex (Iwai et al. 2010). However, these "potential" components of PSI are not handled in this review.

PSI complexes in cyanobacteria usually exist in trimeric form (Shubin et al. 1993), although other forms of PSI such as tetramers were reported in specific species of cyanobacteria (Watanabe et al. 2011). Cyanobacterial PSI comprises only the core part of the reaction center complexes without LHCI, but many cyanobacteria develop stress-induced *isiA* antenna rings under stress conditions, e.g. iron deficiency (Barber et al. 2006). The number of IsiA proteins in the antenna ring was reported to be variable depending on the length of the circumference of the core complexes (Kouril et al. 2003). Thus, only the PSI core must be assembled in the first place, and each IsiA protein binds to the PSI core complexes upon stress. Since one PSI complex contains 12 iron atoms for 3 iron-sulfur centers serving as electron acceptors, the relative PSI content tends to decrease under iron deficiency. Under such conditions, the IsiA ring would serve as additional antenna for highly efficient trapping of light energy (Chauhan et al. 2011) as well as for protection of PSI (Sandström et al. 2001).

B. Biochemical and Genetic Information on the PSI Reaction Center Complex

Although fractionating the PSI reaction center complex was considered to be more difficult compared to PSII (Amunts et al. 2010), the purification of PSI sub-complexes and reconstitution of subunits are possible. One of the earliest analyses of the subunit composition of PSI complexes was carried out with Swiss chard by Nelson's group (Bengis and Nelson 1975), who published the first crystal structure of plant PSI (Ben-Shem et al. 2003). Biochemical analysis revealed that the PSI complexes consist of two large subunits with apparent electrophoretic mobility of 60–65 kDa, and many small subunits in higher plants (Bengis and Nelson 1975, 1977; Thornber et al. 1977) as well as in cyanobacteria (Takahashi et al. 1982; Vierling and Alberte 1983). The large subunits, encoded by *psaA* and *psaB* genes, bind chlorophylls and P700, a reaction center chlorophyll dimer, together with A_0, A_1 and F_X, the electron acceptors of PSI. The minimal complex

with P700 activity is the hetero-dimer of PsaA/B sub-fractionated from cyanobacterial PSI complexes (Takahashi et al. 1982). The PsaA/B hetero-dimer complex could be also isolated from spinach by the use of chaotropic reagents, though the stability of the complex was lower compared with the case of cyanobacteria (Parrett et al. 1989). Although PsaA/B hetero-dimer could be further disassembled into a single subunit chlorophyll a-protein, P700 activity was lost in that preparation (Sonoike and Katoh 1986).

In the early stages of the PSI research, treatment with detergents was the major approach to examine the PSI structure. In cyanobacteria PSI, PsaF, L, K, X and J could be removed by a cationic detergent CTAB, while PsaF, E, X and J (and PsaL and PsaK in some cases) could be removed by the treatment with an anionic detergent SDS (Hatanaka et al. 1993). On the other hand, heat treatment in the presence of ethylene glycol removes all the small subunits with the exception of PsaK in higher plants (Hoshina et al. 1989). By these experiments, some sets of subunits, for example, PsaF, X and J were found to act in a group, suggesting their proximal location in the complex. However, this kind of interpretation of data is not always reliable, and not so much structural information could be drawn from those experiments.

Later, genetic approaches became more common, and analysis of the subunit composition of PSI isolated from the mutants of specific subunits has been carried out. For *Arabidopsis thaliana*, knockout or suppression mutants were created for almost all the PSI subunits (Jensen et al. 2007). The situation is similar for the green alga *Chlamydomonas reinhardtii* (Hippler et al. 2002) or for cyanobacteria. In one of the earliest experiments, PSI complex isolated from the mutant lacking PsaC subunit showed destabilized PsaD, PsaE, and PsaL in *Anabaena variabilis* ATCC29413 (Mannan et al. 1994). From the structural point of view, it is generally observed that lack of PsaA and PsaB results in the strongest phenotype with loss of the PSI complex, while lack of PsaC results in the large perturbations at the stromal side. For other subunits, the structural modification is more or less smaller compared with PsaA, B and C. Since maturation of PsaB mRNA in *Chlamydomonas reinhardtii* requires several factors, the mutants of those factors showed a PsaB-less phenotype, resulting in the total loss of the PSI core complex (Takahashi et al. 2004). In the case of *Arabidopsis thaliana*, however, the mutant of ATAB2, an ortholog of Tab2 working in translation of PsaB in *Chlamydomonas reinhardtii*, showed an albino phenotype, and not only PSI subunits were missing, but also the levels of PSII subunits were largely decreased (Barneche et al. 2006). Generally speaking, the effect of deletion of homologous genes is most prominent in higher plants and relatively small in cyanobacteria with intermediate effect in *Chlamydomonas reinhardtii*.

C. Crystal Structure Information on the PSI Reaction Center Complex

The PSI reaction center complexes were purified from a thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1, and the first crystal structure was reported in 1993 at the resolution of 6 Å (Krauß et al. 1993). The resolution was improved to 4 Å (Krauß et al. 1996), and then 2.5 Å (Jordan et al. 2001). The first report on plant PSI crystal structure appeared in 2003 at 4.4 Å resolution for *Pisum sativum* (Ben-Shem et al. 2003). The resolution has been increased to 3.4 Å (Amunts et al. 2007) and then to 3.3 Å (Amunts et al. 2010). PsaA, PsaB, PsaC, PsaD, PsaE, PsaF, PsaI, PsaJ, PsaK and PsaL consist of the core part of the reaction center complex, and are common to plant and cyanobacterial PSI. Plant PSI additionally contains PsaG, PsaH and PsaO (Knoetzel et al. 2002). Although PsaP was identified in *Arabidopsis thaliana* (Khrouchtchova et al. 2005), it could not be found in the crystal structure. On the other hand, an electron density was observed at the PsaK side of the complex in the crystal structure and tentatively named PsaR (Amunts et al. 2010).

Our past understanding of the structure and function of PSI is improved using the crystal structure as a datum point, and the crystal

structure information is now a universal basis for the studies of PSI. However, it is rather hard to understand the dynamic aspects of PSI, such as the assembling process, solely from the structural information. Analysis of the amino acid contacts between protein subunits in plant PSI crystal structure revealed that the PsaA/PsaB heterodimer is connected with other subunits through extensive interfacial contacts (Amunts et al. 2010). Thus, it is very difficult to estimate the order of assembly of the subunits from crystal structure information alone. More biochemical and genetic information are apparently necessary for the true understanding of the dynamic aspect of PSI.

D. Cofactors

P700, the reaction center chlorophylls of PSI, is the special pair (hetero-dimer) of chlorophyll *a* and chlorophyll *a*', which is an isomer of chlorophyll *a* (Watanabe et al. 1985; Jordan et al. 2001). Upon photooxidation of P700, electrons are transferred to A_0 (chlorophyll *a*), A_1 (naphthoquinone), F_X and finally to F_A and F_B. The last three components are iron-sulfur clusters of [4Fe4S]. The three subunits, PsaA, PsaB and PsaC, bind all the PSI electron transfer components from P700 to F_A/F_B. Other subunits do not bind any electron transfer components, but some of them have been shown to promote the transfer of electrons to the electron carriers outside of PSI complexes. For example, the PsaD subunit promotes electron transfer from F_A/F_B to ferredoxin (Zanetti and Merati 1987; Zilber and Malkin 1988) together with the PsaE subunit (Andersen et al. 1992; Sonoike et al. 1993). The PsaF subunit promotes electron transfer from plastocyanin to P700 (Wynn and Malkin 1988). The PsaC subunit was shown to be an iron-sulfur protein that binds the terminal electron acceptors, F_A/F_B (Hayashida et al. 1987; Høj et al. 1987; Oh-oka et al. 1987). The role of PsaC as F_A/F_B binding protein was elegantly revealed by the reconstitution experiments (Parrett et al. 1990).

As photosynthetic pigments, cyanobacterial PSI contains 96 chlorophylls and 22 carotenoids (Jordan et al. 2001). Together with 6 chlorophylls possibly working for electron transfer, 79 antenna chlorophylls bind to PsaA/B subunits, but other 11 chlorophylls are coordinated to PsaJ, PsaK, PsaL, PsaM, PsaX as well as to lipid molecules. All the carotenoids are in van der Waals contact with chlorophyll a head groups, thus enabling their protective role as a triplet quencher. Most of the carotenoids are assumed to be β-carotene. On the other hand, plant PSI model at 3.3-Å resolution includes 173 chlorophylls and 15 β-carotenes (Amunts et al. 2010). The position of the chlorophylls in the core part of plant PSI is more or less similar to that of cyanobacterial PSI, and chlorophylls specific to plant PSI are present outside of the core part. There are 4 LHCI subunits, and each subunit binds 14 chlorophylls. Additionally, there are about 10 gap chlorophylls located between core and LHCI (Amunts et al. 2007, 2010). It must be emphasized that PSI is a chlorophyll-rich photosystem. The "concentration" of chlorophylls in the PSI complex is much higher than PSII. Moreover, PSI is also an Fe-rich photosystem complex due to its [4Fe4S] clusters serving for electron acceptors. These features are particularly prominent in cyanobacteria, in which about 90% of the chlorophylls in cells are binding to PSI. In higher plants, chlorophylls are distributed almost equally to PSI and PSII.

III. What Helps the Assembly of PSI Complex?

A. General Outline of the Assembly

Although malfunction of many proteins has resulted in the decrease of the PSI content, those proteins could not be regarded as assembly factors simply from such phenotype. PSI content could be affected by many steps other than the defects in assembly process. First, the relative decrease in PSI content (decrease in PSI/PSII ratio) does not necessarily mean a substantial decrease in PSI content. Secondly, PSI content is one of the main targets of light acclimation and is regulated in photosynthetic organisms upon the changes in light intensity or quality. The defects in such regulatory mechanisms

may lead to the decrease or increase of PSI content (Hihara and Sonoike 2001). Thirdly, defects in transcription or translation of genes encoding any PSI subunit, especially the chlorophyll binding PsaA/B subunits, may lead to a decreased accumulation of the PSI complex. This case includes some mutants of PSI subunits themselves as well as the mutants of components that are necessary for the transcription or post-transcriptional regulation of PSI genes. For example, the mutants of the genes required for the processing of the *psaB* gene in *Chlamydomonas reinhardtii* showed PSI-less phenotype (Rochaix 2006). Furthermore, the accumulation of the PSI proteins depends on the presence of chlorophylls or phylloquinones, so that a defect in any enzyme in the biosynthesis pathway of such components may also lead to a decrease in PSI content. Although these factors are essential for the normal accumulation of PSI, these are not regarded as assembly factors.

Several factors were reported to be responsible for the assembly of PSI complex, based on the facts that (1) PSI content decreased in the absence of the factor, and (2) the messenger level of PSI genes were not affected under the same condition. In some cases, it was also demonstrated that (3) the factor was present in a specific stage of the PSI complex, and (4) the factor was not present in mature PSI complex. Those factors, that could be called assembly factors of PSI, are briefly commented below. Although the specific scaffold proteins involved in insertion of the cofactors such as iron-sulfur centers could be also categorized into assembly factors (Shen and Golbeck 2006), those are not handled in this review.

B. Ycf3

Ycf3 is a protein conserved in cyanobacteria, algae and higher plants with three tetratricopeptide repeats (TPR) presumably functioning in protein-protein interaction. In the *ycf3* mutant of tobacco, PSI subunits (PsaC, D, F) were not detected at all in spite of the normal transcription of PSI genes (Ruf et al. 1997). Since initiation of the translation of PSI transcripts is normal, Ycf3 was concluded to be an assembly factor of PSI. In *Chlamydomonas reinhardtii*, *ycf3* forms a transcription unit with *ycf4*. It was possible to inactivate *ycf3* or *ycf4* independently, both resulting in the lack of PSI subunits, but with normal expression of PSI genes (Boudreau et al. 1997). In *Chlamydomonas reinhardtii*, Ycf3 and Ycf4 were present on the surface of thylakoid membranes, and could accumulate in PSI-less mutants. The amounts of Ycf3 and Ycf4 are significantly reduced in etiolated cells. The examination of point mutants of *ycf3* in *Chlamydomonas reinhardtii*, which showed moderate decrease of PSI, revealed that (1) there was interaction of Ycf3 and PSI subunits, (2) the activity of the assembled PSI was normal, (3) the degradation rate of PSI was normal, and (4) the PSI content decreased upon temperature shift, demonstrating that Ycf3 also plays an important role in the assembly of PSI in Chlamydomonas (Naver et al. 2001).

There are several factors that may have some interaction with Ycf3 in *Arabidopsis thaliana*. There is a transcription unit encoding *psaA-psaB-rps14* downstream of the *ycf3* gene. It was reported that the mutant HCF145 with PSI-less phenotype showed a decrease in the levels of the *psaA-psaB-rps14* transcript (Lezhneva and Meurer 2004). However, expression of *ycf3* gene was rather increased, suggesting that Ycf3 and HCF145 could independently affect PSI assembly. Another protein, Y3IP1 (Ycf3-interacting protein 1) was reported for *Nicotiana tabacum* and *Arabidopsis thaliana*. The mutant lacking Y3IP1 showed slow growth and decreased content of PSI, on the chlorophyll basis, to 1/2–1/4 (Albus et al. 2010). Since the amount of Ycf3 was not decreased in this mutant, these two factors could work independently. Another factor is OTP51, which plays some role in the splicing of the *ycf3* intron. The Arabidopsis mutant of OTP51 lacked PSI, PSII and LHCI, and had only LHCII (de Longevialle et al. 2008). This very severe phenotype might be ascribed to the role of OTP51 in the splicing of other introns than that of *ycf3*. It must be noted that

these mutants of PSI assembly factors (e.g. HCF145 or Ycf3) also showed low Fv/Fm, an index of PSII efficiency.

C. Ycf4

The Ycf4 protein is another protein conserved in cyanobacteria, algae and higher plants. The understanding of the role of Ycf4 in PSI assembly recently advanced in *Chlamydomonas reinhardtii*. When either one or a combination of the conserved amino acid residues R120, E179 and E181 of Ycf4 were replaced by alanine or glutamic acid, the quantity of PSI or Ycf4 varied depending on the mutation (Ohnishi and Takahashi 2009). In a particular case, amounts of PSI did not change in the presence of only 20% of Ycf4, suggesting that PSI amount is not regulated by the amount of Ycf4. Although the amount of the Ycf4 protein in the E179/181Q was wild-type level, PSI was not detected in this mutant, suggesting that the E179 and E181 are essential for the function of Ycf4. In this mutant, the 150–170 kDa subcomplex of PSI was detected.

Ycf4-containing complexes could be isolated from a Ycf4-tagged strain of *Chlamydomonas reinhardtii*. This complex was found to contain Ycf4, COP2, PsaA, B, C, D, E and F (Ozawa et al. 2009). COP2 is an opsin-related protein and its role in Chlamydomonas is currently unknown. Although COP2 was co-purified both from Ycf4-containing complexes and PSI complexes, PSI accumulation was not affected by the decrease of COP2 to 10%. Pulse labeling experiments showed that the incorporation of the label into the complexes is quite rapid; both the Ycf4 complex and the PSI complex were labeled in 5 min.

D. Ycf37

Ycf37 is also an assembly factor of PSI. This protein contains three tetratrico-peptide repeats just like Ycf3, and is conserved in cyanobacteria as well as in some algal chloroplasts. A *ycf37* mutant in *Synechocystis* sp. PCC6803 showed decreased PSI content with normal content of PSII (Wilde et al. 2001). Although Ycf37 could not be co-purified with PSI, at least by anion exchange chromatography presumably due to dissociation by the use of detergent, the affinity-purification experiments revealed the interaction of Ycf37 with PsaA, B, C and D (Dühring et al. 2006). Interestingly, the sub-complex of PSI lacking PsaK was not found in the *ycf37* mutant (Dühring et al. 2006). Further analysis showed that three kinds of PSI monomers were present in the wild type of the Synechocystis cells, i.e. full monomer, PsaK-less monomer and PsaK/PsaL-less monomer. The latter two partial complexes were not observed in the *ycf37* mutant (Dühring et al. 2007). The Ycf37 may contribute to the stability of the transient complex during the assembling process in cyanobacteria.

Arabidopsis thaliana and *Chlamydomonas reinhardtii* have a homolog of *ycf37* in the nuclear genome. The thylakoid proteome experiments of Arabidopsis revealed that the amount of Ycf37 homolog increased by high light treatment for 5 days (Giacomelli et al. 2006). The Arabidopsis mutant of the *ycf37* homolog was small and yellow and named *pyg7* (*pale yellow green*). The mutant showed small differences in the transcript level of photosynthesis genes and normal synthesis of PSI reaction center proteins (PsaA/B) but no accumulation of the PSI complex (Stöckel et al. 2006). PSII and Lhca1, 2, 4 proteins were present but the content of the Lhca3 protein was decreased. The Arabidopsis Ycf37 was shown to co-migrate with PSI complexes, suggesting its role in the PSI assembling process.

IV. Where Does the Assembly Process of PSI Take Place?

It is generally considered that most of the assembly process of the photosynthetic complexes takes place in thylakoid membranes immediately after translation in the case of algae and higher plants. For the

pigment protein complexes such as PSI and PSII, absorbed light energy might be harmful in the absence of proper function of the reaction centers. Thus, the photosystems should be assembled in accordance with the development of electron transfer activity, not only of their own but also of other components in the electron transport chain. In that sense, it is reasonable that the assembly process takes place in thylakoid membranes, which are the place of electron transfer systems. Actually, the assembly factors mentioned above, i.e. Ycf3, Ycf4 (Boudreau 1997) and Ycf37 (Stöckel et al. 2006), were reported to be present in thylakoid membrane fractions from *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*.

In the case of the cyanobacterium *Synechocystis* sp. PCC 6803, however, membrane fractionation studies suggested that the initial steps of biogenesis of both photosystems occur in plasma membranes (Zak et al. 2001). Immunoblot analysis showed that the purified plasma membrane contains PsaA, B, C and D, the core reaction center subunits of PSI. The plasma membrane-localized core complexes contained chlorophyll molecules and could perform light-induced charge separations. The PSI assembly factors Ycf3 and Ycf4 were also shown to be localized in plasma membranes (Zak et al. 2001). When plasma membranes were fractionated into the right-side-out and inside-out vesicles, the integral proteins of photosystems including PsaD were enriched in inside-out vesicles (Srivastava et al. 2006). This indicates that the discrete regions of the plasma membranes harbor sites for biogenesis of photosystems in cyanobacteria. Currently, it is proposed that the early biogenesis of cyanobacterial photosystems is performed at the "thylakoid centers" previously described by van de Meene et al. (2006), which are located at the cell periphery, with contact to both plasma and thylakoid membranes (Nickelsen et al. 2011). There is one cyanobacterial factor called BtpA, which seems to contribute to the stability of PSI (Bartsevich and Pakrasi 1997). Since BtpA (Zak et al. 1999), as well as the late assembly factor Ycf37 (Dühring et al. 2005), are localized in thylakoid membranes in cyanobacteria, later steps of PSI assembly seem to take place in thylakoid membranes in cyanobacteria as well as in eukaryotes.

V. How Is the PSI Complex Assembled?

For the detailed elucidation of the assembly process of PSI, the genetic approach is not enough and a direct biochemical approach is necessary. Thus far, only a few groups have been successful in the difficult task such as isolation of intermediate complexes during assembly of PSI. One excellent example is the work of Ozawa et al. (2010), who detected PSI intermediate complexes by pulse labeling of *Chlamydomonas reinhardtii* cells in logarithmic phase (Ozawa et al. 2010). Separation by sucrose density gradient centrifugation revealed that the transient form of the PSI complex lacking PsaG and PsaK was labeled by the short (1 min) labeling, while the label moved to the mature PSI complex after 2 h. LHCI was not detected in the transient form of PSI complexes, at least after isolation. The rate of $NADP^+$ photoreduction by the transient PSI complexes was intact while the re-reduction of P700 became slow possibly due to the partial loss of PsaF. From these results, Ozawa et al. (2010) proposed a working hypothesis of the PSI assembly in Chlamydomonas. According to this hypothesis, the subunits of PSI are incorporated into the complex in the order of (1) PsaB, (2) PsaA, (3) PsaC, D, E, H, L, I, (4) PsaF, J, O, (5) LHCI subunits, and finally, (6) PsaK, G, N, R (Fig. 5.1).

As for cyanobacterial PSI, three kinds of monomeric PSI complexes, namely full monomer, PsaK-less monomer and PsaK/PsaL-less monomer, could be isolated in addition to the trimeric complex of PSI (Dühring et al. 2007). The PsaK- and PsaK/L-less monomers are the transient forms of the PSI complex during assembly, since they were mainly labeled by the short (20 min) labeling, while the label moved to the mature

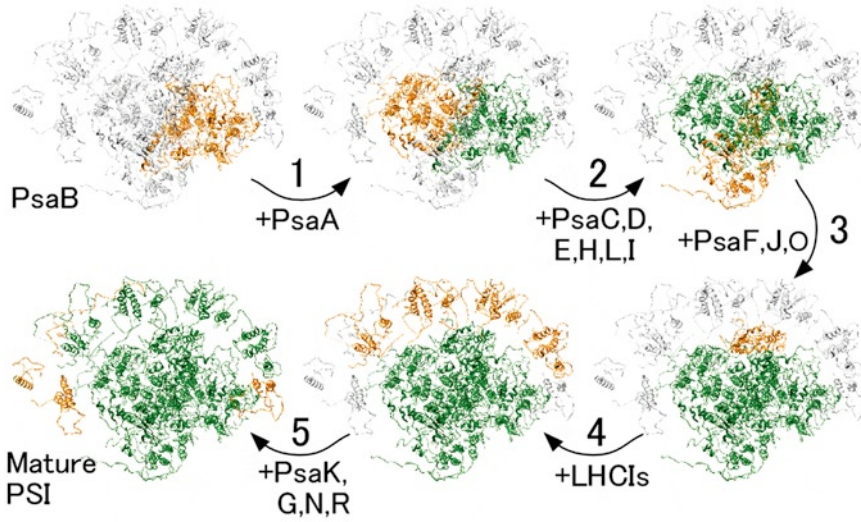

Fig. 5.1. Hypothetical five steps of plant PSI assembly based on Ozawa et al. (2010) expressed by the crystal structure (PDB ID: 3LW5) reported by Amunts et al. (2010). PsaO, which was not found in the crystal structure, might be assembled in step 3. A putative PSI subunit in the crystal structure, PsaR, is assumed to be assembled in step 5, because of its location near PsaK. The newly assembled subunits in each step were colored by *green*, while the subunits already assembled are colored by *orange*.

PSI after 4 h. Apparently, PsaL is assembled in a relatively early stage in eukaryotes, while in the late stage in prokaryotes. On the other hand, PsaK is one of the last subunits assembled into the PSI complex, both in eukaryotes and prokaryotes. PsaK was speculated to be involved in antenna function of PSI based on the parallel loss of LHCI and PsaK under iron deficiency condition in *Chlamydomonas reinhardtii* (Moseley et al. 2002). On the other hand, there are two *psaK* genes, i.e. *psaK1* and *psaK2*, in *Synechocystis* sp. PCC6803. *psaK2* is the only gene that is up-regulated under high light conditions among PSI genes (Hihara et al. 2001), and PsaK2 in Synechocystis is incorporated into the PSI complex promoting the high light-induced state transition, i.e. regulation of energy flow from phycobilisome antenna to PSI core (Fujimori et al. 2005). Thus, PsaK is functioning in the antenna-core relationship both in prokaryotes and eukaryotes. It could be speculated that the adjustment of the antenna-core relationship is the last step of PSI assembly in both types of organisms, although the antenna systems are totally different between chlorophyll *b* containing higher plants/green algae and phycobilisome containing cyanobacteria.

VI. Under Which Circumstance the Assembly of PSI Complex Is Necessary?

A. Greening and Initial Synthesis

In mature leaves of higher plants, the turnover rate of PSI complexes is very slow, so that it is hard to analyze the assembly process and to detect assembly intermediates of PSI. Upon the illumination of the dark-grown plants, however, rapid synthesis of chlorophyll (greening) takes place and PSI proteins as well as PSII proteins are synthesized and assembled into complexes. There are only few studies focused on the PSI accumulation during the greening process. It seems that PSI appeared a bit earlier than PSII but the PSI/PSII ratio is more or less constant during the later stage of the greening (Ohashi et al. 1992).

In contrast to angiosperms having only light-dependent protochlorophyllide reductase

(LPOR), other chlorophyll *a*-containing organisms have light-independent protochlorophyllide reductase (DPOR) as well as LPOR, and thus can synthesize chlorophyll in the dark (Reinbothe et al. 2010). In these organisms, PSI must be continuously synthesized and assembled especially during the active growth phase. It was actually shown that intermediate PSI sub-complexes during assembly exist only in the logarithmic phase, but not in the stationary phase in *Chlamydomonas reinhardtii* (Ozawa et al. 2010).

Kada et al. (2003) observed the etiolation process in cyanobacteria using the *chlL* mutant of *Plectonema boryanum* lacking one subunit of DPOR. In this mutant, chlorophyll synthesis is light-dependent as in angiosperms. When the mutant cells were cultivated heterotrophically in the dark, the chlorophyll content decreased to less than 0.5% of the original level. In parallel to this, a decrease in the PSI activity and the amounts of PSI subunits, PsaA/PsaB and PsaC, was observed. In contrast, PSII was maintained to a significant extent in terms of activity and protein levels until a late stage of the etiolation. It seems that the pre-existing chlorophyll molecules in the periphery of PSI could be released and re-distributed for PSII biosynthesis in the etiolating cyanobacterial cells. Similarly, Xu et al. (2004) reported a decrease in the PSI/PSII ratio following the addition of gabaculin, an inhibitor of chlorophyll synthesis, to wild-type cells of *Synechocystis* sp. PCC 6803. Selective repression of PSI content was observed in *Synechocystis* sp. PCC 6803 under high-light conditions where chlorophyll biosynthesis is arrested (Hihara et al. 1998; Muramatsu et al. 2009). These results suggest that biogenesis of PSI in cyanobacteria is more closely linked to de novo synthesis of chlorophyll compared to that of PSII.

B. Regulation of PSI Quantity upon Acclimatory Process

In spite of its general static image, PSI is the target of regulation during acclimatory processes to different environments (Hihara and Sonoike 2001). The most well-known acclimation is the alteration of photosystem stoichiometry (PSI/PSII ratio) upon changes in light intensity or light quality. Although the regulation of photosystem stoichiometry is also observed in higher plants, algae and cyanobacteria, the most striking changes are observed in phycobilisome containing organisms such as red algae and cyanobacteria (Fujita 1997). This is quite reasonable, since PSI with chlorophyll antenna and PSII with phycobilisome antenna should absorb a totally different quality of light in these organisms, and the regulation of the ratio between the two photosystems would be essential upon the changes of light quality. The change of photosystem stoichiometry is also observed upon the change of light intensity. Under high light condition, the quantities of both PSI and PSII decrease with a larger change in PSI, resulting in the decrease of PSI/PSII ratio in many photosynthetic organisms. At least in cyanobacteria, such a high light-induced change in photosystem stoichiometry is essential for the survival under high light stress conditions (Sonoike et al. 2001). Although many genes were identified to affect the high light-induced modification of photosystem stoichiometry in cyanobacteria (Ozaki et al. 2007; Ozaki and Sonoike 2009), their precise functions are largely unknown. One of such genes, *pmgA*, was extensively characterized. A *pmgA*-disrupted mutant of *Synechocystis* sp. PCC 6803 had a defect in suppressing PSI content at a low level under high light conditions, whereas its PSII content is regulated normally (Hihara et al. 1998). A *pmgA*-mediated regulatory mechanism seems to suppress the activities of *psaAB* transcription and of chlorophyll biosynthesis at low levels under prolonged high light conditions (Muramatsu and Hihara 2003, 2012; Muramatsu et al. 2009). Coordinated synthesis of chlorophyll and chlorophyll-binding proteins is essential for photosynthetic organisms, because accumulation of free chlorophylls or chlorophyll intermediates would cause severe photooxidative damage to cellular components. As for light-intensity dependent transcriptional regulation of PSI

genes, a response regulator RpaB was shown to be a critical factor. RpaB binds to the HLR1 sequence commonly located just upstream of the core promoter region of PSI genes and acts as a transcriptional activator under low light conditions, whereas its activity is lost upon the shift to high light conditions (Muramatsu and Hihara 2006, 2007; Seino et al. 2009; Takahashi et al. 2010).

Since cyclic electron transfer around PSI is the source of ATP for the energy demanding processes, the increase of energy demand due to environmental changes would lead to the de novo synthesis of PSI. For example, C_4 photosynthesis requires additional ATP for the C_4 cycle at pyruvate orthophosphate dikinase (PPDK). It is also known that bundle sheath cells of C_4 plants, especially those of NADP-malic enzyme (NADP-ME) subtype plants such as maize, have chloroplasts with high PSI/PSII ratio, since the reducing power (NADPH) and CO_2 are supplied to the cells as malate, and only ATP is necessary for the operation of the Calvin-Benson cycle. PSI must be synthesized and assembled in need of ATP under such conditions. In the case of cyanobacteria, increases of the content of PSI and of the activity of cyclic electron transfer have been reported in several different environmental conditions. PSI content is affected by salinity stress, since the active export of sodium ions and the synthesis of compatible solutes require energy (Joset et al. 1996) that should be supplied by the cyclic electron transport around PSI (Jeanjean et al. 1993; Hibino et al. 1996). Similarly, PSI content in cyanobacteria is affected by the change in the concentration of CO_2, a substrate of photosynthesis (Manodori and Melis 1984; Murakami et al. 1997), since the active import of CO_2 also requires energy.

C. Recovery of PSI After Environmental Stress

Although PSI has long been considered as tolerant to photoinhibition, it is now established that PSI can be the site of photoinhibition especially under low light/low temperature condition (Sonoike 2006, 2011). The mechanism of photoinhibition of PSI was extensively studied in cucumber, a chilling sensitive plant to explore the mechanism of chilling sensitivity (Sonoike 1998). The photoinhibition of PSI is a universal phenomenon that is observed both in chilling tolerant and chilling sensitive plants, although the selective photoinhibition of PSI could only be induced in some chilling sensitive species. The initial event in PSI photoinhibition is the destruction of iron-sulfur centers on the reducing side of PSI (Sonoike et al. 1995). Although protein degradation is not so prominent just after the photoinhibitory treatment (Sonoike and Terashima 1994), PsaA/B subunits, together with their binding chlorophylls, were degraded to the half of the initial level in subsequent 3 days under normal growth condition (Kudoh and Sonoike 2002). The extent of the bleaching is independent of the light intensity during the process, excluding the possibility that the process is a passive photo-destruction of the pigments (Kudoh and Sonoike 2002). Similarly, the degradation of subunits is also an active process, since low temperature suppresses the degradation indicating the involvement of enzymatic steps (Sonoike, unpublished data). Thus, the degradation of the PSI complex following photoinhibitory treatment is not the process of damage, but a kind of emergency measure that protects plants by removing the inactive but still energy-absorbing PSI reaction center complex.

In photoinhibited cucumber leaves at chilling temperature, P700 activity per chlorophyll content recovered to almost the level before the treatment due to the decrease of chlorophyll content by degradation of inactive PSI. On the other hand, the recovery is very slow if P700 is expressed per leaf area basis: After 6 days of recovery from the 4°C treatment under growth light, half of P700 is still inactive and most of the inhibition of PSI seems to be irreversible. Still, P700 per leaf area showed the recovery in 6 days by 20–30% of its original level. At least for that part, PSI must be newly synthesized and assembled. Although the selective photoinhibition of PSI can be observed for specific plant species under specific conditions, the inhibition of PSI itself is observed in more

general conditions. The synthesis and assembly of PSI must take place generally after such inhibition of PSI.

VII. Concluding Remarks: Why Is the Assembly of PSI Important?

Although the precise mechanism of PSI assembly is far from being elucidated, many components involved in the process have been investigated. PSI assembly is not a simple one step process but multiple processes that take place in the distinct order. For the assembly of PSI and PSII complexes that handle light energy, the timing of the insertion of pigments and redox components must be strictly controlled. As a low redox photosystem, the most dangerous components of PSI are the electron acceptors on the reducing side. Reduced iron sulfur centers of PSI are not only the source of superoxide anion radicals via oxygen reduction, but also the catalytic site of the Fenton reaction that produces hydroxyl radicals, the most dangerous reactive oxygen species (Sonoike 1996). The fact that the subunits of PsaC/PsaD/PsaE on the stromal side of PSI are the first ones to be assembled is quite consistent with the importance of the proper structure of the reducing side during PSI assembly. Proper assembly of PSI is very important, since malfunction of PSI at the last step of electron transfer results in the congestion of the whole electron transfer chain possibly inducing photoinhibition of PSII. In that sense, PSI is the key component in photosynthetic electron transfer. Absence of PSII is troublesome but not dangerous compared with that of PSI. It is indispensable to pursue the elucidation of the assembly process of PSI, especially through a biochemical approach such as isolation of intermediate assembly complexes.

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Chapter 6

Rubisco Assembly: A Research Memoir

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Summary

Rubisco is responsible for net carbon dioxide fixation. Due to the high concentration of oxygen in the atmosphere and the relatively low concentration of carbon dioxide, Rubisco "misfires" frequently, splitting a molecule of ribulose bisphosphate rather than adding carbon to it. Evolution has worked to minimize this tendency, but the strategies have been varied, from slight changes in kinetic properties to wholesale re-organization of leaf anatomy. Rubisco consists of two types of subunits in higher plants, green algae, and certain cyanobacteria. The large (L) subunit is encoded in chloroplast DNA and the small (S) subunit in the nucleus. The discovery that Rubisco is encoded by genes in both the chloroplast and the nucleus of higher plants and green algae has motivated considerable research on the biogenesis and biochemistry of Rubisco. This article describes the role of my laboratory in the study of the assembly mechanism of this important enzyme in higher plants.

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I. Introduction

The photosynthetic enzyme Rubisco, (ribulose 1,5 bisphosphate carboxylase/oxygenase, (EC 4.1.1.39)), is the entry point for CO_2 in the photosynthetic carbon reduction, or Calvin Benson cycle, a pathway in bacteria, algae, and plants. It catalyzes the addition of CO_2 to the substrate ribulose 1,5 bisphosphate to produce two molecules of 3-phosphoglycerate. However, it also catalyzes the oxygenation of this substrate, leading to cleavage of a carbon – carbon bond and initiation of the photorespiration pathway, which actually loses energy and carbon (Bowes et al. 1971). The relative rates of these two reactions vary somewhat among organisms. Under certain circumstances the oxygenase reaction can lead to severe limitations or even cessation of net carbon assimilation by an organism. It has been therefore the target of considerable study for over half a century, and this review can hardly scratch the surface even of the reviews of its literature (Roy and Andrews 2000; Spreitzer and Salvucci 2003; Eberhard et al. 2008; Tabita et al. 2008; Andersson 2008; Whitney et al. 2011). I offer my apologies to those of my colleagues whose work I cannot describe here. I have to limit myself to a small task – simply to give a brief account of my perspective on the assembly of this enzyme. Without any purpose of providing reviews, I have however added references to works on Rubisco expression in foreign hosts, synthesis and development that I found interesting.

Abbreviations: CABP – Carboxy – arabinitol – bisphosphate; (n-)Cpn(x) – Chaperonin (n-) represents a biological source such as a *ch*loroplast, and x is the approximate molecular weight in kDa; GroEL – Any chaperonin homologous to the E. coli form a tetradecamer of 60 kDa subunits; GroES – The co-chaperonin of GroEL that binds to GroEL and facilitates protein folding; L-subunit or RbcL – The larger of two subunits of Rubisco of eukaryotes or the catalytic subunit of any Rubisco; Rubisco – Ribulose 1,5 bisphosphate carboxylase/oxygenase (EC 4.1.1.39); S-subunit or RbcS – The smaller of two subunits of Rubisco found in eukaryotic organisms

II. Raising the Curtain on the Problem

Rubisco (known in the 1960s as carboxydismutase) was a contaminant in early preparations of the chloroplast coupling factor in my work at Johns Hopkins in the laboratory of Van Moudrianakis. The subject of Rubisco development came to my attention as the result of a visit by R.P. Levine to Johns Hopkins in the late 1960s. He had been invited by a committee of Biology graduate students. What he had to say about the light-dependent development of photosynthetic enzymes in Chlamydomonas fascinated me at the time, and I later applied to work with Levine on these problems before choosing to accept André Jagendorf's offer of a position as a postdoctoral fellow at Cornell. (I had decided early on that Rubisco was the "hemoglobin" of plants. I also thought that nobody else would figure this out, which would avoid too much competition. The first thought was right; the second wildly off. Rubisco is so abundant that it wound up being either a problem or an opportunity for almost everyone working on plants or algae).

Today's understanding of the origins of chloroplasts and mitochondria is very different from the current view in the early 1960s (Kirk 1986; Mounolou and Lacroute 2005). The discovery of DNA in these organelles and the documentation that they could carry out protein synthesis on 70S ribosomes were really unexpected, and they led rapidly to a host of investigations. Levine et al. (1971) showed that in the unicellular green alga *Chlamydomonas reinhardtii*, the synthesis of Rubisco could be blocked by inhibiting protein synthesis by either cytoplasmic or organellar ribosomes. This was consistent with the fact that the enzyme consisted of two subunit types, called the large subunit (or Rbc-L) and the small subunit (or Rbc-S)(Rutner and Lane 1967). Rubiscos that contain small subunits are now called form I Rubisco. Working with a higher plant, Criddle et al. (1970) showed that inhibitors of chloroplast and cytosolic ribosomes had differential effects on the synthesis of the two subunits of this enzyme, indicating that the large subunit was

synthesized in the chloroplast but the small subunit was synthesized in the cytosol.

In the early 1970s, RJ Ellis working in England, AT Jagendorf in the USA, and a number of others began studying this issue as well. As a postdoctoral fellow in the Jagendorf lab I worked with Linda Gooding, then a graduate student, on identifying the ribosomes engaged in the synthesis of these subunits, using antibodies against wheat Rubisco. We confirmed the synthesis of the large subunit in chloroplasts by showing specific precipitation of radioactive peptidyl-puromycins released from 70S ribosomes using the antibody against the large subunit, but not the small subunit of Rubisco. One curious fact was that although the antibodies were specific for the subunits, both anti-large subunit and anti-small subunit precipitated the same amount of peptidyl puromycins released from 80S ribosomes. This suggested to us that free large subunits might bind to nascent small subunits (Roy et al. 1973). In retrospect this only could have occurred during or after homogenization of the plant tissue. It was a clue, entirely ignored, that soluble large subunits existed in plant leaves. Later, Blair and Ellis found that the large subunit, but not the small subunit, of Rubisco was synthesized by isolated pea chloroplasts (Blair and Ellis 1976). In the Jagendorf lab Richard Patterson and I immunoprecipitated in vitro-completed soluble polypeptides using antibodies against the small subunit of pea Rubisco. These migrated in two bands in SDS polyacrylamide gels, one the same length as the native small subunit, the other about 20 kDa. We were able to identify only the smaller one by tryptic peptide analysis, as an accident prevented analysis of the larger one. We only repeated the analysis on the smaller band and contented ourselves with the suggestion that the bigger one might be a precursor (Roy et al. 1976). Thus we almost discovered the precursor of the small subunit, later found by Nam-Hai Chua and Gregory Schmidt (1978), who were the first to make a definitive identification of the product. Their work opened up the very fruitful investigation of the import of proteins into chloroplasts – a vast field that I cannot cover (see Strittmatter et al. 2010 for a recent review).

A side observation during the course of our work with Jagendorf arose from the behavior of the subunits of Rubisco that we prepared in order to make antibodies. We found that after they were denatured in 8 M urea for example, they would form an insoluble clot when dialysed back into neutral aqueous buffers. Thus, to get antibodies, we had to inject the subunits in urea solution. It was a surprise to us that the subunits did not renature spontaneously, even at low temperatures. I later learned that most workers on the enzyme had tried similar experiments with the same negative results. The enzyme, once denatured, was dead forever. Given the results of many renaturation experiments with other enzymes, this was unexpected, and remained unclarified for some time.

III. The Behavior of Unassembled Rubisco Subunits

Shortly after establishing a laboratory at Rensselaer in 1976, I returned to this problem and decided to take an in vivo approach. I fed radioactive methionine of the highest specific activity available directly to cut seedling tips of peas, using a cool stream of air to assist in the uptake. After short periods of labeling, I homogenized these with some carrier leaves, and then centrifuged the soluble proteins on a sucrose gradient. Most of the radioactivity co-sedimented with the 18S peak of native Rubisco. This I interpreted to mean that many subunits had already assembled. But I also found both subunit types in low molecular weight fractions, which I attributed to free, recently completed (and soluble!) forms, since they were not found when the purified native enzyme was centrifuged in control experiments. Experiments with cycloheximide and chloramphenicol, similar to the ones done much earlier by Criddle et al. (1970), showed that the synthesis of the small subunit was differentially sensitive to cycloheximide, and the large subunit differentially sensitive

to chloramphenicol (Roy et al. 1978, 1979). It seemed that in vivo, these subunits were soluble, and in that respect different from those produced by urea denaturation of the native enzyme. Thus, at least the plant had solved the problem of how to make soluble subunits of this enzyme. But why the difference? What mechanism was responsible for their "good" behavior in vivo?

IV. The Discovery of the Large Subunit Binding Protein, Chaperonin 60

The first clue came as it so often does from work in another laboratory. Roger Barraclough and RJ Ellis (1980), using a new method for isolating intact pea chloroplasts, showed that when protein synthesis was carried out in the light, the large subunit of Rubisco was soluble, but that the bulk of it migrated separately from Rubisco itself during nondenaturing polyacrylamide gel electrophoresis. Not only that, but also the radioactive band was stainable with Coomassie Blue. Barraclough and Ellis showed that this band contained subunits of about 60 kDa and dubbed the high molecular weight complex as the large subunit binding protein. (This is now called the chloroplast chaperonin 60). However the chaperonin 60-large subunit complexes were found to be much bigger than even the fully assembled Rubisco. On prolonged illumination, the radioactive large subunits began to appear in the native Rubisco band, indicating they were assembly competent. Apparently the binding protein was keeping the large subunit soluble long enough to enable it to assemble. The nondenaturing electrophoresis method did not allow resolution of soluble large subunits that were not bound to the chaperonin 60 complex (they migrate as a smear). So Barraclough and Ellis did not see those.

V. In Vitro Synthesis and Assembly of Various Rubiscos

I kicked myself later when I realized that the binding protein, for me, was yet another "almost" discovery! The reason I had missed the chaperonin 60 protein was that I had not used the nondenaturing gel electrophoresis method in my pulse labeling experiments. Chloroplast chaperonin 60-large subunit complexes and Rubisco holoenzyme did not separate well enough on my sucrose gradients to resolve them. We quickly confirmed Barraclough and Ellis' observations (Roy et al. 1982), and then showed, with Mark Bloom and Patrice Milos, that the incorporation of newly made large subunits into Rubisco in chloroplasts could not take place in the dark. The energy requirement we figured must be due either to reduced substrates or ATP produced by photosynthesis. We found that we could drive the post-translational assembly of most of the large subunits in chloroplast extracts when we added ATP and Mg^{2+} (Bloom et al. 1983; Milos and Roy 1984). Both we and the Ellis lab experienced considerable skepticism about our respective results. But, to paraphrase Stephen J Gould, data are data. In my lab Bloom had discovered that the large subunits associated with chaperonin 60 could be released by ATP, which caused the complete dissociation of the chaperonin 60 at the same time. Once released the majority of these were able to assemble into Rubisco in vitro. This established the paradigm for the mechanism of action of this class of protein. Antibodies directed against the chloroplast chaperonin, and provided to us by the Ellis laboratory, were able to inhibit this assembly (Cannon et al. 1986). This result showed that the chaperonins were involved in Rubisco assembly. The apparent involvement of a second protein in the assembly of Rubisco was very surprising to most investigators. Considerable light was shed on the generality of all this when the Ellis laboratory established the homology of the chloroplast chaperonin with the *E. coli* GroE protein, which had for some time been known to be important for the assembly of viruses in that organism (Hemmingsen et al. 1988). Skepticism was allayed further when Goloubinoff et al. (1989) showed that the GroE protein could support the formation of the dimeric (form II) Rubisco from *Rhodospirillum rubrum* in *E. coli*. This was

the origin of the huge field of chaperone research, which also cannot be covered here in any detail. Today chaperones are known to be involved in countless processes in cells of all types. But nobody ever predicted the occurrence of such proteins (see Fink 1999 for a general review of chaperones).

VI. Characterizing the Process and the Product

Later, working with Panna Chaudhari (Roy et al. 1988b) and Alan Hubbs (Roy et al. 1988a), we worked out additional details of the assembly process, showing that it could take place even when the large subunits were synthesized outside chloroplasts by soluble polyribosomes. In these experiments we also were able to document the dependence of assembly on added small subunits (Hubbs and Roy 1992a). We found that concentrating the chloroplast chaperonin would prevent its dissociation in the presence of ATP, which thus explained how the molecule could exist in oligomeric form in vivo (Hubbs and Roy 1992b). Hubbs was also able to show that under limiting conditions, large subunits would accumulate in a soluble high molecular weight aggregate (which we called "Z") that had characteristics expected of an intermediate in the assembly process, possibly a large subunit octamer (Hubbs and Roy 1993). This was too low in abundance to show up with Coomassie Blue staining. Another student, Michele Gilson, and my postdoctoral fellow Balaji documented that the Rubisco assembled in vitro after synthesis on chloroplast ribosomes could bind the transition state analog CABP (Balaji et al. 2006). This showed that the in vitro assembled enzyme had a native character. This was important because the amounts of Rubisco synthesized in our experiments were too small to cause any significant changes in the level of Rubisco catalytic activity. A model for Rubisco assembly consistent with our observations was that large subunits after synthesis were complexed with chaperonin 60, and in the presence of ATP and Mg^{2+}, released in assembly-competent form, after which they formed dimers and then octamers, followed by small subunit binding. However, the exact sequence of events was not resolved and it was not clear if chaperonin 60 was obligatory or sufficient for Rubisco assembly in chloroplasts. This required a well defined in vitro system where each required component could be controlled.

VII. Mechanism of Chaperonin Action

The detailed mechanism of chaperonin action was largely worked out by many researchers using the E. coli GroEL-GroES system (Thirumalai and Lorimer 2001; Horwich et al. 2007). These proteins are noted for having sevenfold rotational symmetry, and are able to accelerate folding of many different proteins in vitro, mostly by suppressing the aggregation of monomers in free solution (Apetri and Horwich 2008). The basic chaperonin 60 component consists of two stacked rings of 7 subunits each. The GroES protein, a heptamer, can reside on either or both ends of the chaperonin 60 tetradecamer. The chaperonin 60 rings and the GroES complexes surround a large cavity where unfolded proteins bind and where, as a result of ATP-induced conformational changes, their folding is actually facilitated. It appears that when ATP binds first to GroEL, folding of prokaryotic Rubisco is more rapid than otherwise (Tyagi et al. 2009). Curiously, the chloroplast co-chaperonin 21 (ch-cpn21), which contains a tandem repeat homologous to GroES, was reported to form a tetramer in solution (Koumoto et al. 1999). However, more recent work has uncovered a second chloroplast GroES homologue ch-cpn10, that forms heptamers in solution. It appears that the aggregation states of ch-cpn 21 are much more complex than simply tetramers (Sharkia et al. 2003). With both forms of co-chaperonin (ch-cpn10 and ch-cpn21), only one species of complex forms with chaperonin 60, which is a tetradecamer (Dickson et al. 2000). The chloroplast chaperonin 60 differs from the E coli GroEL in that it has two slightly different subunits, α and β (Hemmingsen and Ellis 1986). Dickson et al. (2000) showed

interesting differences between chloroplast Cpn60 molecules reconstituted from β subunits and mixtures of α and β subunits. While the former could interact with mitochondrial cpn10, the latter was more catholic in being able to interact with chloroplast, bacterial, and mitochondrial cpn10. Formation of tetradecamers from β subunits, but not α subunits, could occur in the presence of ATP; however assembly of α subunits into cpn60 tetradecamers depended on β subunits. It was suggested that the different types of subunits might interact with the different domains of the chloroplast cpn21 protein, but clearly more work needs to be done to characterize the chloroplast system. Does each ring of ch-cpn60 have the same number of each subunit type, or are they different? In complexing with chaperonin 60, does chaperonin 21 have an unpaired subunit domain "sticking out"? Further structural studies of this system are needed.

VIII. The Holy Grail of In Vitro Rubisco Assembly

By the mid 1990s it was clear that Rubisco large subunits were associated with chaperonin 60, and were released by ATP. It seemed probable that after release they formed dimers that then associated to form a core particle which could bind small subunits. But we never were able to find the Holy Grail, i.e., synthesize and assemble higher plant Rubisco using mRNA as a starting material in E. coli extracts. The subunits could be made, but they would bind unproductively to the *E. coli* GroEL protein, which had been shown to be homologous to the large subunit binding protein of chloroplasts. Addition of ATP and small subunits of Rubisco or even the chloroplast chaperonin 60 could not coax these subunits to assemble into Rubisco. Indeed, many attempts to express form I Rubisco in foreign environments failed. For example, Whitney et al. (2001) found that expression of the genes for non-green algal Rubisco subunits did not lead to their assembly in tobacco chloroplasts. This seemed a general result (Whitney and Andrews 2001; Andrews and Whitney 2003). The difficulties all these workers experienced in this regard led us to suppose that there were additional factors besides the GroEL/GroES system involved in the assembly of Rubisco in plants (reviewed in Roy and Andrews 2000). These conclusions appear to have been justified: indeed, new proteins involved in Rubisco assembly, BSD 2 (*b*undle *s*heath *d*efective 2) and RbcX, have since been found (Brutnell et al. 1999; Saschenbrecker et al. 2007). Liu et al. (2010) have succeeded in carrying out the in vitro reconstitution of cyanobacterial form I Rubisco using chaperonin 60 and one of these newly discovered proteins, $RbcX_2$. This works by binding to the large subunit after it has dissociated from the chaperonin 60 complex. $RbcX_2$ binds to a cleft on the molecule that is not found in form II Rubiscos. This stabilizes the RbcL subunits as dimers. Fully folded small subunits can then bind to these by displacing $RbcX_2$. Apparently, when the form I large subunit is released from chaperonin 60 it still has a strong tendency to aggregate; $RbcX_2$ binds to the large subunit and blocks this aggregation allowing small subunits to bind later, rendering the products fully soluble (Fig. 6.1 from Saschenbrecker et al. 2007). These conclusions are consistent with the observations in the chloroplast system, particularly the properties of the unbound large subunits, some of which sediment about 7S in sucrose gradients, as expected of dimers, and others of which have a strong tendency to aggregate at the top of gel lanes in nondenaturing electrophoresis (Roy et al. 1982). RbcX is related to several other chaperones, mitochondrial Tim9-10, prefoldin, and Skp (Shiho et al. 2007). It is not clear what the relationship between these and BSD 2 might be. The proteins are quite different based on their sequences, and it has been proposed that BSD 2 acts before chaperonin 60 in the assembly pathway (Brutnell et al. 1999). It has been found that deletion of RbcX still does not prevent Rubisco formation in *Synechococcus* PCC7942 (Emlyn-Jones et al. 2006).

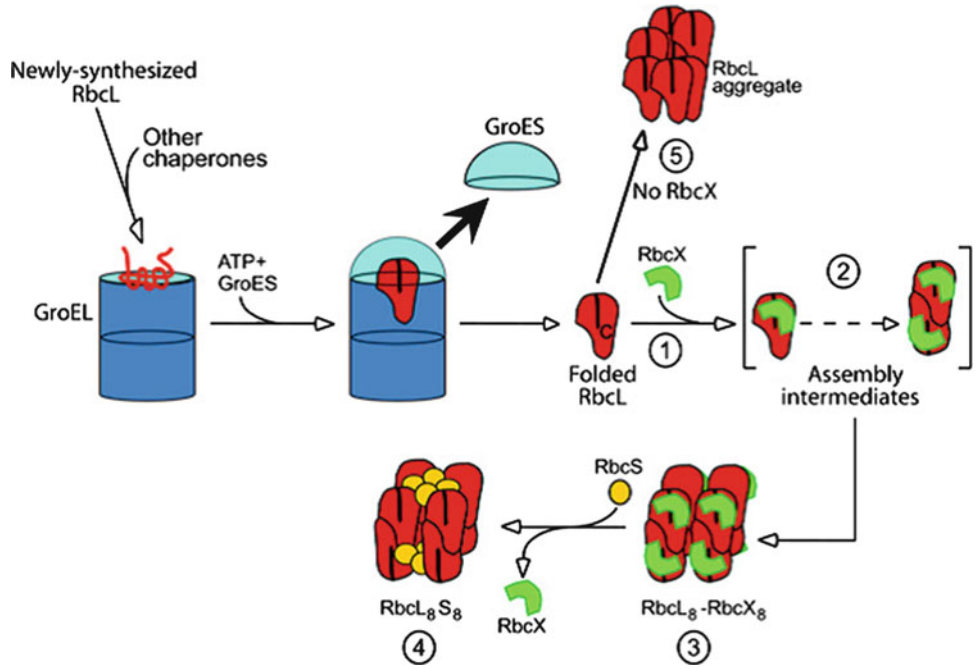

Fig. 6.1. This model is consistent with prior data and incorporates new information on the role of RbcX in the assembly of L subunits into Rubisco. RbcX appears as an assembly factor after folding is mediated by the chloroplast Cpn60. (Modified from Saschenbrecker et al. 2007).

IX. Expressing Foreign Rubisco

Work is ongoing taking advantage of the ability to engineer higher plant Rubisco using plant transformation procedures (Karkehabadi et al. 2005; Wostrikoff and Stern 2007; Whitney and Sharwood 2008; Whitney et al. 2009). The work of Whitney et al. (2009) is of particular interest regarding the mechanism of Rubisco assembly, because they showed that an active enzyme could be formed from polypeptides that consisted of tandemly linked tobacco L and S subunits. These are probably too big to fit into the cavity of the chloroplast cpn60 tetradecamer, and indeed a large proportion of them aggregate and fail to assemble. It is well known that small subunits can bind to large subunit octamers in vitro to form active Rubisco (Andrews and Ballment 1983). The fact that tandemly linked L-S polypeptides can also assemble into an active product shows that there is more than one path to formation of the L_8S_8 enzyme. This is entirely consistent with the underlying rationale of self-assembly, but of course with the added provison that folding or assembly or both may require assistance. The discovery of the action of the chloroplast chaperonin in the formation of Rubisco triggered the field of chaperone research and established a major modification of the principle of self-assembly. In addition to the fact that Rubisco requires assistance to assemble, it also requires assistance by another protein, Rubisco activase, to carry out efficient catalysis in higher plants (Portis et al. 2008), and it is subject to a host of post-translational modifications mostly of yet-unknown function (Houtz et al. 2008). Considered in this light, Rubisco appears to be a very "needy" enzyme. This apparently results from the extremely close connection between the biochemistry of the enzyme and the contents of the physical environment in nature, particularly CO_2 and O_2.

X. Cell Biology of Rubisco Synthesis

In both the cytoplasm and in the chloroplast there are both membrane-bound and soluble polyribosomes (Chua et al. 1973; Margulies and Michaels 1974). The general expectation is that the former are making membrane proteins and the latter are making soluble proteins. However, the literature on Rubisco presents a confusing picture, because there is evidence suggesting that both subunits of Rubisco are made by both membrane bound and soluble ribosomes (Gooding et al. 1973; Hattori and Margulies 1986). On the other hand Ellis (1981) stated that the large subunits are made by soluble ribosomes. We used soluble polyribosomes to carry out in vitro synthesis of the large subunit of pea Rubisco (Hubbs and Roy 1992a, b, 1993). Minami et al. (1988) reported that the L subunit mRNA bound to spinach thylakoids was not translated. Indeed, it is not clear why either Rubisco subunit should be made on membrane-bound ribosomes. It is thought that nascent large subunit chains of Rubisco can bind directly to RNA and that this is an important factor in translational control of Rubisco synthesis (Cohen et al. 2005, 2006). But it also might explain why Rubisco mRNA might wind up indirectly associated with membranes.

In C-4 plants like maize, the Calvin Benson cycle is restricted to bundle sheath cells while the mesophyll cells are given over to supplementary mechanisms for temporary fixation of CO_2 (see Edwards et al. 2004 for a recent review). In Crassulacean metabolism, there is a diurnal regulation of CO_2 fixation that conserves water, allowing gas exchange and temporary CO_2 fixation at night, followed by isolation from the air during the day and ultimate fixation of CO_2 by Rubisco (see review by Bohnert and Cushman 1999). These different developmental and physiological pathways appear to have evolved to deal with the high oxygenase activity of Rubisco, which basically destroys a substantial fraction of the fixed carbon at every turn of the Calvin Benson cycle. Moreover, they have evolved independently in many different lineages of plants and show remarkable diversity. In some of these plants Rubisco is restricted to bundle sheath cells, which receive dicarboxylic acids from the mesophyll cells and decarboxylate them before Rubisco re-fixes the CO_2. In others there are subcellular differentiations that separate Rubisco from the ambient air. Why not "simply" evolve a more efficient Rubisco? Perhaps part of the problem is that there are so many copies of the Rubisco L subunit gene that "better" mutants never get a chance to be expressed at a high enough level to have an effect on survival. Alternately, there may not be sufficient scope for improvement given the basic structure of the molecule. After all, the range of CO_2/O_2 specificity of Rubisco in nature is not very great – less than two orders of magnitude includes them all, both eukaryotic and prokaryotic. A fascinating example of what selection can do is provided by a study of an unique nuclear-coded dinoflagellate form II Rubisco, which has a specificity factor about twice as great as that of the anaerobic *R. rubrum*, still less than that of the form I Rubisco from the alga Synechococcus (Whitney and Andrews 1998). The small subunits do influence conformation, levels of activity and kinetic parameters considerably in form I Rubisco (Roy and Andrews 2000).

XI. Developmental Control

Rubisco is part of the total ensemble of chloroplast proteins, and its activity and abundance change as a function of developmental (Leon and Arroyo 1998) and physiological (Geiger and Servaites 1994) factors. Because the protein is so abundant, it has been a leading marker for developmental studies since the 1960s (Ellis 1981). The amount of chloroplast protein is quite low in germinated seedlings, in which the chloroplasts exist only as proplastids, containing but a precursor network of membranes, that over the course of normal growth elaborate into the thylakoid system (Mullet 1988; Leon and Arroyo 1998). This is accompanied by an

increase in the quantity of Rubisco and the onset of a daily rhythm of Rubisco activity which is controlled by light (Geiger and Servaites 1994). In higher plants it is possible to keep seedlings in the dark for prolonged periods, during which they develop in an etiolated state, that is without turning green. The proplastids develop partially forming what are called etioplasts. When the etiolated seedlings are exposed to light, the etioplasts transform rapidly into chloroplasts and protein synthesis increases (Drum and Margulies 1969). Rubisco accumulates under these conditions (Ellis 1981). In Chlamydomonas, the famous *Y-1* mutant is yellow when grown in the dark but turns green in the light and accumulates Rubisco and many other components of the photosynthetic apparatus (Bar-Nun et al. 1977). Yellow plants (mutant or etiolated) and algae therefore have served as interesting systems to investigate the control of chloroplast development, and Rubisco has served as a marker for this process. A broad issue has emerged from all this, namely the role played by transcriptional vs. translational control (Mullet 1988). Related questions pertain to the signaling systems whereby light brings about changes in the proplastids or etioplasts (Leon and Arroyo 1998; Sakamoto et al. 2008). The phytochrome system is involved in higher plants, and there are definite changes in the amount of transcription of the chloroplast genome caused by light. Although for the most part this process is controlled by the action of nuclear-coded genes, there is some feedback from the chloroplast that affects nuclear transcription. In algae, drugs that block chloroplast nuclear transcription interfere with the greening process. However, it has become apparent since the development of refined molecular methods for identifying transcripts that differential transcription of chloroplast genes is not very pronounced (Deng et al. 1989; see Cohen and Mayfield 1997). Instead, the different transcripts of the chloroplast genome show rather constant ratios one to the other as development proceeds, even though the protein products may show marked changes relative to one another. This has focused attention on the mechanism of translational control. This has been established quite well for a number of thylakoid proteins and recently for Rubisco, where it appears that the accumulation of excess large subunits leads to inhibition of translation of large subunits without affecting transcription specifically (Cohen et al. 2005, 2006; Wostrikoff and Stern 2007; Whitney et al. 2009). This fits with the observation that an excess of the small subunit leads to impairment of Rubisco large subunit synthesis (Rodermel et al. 1988). In the converse situation, where large subunits are limiting, small subunits continue to be synthesized and imported into the chloroplasts, where they are degraded in the absence of an assembly partner (Schmidt and Mishkind 1983). Is there some other control on small subunit synthesis? Suzuki et al. (2009) have found that overexpression of S subunits in rice leads to increased accumulation of L subunit mRNA and increased accumulation of Rubisco in fully expanded leaves but not in lower leaves, with the overall result that no increase in photosynthetic capacity took place. Uchida et al. (2005) overexpressed the L subunit mRNA in Chlamydomonas, which brought about increases in S subunit mRNA, but no changes in the amount of Rubisco took place. This indicates cross-talk about transcription levels but overall control by post-transcriptional mechanisms. Similar conclusions came from other studies in higher plants (Tang et al. 2003). Questions like this can be asked about any of the many chloroplast protein complexes that contain subunits encoded in both the chloroplast and nuclear genomes (Leon and Arroyo 1998). Recently a protein, MRL1, has been identified that binds to the 5′ end of the single *rbcL* mRNA that is formed in Chlamydomonas and prevents its nucleolytic degradation in that organism, thus being required for Rubisco synthesis. A similar protein in Arabidopsis binds only to the processed form of the *rbcL* mRNA and is not essential for Rubisco biogenesis (Johnson et al. 2010). It thus appears that the biogenesis of Rubisco

varies considerably in different taxa, indicating a profound sensitivity of Rubisco to evolutionary pressures.

Although most work on Rubisco in chloroplast development has concentrated on the greening process, equally dramatic processes occur when conditions change and photosynthesis is down-regulated. Rubisco is then degraded and its components are largely remobilized by the plant. There is a considerable literature on Rubisco in relation to proteases of the chloroplast (Vierstra 1993; Feller et al. 2008; see also Costa et al. Chap. 18; Wada and Ishida, Chap. 19).

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Chapter 7

Glycerolipid Biosynthesis and Chloroplast Biogenesis

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Summary

Chloroplast membranes are enriched with galactoglycerolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These galactolipids do not contain phosphorus and chloroplast membranes are therefore very poor in phospholipids, primarily represented as a special *trans* Δ3-hexadecenoic acid-containing phosphatidylglycerol (PG), a finely compartmentalized amount of phosphatidylcholine (PC) and a very low and transitory

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level of phosphatidic acid (PA). The biogenesis of chloroplasts requires a highly efficient glycerolipid-synthesis system for the development and functioning of both the chloroplast envelope and the thylakoids. Photosynthesis notably relies on the presence of galactolipids and PG. In this chapter, we review the properties of these glycerolipids, their role in photosynthesis and the characteristics of their synthetic pathways. We focus on the role of MGDG synthase in chloroplast biogenesis, the enzyme functioning in the chloroplast envelope membrane, and different mechanisms involved in its regulation. The regulation of MGDG synthase by the phospholipids, PG and PA, is discussed in terms of membrane homeostasis and plant cell biology.

I. Introduction

Chloroplasts are the plastids dedicated to photosynthesis. In plants, these organelles are bound by an envelope consisting of two membranes with different functions. The envelope constitutes a frontier between cytosol and chloroplast and also a source of lipids to build membranes. The envelope is notably the primary source of lipids for thylakoids, an extended network of membranes structured efficiently for photosynthesis.

The envelope membranes form a very lipid-rich structure and are as a result low density membranes compared to thylakoid or mitochondria membranes. The outer envelope membrane has the highest lipid to protein ratio among plant cell membranes (2.5–3 mg lipids/mg proteins), and this property is responsible for its very low density (1.08 g/cm^3, Block et al. 1983a). The lipid to protein ratio of the inner membrane is rather high (1–1.2 mg lipids/mg proteins), corresponding to a density of 1.13 g/cm^3 (Block et al. 1983a).

A characteristic of chloroplast membranes is their specific enrichment in galactoglycerolipids, MGDG and DGDG. These galactolipids do not contain phosphorus and therefore chloroplast membranes are very poor in phospholipids, primarily represented as a special *trans* Δ3-hexadecenoic acid-containing PG, a finely compartmentalized amount of PC and a very low and transitory level of PA.

The biogenesis of chloroplasts requires a highly efficient system for the manufacture of membranes. A basic step in this process is the generation of the lipid bilayer enriched in galactolipids and PG. This newly formed bilayer is necessary for the extension of the envelope membranes and for the development of thylakoids. A second important step in the process is the proper assembling of the lipid components with specific protein components in order to get functional protein complexes. Then, different steps of coordination and regulation must be considered: formation of the chloroplast membranes is partly dependent on formation of the other cellular membranes and chloroplast biogenesis is dependent on growth and development of the plant in its environmental context. Lipid synthesis in plants is initiated by fatty acid synthesis in chloroplasts, and one part of the chloroplast galactolipids is dependent on hydrolysis of ER-synthesized PC. This

Abbreviations: ACP – Acyl carrier protein; C16:0 – Palmitic acid; C16:3 – Hexadecatrienoic acid; C18:0 – Stearic acid; C18:1 – Oleic acid; C18:2 – Linoleic acid; DAG – Diacylglycerol; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; G3P – Glycerol-3-Phosphate; MGDG – Monogalactosyldiacylglycerol; PA – Phosphatidic acid; PAP – Phosphatidic acid phosphatase; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG–Phosphatidylglycerol; PGP–Phosphatidylglycerol phosphate; PI – Phosphatidylinositol; PLC – Phospholipase C; PLD – Phospholipase D; PS – Phosphatidylserine; PS II – Photosystem II; SQDG – Sulfoquinovosyldiacylglycerol; TAG – Triacylglycerol; TetraGDG – Tetragalactosyldiacylglycerol; TriGDG – Trigalactosyldiacylglycerol;

Table 7.1. Glycerolipid composition of spinach chloroplast membranes as reported by Block et al. (2001)

	Outer envelope membrane	Inner envelope membrane	Total envelope membranes	Thylakoids
Total polar lipids (mg/mg protein)	2.5–3	1	1.2–1.5	0.6–0.8
Polar lipids (% of total)				
MGDG	17	55	32	57
DGDG	29	29	30	27
SQDG	6	5	6	7
PC	32	0	20	0
PG	10	9	9	7
PI	5	1	4	1
PE	0	0	0	0

indicates that there is a finely intermingled network of regulation between the lipid-synthesizing enzymes located in the chloroplast and those located outside the chloroplast. Furthermore, some intermediates in lipid synthesis such as PA are also important mediators of cellular signaling suggesting complexity of regulation of chloroplast lipid synthesis.

In this chapter, we will review glycerolipids specific to chloroplast membranes. We will discuss how they are synthesized, regulated and coordinated.

II. Chloroplast Glycerolipids

Chloroplast membranes are built on glycerolipid bilayers (Table 7.1). These glycerolipids are very different from those usually found in non-plastidial membranes. They are mainly non-phosphorous lipids with a very high proportion of galactolipids, up to 80% of the total lipids. MGDG and DGDG represent respectively 50% and 30% of the chloroplast lipids. PG is the main chloroplast phospholipid and is a clear chloroplast-distinctive lipid due to a unique structure with a *trans* Δ3-hexadecenoic acid at the *sn*-2 position of the glycerol backbone (Dubacq and Trémolières 1983). It represents from 7% to 9% of the chloroplast lipids. Another specific glycolipid, sulfoquinovosyldiacylglycerol (SQDG), is present in the chloroplast membranes although at a minor level, from 3% to 10% of the chloroplast membrane lipids, at a concentration increasing with the stage of maturation of the leaf and/or when phosphate becomes less available in the plant.

It should be noted that PC is present in chloroplast, but exclusively on the organelle surface, in the outer layer of the outer envelope membrane where it is concentrated (Dorne et al. 1985). Thylakoids normally do not contain PC (Block et al. 1983b; Fritz et al. 2007). Altogether, the lipid composition of the thylakoids and the inner envelope membrane are very similar: they mostly contain the two galactolipids (MGDG and DGDG), PG, and SQDG (Table 7.1). With the presence of PC, the outer envelope membrane differs notably from the other chloroplast membranes. It, however, also contains galactolipids and SQDG, which were demonstrated to partly face cytosol (Billecocq et al. 1972; Billecocq 1975). Finally, chloroplast membranes indistinctly contain also a low proportion of phosphatidylinositol (PI), whose origin, role and metabolism remain rather imprecise in chloroplasts.

The role of galactolipids in chloroplast biogenesis is related to their specific molecular properties. MGDG has a high content in polyunsaturated fatty acids. It is mostly linolenic acid (C18:3), although a high proportion of hexadecatrienoic acid (C16:3) can be present in some plants, in this case exclusively at the *sn-2* position. Due to the

high content in unsaturated fatty acids and the restricted size of the polar head with only one galactose, MGDG has a cone shape whereas DGDG, with two galactoses and less unsaturated fatty acids, has a cylindrical shape. Due to this shape, DGDG is primarily favourable to bilayer formation. In contrast, MGDG is an important modulator of the fine membrane organization. It organizes in a hexagonal II phase in pure water but favours formation of large ordered lamellar structures in photosynthetic membranes (Simidjiev et al. 2000). It is expected that modification of the membrane organization affects membrane activity. It has been observed that accumulation of MGDG in *E. coli* membranes induces elongation of the bacteria, probably through inhibition of the cell division machinery (Gad et al. 2001).

Analysis of mutants devoid of MGDG or DGDG contributed to the conclusion that these lipids are essential for chloroplast biogenesis. As a straight example, the *mgd1-2* mutant (see below) that is impaired in MGDG synthesis has a drastic reduction of MGDG content and exhibits a complete loss of chlorophyll and photosynthetic activity (Kobayashi et al. 2007). Similarly, the *dgd1, dgd2* mutant, lacking two DGDG synthases, is completely devoid of DGDG and severely impaired in growth, while the *dgd1* mutant having only 10% of the wild type-DGDG content has an altered thylakoid membrane ultrastructure and reduced photosynthetic capabilities (Dörmann et al. 1995; Kelly et al. 2003). The effect of partial MGDG deficiency, as observed in the *mgd1-1* mutant, indicated that MGDG directly or indirectly contributes to efficient protection of photosynthesis at high light intensities through the xanthophyll cycle (Aronsson et al. 2008). The reduced level of MGDG induced an increased conductivity of the thylakoid membranes at high light intensities, so that the proton motive force was reduced and the thylakoid lumen became less acidic. Consequently, the pH-dependent activation of both the violaxanthin de-epoxidase and the PsbS protein was impaired. The specific role of DGDG was studied by introduction of the bacterial glycolipid glucosylgalactosyldiacylglycerol in the *dgd1, dgd2* mutant (Holzl et al. 2009). This experiment showed that the bacterial glycolipid is sufficient to restore thylakoid ultrastructure but insufficient to restore maximal PS II quantum efficiency suggesting that the second galactose of DGDG is required for optimal PS II functioning. In another study, circular-dichroism measurements on the *dgd1* mutant revealed that DGDG-deficiency hampers the formation of chirally organized membrane macrodomains that contain the main chlorophyll a/b light-harvesting complexes (Krumova et al. 2010).

The phospholipid PG is a bilayer forming phospholipid. In thylakoids, the role of PG is, however, not restricted to the organization of the lamellar phase domains since a separate pool of PG molecules is involved in formation of a non-bilayer isotropic phase that co-exists with the bilayer phase. These non-bilayer structures remain in the membrane and the exchange of lipids between them and the lamellae phase is increased at high temperature (Krumova et al. 2008). A number of experiments showed that mutants affected in the biosynthesis of chloroplast PG are severely affected in chloroplast development and photosystem assembly (Dubertret et al. 1994; Sato et al. 2000; Babiychuk et al. 2003; Frentzen 2004; Pineau et al. 2004; Wu et al. 2006). The role of PG in chloroplast membrane biology is based on two specific features. It is a negatively charged lipid like SQDG and it contains a specific *trans* Δ3-hexadecenoic fatty acid. Evidence that *trans* Δ3-hexadecenoic acid-containing PG is specifically involved in biogenesis of the light-harvesting chlorophyll a/b-protein complexes was early brought by in vivo manipulations of lipid composition in *Chlamydomonas reinhardtii* mutants (Dubertret et al. 1994). In addition, it has been shown that fatty acid unsaturation of PG has a critical role in stabilization of the photosynthetic machinery against low-temperature

photoinhibition by accelerating the recovery of the PS II protein complex (Moon et al. 1995). More recently, electric light scattering technique and 77K fluorescence analysis of chlorophyll were used to analyze properties of thylakoid membranes in a cyanobacterium PG mutant. Results suggested that PG depletion significantly perturbs the surface charge of the membranes, probably in relation to the negative charge of the lipid (Apostolova et al. 2008).

Anionic lipids are indeed required for chloroplast structure and function (Yu and Benning 2003; Sato 2004). SQDG is the second anionic lipid present in chloroplasts. One of the functions of SQDG could be the maintenance of negative charges in the membrane, especially when PG becomes insufficient such as when phosphate availability is limited (Benning 2007).

Numerous detailed structural analyses of photosystems enabled to position precisely the lipid molecules in the photosynthetic complexes and to have a refined look at the role of each lipid. For instance, a recent analysis of the cyanobacterial PS II at 2.9 Å-resolution indicated different roles of lipids in PS II assembly and functioning (Guskov et al. 2009). To summarize, this analysis indicated that lipids bring some flexibility into the structures and help movement of protein subunits against each other in the membrane milieu. More specifically, they facilitate D1 monomer turnover. They help PS II to get in contact with the plastoquinone pool and make a lid-like structure on the cytosolic side of PS II to control plastoquinone influx. They draw diffusion paths for O_2 or protons in the membrane keeping some sensitive part of the protein complex protected. These functions lie on specific interaction between amino acids and the polar head group of each lipid (Loll et al. 2007; Domonkos et al. 2008) although we can not exclude that the fatty acid part of the interacting lipid does not play also some role (Wada and Murata 1990).

III. Biosynthesis of Chloroplast Glycerolipids

A. Building the Diacylglycerol Backbone of Chloroplast Glycerolipids

All glycerolipids are built from PA that results from acylation of glycerol-3-phosphate (Fig. 7.1). These acylation steps are essential for formation of the DAG backbone of glycerolipids. A specificity of the plant cell is that fatty acid units involved in acylation are formed in the plastid as Acyl Carrier Protein Thioesters (ACP). From acetyl-CoA units, the complex plastid fatty acid synthetic machinery leads to the formation of (C16:0) palmitoyl-ACP and (C18:0) stearoyl-ACP desaturated into (C18:1) oleoyl-ACP by a stearoyl-ACP Δ9 desaturase (Ohlrogge et al. 2000; Rawsthorne 2002). The fatty acyl units can be either directly transferred onto glycerol-3-phosphate in the plastid or transthioesterified from ACP to Coenzyme A on the plastid envelope where they will be exported outside the plastid before integration into the glycerolipid backbone. From this point, two different pathways for chloroplast glycerolipid formation are possible. We'll first describe the synthesis of PA occuring in the plastid.

In chloroplasts, two different acyltransferases called ATS1 and ATS2 are involved in successive acylation of glycerol-3-phosphate from acyl-ACP. ATS1 is specific for C18:1 chain and *sn-1* position on glycerol-3-phosphate (Nishida et al. 1993). This enzyme is present in the plastid stroma but strongly interacts with the inner envelope membrane (Joyard and Douce 1977). In contrast, ATS2 is specific for C16:0 chain and Lyso-PA (Frentzen et al. 1983; Yu et al. 2004) and is strictly a chloroplast envelope protein (Ferro et al. 2003). Final structure of PA formed in the chloroplast envelope is then C18:1-*sn-1*, C16:0-*sn-2*. This is typically a prokaryotic structure because of the exclusive occurrence of C16:0 at *sn-2* position of glycerol. Glycerolipids formed from this type of PA are therefore called prokaryotic lipids. PA is

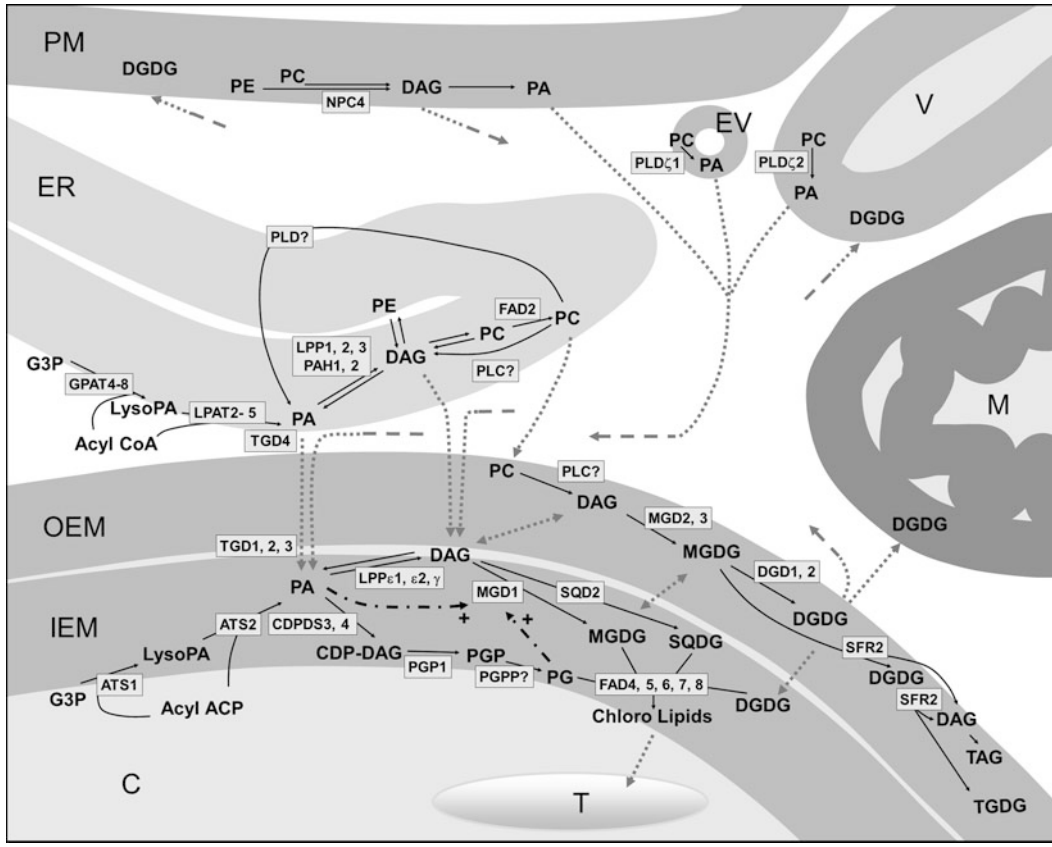

Fig. 7.1. Subcellular localization of the metabolic pathways leading to formation of chloroplast lipids. MGDG, DGDG, SQDG and PG are formed via two major sources of PA, a chloroplastic source and a ER source. The ER *de novo* synthesized PA is then converted to phospholipids mainly PC and PE while fatty acids are desaturated. The enzymatic steps between the ER formation of PA and galactosylation of DAG by MGDs and DGDs or sulfoquinovosylation of DAG by SQD2 in the chloroplast envelope are still under investigation. Under phosphate deprivation, some enzymes such as NPC4, a plasma membrane PLC, or PLDζ1 and PLDζ2, vesicular endomembrane and vacuole membrane PLDs, and the ER PAPs, PAH1 and PAH2, play a role in galactolipid formation. Several lipid transports are involved in the pathways but most actors remain unidentified except TGDs involved in lipid trafficking from ER to chloroplast. Under phosphate deprivation, transfer of diacylglycerol backbone from phospholipids of extraplastidic origins to chloroplast lipids may be coupled with replacement of phospholipids by DGDG in extraplastidic membranes. Once synthesized and desaturated in the envelope, chloroplast lipids, i.e., MGDG, DGDG, PG, and also SQDG, are transported to the thylakoids. Subcellular compartments are PM for plasma membrane, V for vacuole, EV for endomembrane vesicles, ER for endoplasmic reticulum, M for mitochondria, C for chloroplast, T for thylakoids, OEM for outer envelope membrane and IEM for inner envelope membrane. Enzyme names are framed. Solid lines indicate enzyme reaction, *dotted lines* indicate lipid transports and *semi-dotted lines* indicate enzyme regulation. (This scheme is adapted from Fig. 1 of Dubots et al. (2012).)

then either converted to CDP-DAG, before incorporation into plastid PG, or alternatively it is dephosphorylated to DAG before incorporation into plastid prokaryotic glycolipids that are galactolipids or SQDG. The importance of prokaryotic PA formation was first indicated by full invalidation of *ATS2* in Arabidopsis. The *ats2* knock out mutant is not viable and shows embryo development arrest at the stage of chloroplast formation (Kim and Huang 2004; Yu et al. 2004). The lipid phenotype of *ats1* mutants also illustrates this pathway since these mutants show a massive loss of prokaryotic lipids together

with a strong developmental defect (Xu et al. 2006). However, in contrast to the *ats2* mutant, the *ats1* mutants remain viable due to a residual activity of the mutated ATS1 proteins. Detailed lipid analysis indicated a complete loss of prokaryotic glycolipids and only a partial loss of plastid PG. This difference in glycolipid over PG synthesis suggests that plastid PG synthesis is dominant over prokaryotic glycolipid synthesis when synthesis of prokaryotic PA is limited.

In an alternative process, fatty acids are exported from the plastid before their integration into glycerolipids in the endoplasmic reticulum. This process is important for the formation of eukaryotic galactolipids. Koo et al. (2004) estimated the pool of exported fatty acid to 15–55 pmol C18 fatty acids/s/mg chlorophyll, representing 50–90% of newly synthesized fatty acids in leaves. The transfer mechanism of fatty acyl export through the chloroplast envelope membranes remains elusive. It probably combines the activity of an acyl-ACP thioesterase positioned in the inner membrane and an acyl-CoA synthetase in the outer membrane. From the analysis of the flux of fatty acids exported from the chloroplast, Koo et al. (2004) proposed that the movement of free fatty acids is a transporter-facilitated process with an intermediate protein between the acyl-ACP thioesterase and the acyl-CoA synthetase. Among the various long acyl CoA synthetases (LACSs) present in Arabidopsis, LACS9 could be involved in this transport since it is the preponderant long acyl CoA synthetase in plastids, but residual levels of fatty acid export and of long acyl CoA synthetase activity in chloroplast of a *lacs9* deletion mutant indicate that at least another acyl CoA synthetase should also be involved (Schnurr et al. 2002).

Exported acyl-CoAs are then incorporated into a large array of lipids such as glycerolipids, triglycerides, waxes, cutins and oxylipins. Among the resulting glycerolipids formed in this pathway, PC is a key intermediate in the formation of chloroplast eukaryotic galactolipids as was first demonstrated by Heinz and Harwood (1977) and Slack et al. (1977). PC has the typical eukaryotic structure, i.e., with C18 fatty acids at the *sn-2* position of glycerol, which is then transferred in about half of the chloroplast galactolipids in Arabidopsis. This structure is the consequence of the ER acyltransferase specificity for acyl-CoA during formation of PA (Frentzen et al. 1990). A number of genes encoding putative acyltransferases present in ER have been identified in Arabidopsis but not all have been characterized. *LPAT2* encodes the ubiquitous ER-located lysophosphatidic acid-acyl CoA acyltransferase and is essential for viability of the plant (Kim et al. 2005). Although not essential, *LPAT3* could replace *LPAT2* at least in some tissues such as the male gametophyte (Kim et al. 2005). It is, however, not clear whether LPAT2 or LPAT3 play a role in synthesis of a specific pool of PC that would specifically affects galactolipid synthesis. As highlighted by Benning (2009), it is important to note that PC fatty acid composition is also highly dependent on the extremely dynamic direct exchange between acyl-CoA and PC which is carried out by diverse phospholipases A, acyl-CoA synthetases and acyltransferases. These enzymes are highly responsive to the condition of plant growth and to the stage of plant development and this gives to PC synthesis a high degree of complexity. We do not know yet the full story of this PC fatty acid recycling on chloroplast galactolipid synthesis. However, it has been clearly shown by different studies on Arabidopsis mutants that PC desaturation is important for eukaryotic galactolipid formation with a specific requirement in the FAD2 C18:1 desaturase present in the ER (Okuley et al. 1994; Yang et al. 2006).

As we will see in the following paragraphs, the eukaryotic or prokaryotic galactolipids are initially formed by galactosylation of diacylglycerol (DAG) with UDP-galactose. In addition, MGDG synthases that catalyze the first galactosylation reaction are present in the chloroplast envelope. These characteristics of MGDG formation raise two linked questions. How is eukaryotic DAG backbone transferred from the PC of the ER to the chloroplast envelope MGDG? How does this mechanism

interact with formation of prokaryotic PA in the envelope?

The eukaryotic DAG formation results from hydrolysis of PC by either a phospholipase C (PLC), or a phospholipase D (PLD) coupled with a phosphatic acid phosphatase (PAP) (Andersson et al. 2004). Identification of these enzymes is fairly difficult because numerous different proteins have redundant activity and, in most conditions, adjustment of their expression can compensate for absence of others. Additionally, the PLD/PAP steps lead to a transient formation of PA that has a regulatory role in many different cell processes and can alter membrane biogenesis. This can indirectly modify chloroplast lipid composition. Analysis of Arabidopsis mutants and of plants grown under phosphate limitation, which enhances the eukaryotic galactolipid formation leading to replacement of phospholipids by DGDG in extra-plastidial membranes, indicated that a PLC, NPC4 (Nakamura et al. 2005), and two PLDs, PLDζ1 and PLDζ2 (Cruz-Ramirez et al. 2006; Li et al. 2006a, b), could play a role in the formation of eukaryotic galactolipids. However NPC4, which is a plasma membrane protein, has a low expression in leaves and is mostly involved in catabolism of plasma membrane PC under phosphate limitation (Nakamura et al. 2005). Similarly PLDζs are mostly expressed under phosphate deprivation and their role in supply of intermediates for eukaryotic lipid formation is limited to roots under phosphate deprivation (Cruz-Ramirez et al. 2006; Li et al. 2006a, b). PLDζ1 was localized in vesicles underlying plasma membrane (Ohashi et al. 2003) and PLDζ2 in tonoplast (Yamaryo et al. 2008), which suggests that they hydrolyze specific pools of PC in the cell probably outside of ER (Figs. 7.1 and 7.2a). Downstream of PLDs, a set of PAPs called PAH1 and PAH2 were shown to mediate eukaryotic galactolipid formation (Nakamura et al. 2009b). These proteins are also overexpressed in plants under phosphate limitation (Morcuende et al. 2007) and in this situation they cope critically with galactolipid formation (Nakamura et al. 2009b). On the other side, PAH1 and PAH2 have a regulatory role in activation of phospholipid synthesis at the ER and the activation of PC formation should likely also results in enhancing eukaryotic galactolipid formation (Eastmond et al. 2010).

Another PAP enzyme plays a role in chloroplast lipid formation. This enzyme is present in the chloroplast envelope membrane (Figs. 7.1 and 7.2a). It was initially proposed to function exclusively in prokaryotic galactolipid formation because the activity was detected in chloroplasts or chloroplast envelopes isolated from plants specifically containing prokaryotic galactolipids (Andrews et al. 1985). This enzyme has been characterized using isolated spinach chloroplast envelope (Joyard and Douce 1977, 1979; Block et al. 1983a; Malherbe et al. 1992). It is clearly different from other PAPs detected in the cell. It is a membrane bound enzyme tightly associated with the inner envelope membrane and it shows an alkaline optimum pH and a drastic inhibition by Mg^{2+} suggesting a link with photosynthetic activity. It is also highly retro-inhibited by DAG indicating a strong regulation between PA hydrolysis and DAG consumption in the membrane (Malherbe et al. 1992). Three Arabidopsis genes encoding chloroplast located PAPs, LPPγ, LPPε1, and LPPε2, have been identified (Nakamura et al. 2007). Only LPPγ has enzymatic properties matching to the previously characterized envelope enzyme. So far, none of the identified Arabidopsis PAP proteins has been found in the envelope protein repertoire (Joyard et al. 2010). Altogether the data indicate that these enzymes are present in minor amounts and active in specific pathways. Depletion of the *LPPγ* gene led to a lethal mutant, whereas the double knock out mutant *lppε1, lppε2* exhibited no lipid phenotype (Nakamura et al. 2007). This suggests that LPPγ plays a determinant role in hydrolysis of PA in the chloroplast inner envelope membrane and that accumulation of PA in chloroplast is toxic. However, more work is needed to understand whether LPPγ does play a role in chloroplast lipid formation and whether it is

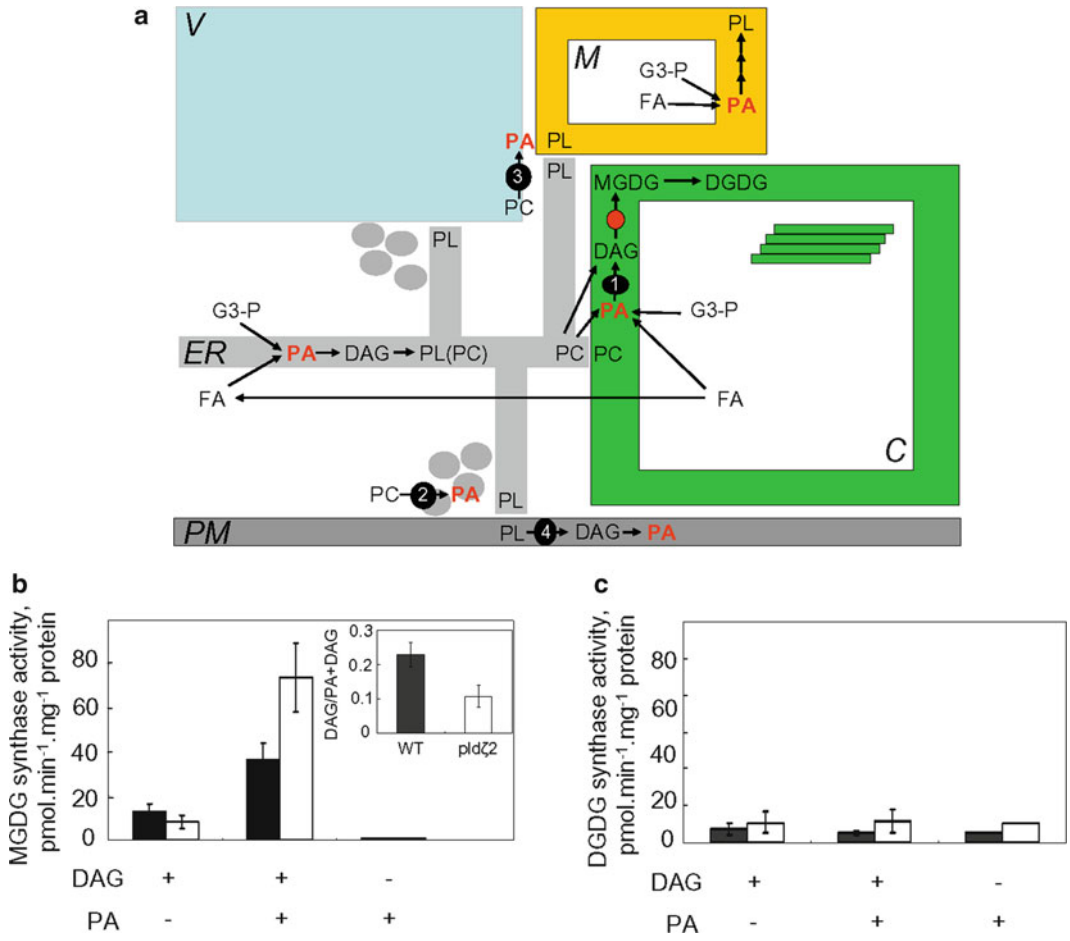

Fig. 7.2. Relationship between MGDG synthesis and PA formation. (**a**) Comparative localization of the MGDG synthesis and of the main spots of PA formation in plant cell on a schematic representation of the plant cell. MGDG synthase represented as a *red circle* is present in the chloroplast envelope. It catalyzes galactosylation of DAG issued from one part from PA synthesized inside chloroplast and for the other part from PC conversion. PC is synthesized from PA in the ER. The chloroplast envelope PAP (1) plays some role in the conversion of PA into MGDG. It is a regulated enzyme highly inhibited by DAG and Mg^{2+} suggesting links between photosynthesis activity and chloroplast lipid biogenesis. The PLDs, PLDζ1 (2) in vesicles underlying the plasma membrane, PLDζ2 (3) in the vacuole membrane, and the PLC NPC4 (4) in the plasma membrane play some role in the conversion of PC into MGDG. *C* chloroplast, *M* mitochondria, *V* vacuole, *ER* endoplasmic reticulum, *PM* plasma membrane. (**b** and **c**) Activation of MGDG synthase by PA in Arabidopsis leaf homogenates as reported by Dubots et al. (2010). MGDG synthase activity (**b**) and DGDG synthase activity (**c**) in wild-type and in the *pldζ2* knock out mutant. PA (1.5 mol%), DAG (7 mol%) were added as indicated. The *inset* shows the ratio of the activities measured in the absence of PA versus in the presence of PA.

involved in hydrolysis of both prokaryotic and eukaryotic PAs.

The transfer of the DAG-containing lipid intermediates from the ER to the chloroplast is critical in the formation of eukaryotic galactolipids. Inter-membrane transport of lipid molecules through an aqueous phase, a general problem in cell biology, is now resolved in only a few specific cases (for a review see Jouhet et al. 2007). In the case of chloroplast galactolipid formation, it is still discussed what kind of molecule is shuttled between ER and chloroplast. Since PC is present in the outer layer of the chloroplast

envelope, it has been initially proposed that PC (or Lyso-PC) is the transported intermediate (Miquel et al. 1988; Bessoule et al. 1995). However, the absence of detectable phospholipase activity in the chloroplast envelope questions this hypothesis. The extra-chloroplast location of phospholipases further suggests that the transported intermediate is rather PA or/and DAG. Jouhet and coworkers indeed observed that DAG, with a similar fatty acid composition as PC, increases under phosphate starvation, whereas it does not accumulate in plastids (Jouhet et al. 2003). On the other hand, recent work on a set of TGD proteins indicates that a PA interacting complex present in the envelope is involved in formation of eukaryotic galactolipids. The TGD proteins assemble in a ABC-type complex associated with the chloroplast envelope and one subunit, TGD2, contains a PA-binding site (Xu et al. 2003, 2008, 2010; Awai et al. 2006; Lu et al. 2007). This complex possibly supplies PA for DAG formation in the envelope. Introduction of PA in a membrane can induce modification of membrane curvature and enhance membrane fusion. TGD complexes might possibly also stimulate membrane fusion between ER and chloroplast membrane and contribute to the transfer of other lipid intermediates that remain to be identified. As a support of this hypothesis, by confocal microscopy combining laser scalpel and optical tweezers manipulation, Andersson et al. (2007) observed resistant membrane contact sites between the ER network and the chloroplast surface (Andersson et al. 2007). The challenge is now to identify these membrane contact sites.

B. Monogalactosyldiacylglycerol Synthesis

MGDG synthesis is catalyzed by the UDP-galactose:DAG galactosyltransferase, also called MGDG synthase. This activity is uniquely detected in the chloroplast envelope (Douce 1974; Douce and Joyard 1990; Block et al. 2001) and was reported in the inner membrane in *Spinacia oleracea* (Block et al. 1983b) and in the outer membrane in *Pisum sativum* (Cline and Keegstra 1983). Shimojima et al. (1997) reported the first identification of an MGDG synthase and several other MGDG synthases were subsequently identified (Miège et al. 1999; Awai et al. 2001). The proteins are GT28 glycosyltransferases that link galactose onto glycerol by a β-glycosidic bond. They can be classified in two families: type A and type B MGDG synthases (Awai et al. 2001). In *A. thaliana,* there are two genes: *MGD1* that belongs to type A, and *MGD2* and *MGD3* to type B. The two families differ by (1) the presence of a classical chloroplast transit peptide in type A, but its absence in type B, (2) the localization of type A in the inner envelope membrane whereas type B is in the outer membrane, (3) the major expression of type A in photosynthetic tissues, (4) the higher expression of type B in roots and flowers and their overexpression in phosphate limiting conditions (Awai et al. 2001).

The fundamental role of MGDG synthase 1(MGD1) in biosynthesis of chloroplast membranes was demonstrated by analysis of several Arabidopsis *mgd1* mutants. The *mgd1-1* mutant has a yellow-green phenotype and shows a 75% reduction in *MGD1* mRNA abundance which correlates with reduction of the level of MGDG synthase activity in chloroplasts; the abundance of MGDG in mature leaves is therefore reduced by 42% (Jarvis et al. 2000). The vital role of MGD1 in the formation of chloroplast membranes was further demonstrated by analysis of the knock out *mgd1-2* mutant where the complete suppression of MGD1 induces a drastic reduction of MGDG and DGDG content and an impairment of photosynthetic growth (Kobayashi et al. 2007). The chloroplast internal membrane network was severely underdeveloped, and conversely an abundant invagination development of the inner envelope membrane was observed suggesting a temporary mechanical interaction between the inner envelope membrane and the thylakoid network with a blockage of their interaction. Interestingly, the double-membrane structure of the chloroplast

envelope looked normal despite the very low level of galactolipids in the mutant. This suggests that biogenesis of the chloroplast envelope either doesn't require galactolipids or more likely can be carried by type B MGDG synthases. The role of type B MGDG synthases is actually complex. Their expression is restricted to some parts of tissues, such as leaf tips, flower tips or pistils. In flowers, galactolipid synthesis is activated during development of pistils and pollen tubes suggesting a role in pollen tube elongation although fertility was apparently normal in the double mutant *mgd2, mgd3* (Kobayashi et al. 2004; Nakamura et al. 2009a). Type B MGDG synthases likely contribute to supply MGDG as a precursor for DGDG synthesis as was demonstrated in the case of plants grown under phosphate limitation (Kobayashi et al. 2009). Reduction in fresh weight, root growth and photosynthetic performance in the double mutant *mgd2, mgd3* under phosphate starved conditions indicated that type B MGDs contribute to the adaptation of plants to specific environmental conditions (Kobayashi et al. 2009).

Based on a high throughput chemical screen, a MGD inhibitor, called Galvestine-1, has been recently characterized (Botté et al. 2011). Inhibition of the different types of MGDs in Arabidopsis plants caused a reduction of MGDG content and an impairment of chloroplast development with a dose-dependent manner. In transmission electron micrographs of mesophyll cells, chloroplasts looked smaller and had less thylakoids with increasing doses of Galvestine-1. Like in the Arabidopsis *mgd1-2* null mutant, the inner envelope membrane of chloroplasts showed invaginations. Although Galvestine-1 treatment did not affect pollen grain germination rate, it showed a striking effect on pollen tube growth, marked by a partial inhibition of the elongation, which is consistent with a contribution of MGD2 and MGD3 to half of the membrane expansion required for pollen tube elongation, a process known as one of the fastest cellular growth in nature. Effects of Galvestine-1 on whole-genome transcriptional response of Arabidopsis finally pointed to potential benefits of Galvestine-1 as a novel tool to functionally study lipid homeostasis in plants.

C. Digalactosyldiacylglycerol Synthesis

The characterization of the *A. thaliana dgd1* mutant, which is severely impaired in DGDG synthesis led to the first identification of a DGDG synthase gene (Dörmann et al. 1995). DGD1 is composed of two distinct domains: a C-terminal glycosyltransferase domain and an N-terminal domain with a probable regulatory function. DGD1 is the main MGDG-UDP-galactose galactosyltransferase in plants. By similarity to *DGD1*, a second gene, *DGD2*, was identified (Dörmann et al. 1999). The DGD2 protein possesses only the glycosyltransferase domain. The *DGD2* gene is not essential for DGDG synthesis under optimal growth conditions and encodes, as *DGD1,* a MGDG-UDP-galactose galactosyltransferase (Kelly and Dormann 2002; Kelly et al. 2003). Refined analyses of the *dgd1* and *dgd2* mutants indicated that DGD1 preferentially acts on MGDG molecular species with C18 fatty acids at both *sn*-positions of glycerol whereas DGD2 seems to preferentially function on MGDG with C16 fatty acids at the *sn-1* position, and C18 fatty acids at *sn-2* position (Kelly et al. 2003). Both enzymes are located on the outer surface of the chloroplast envelope (Froehlich et al. 2001; Kelly et al. 2003), but an additional mitochondrial association is possible for DGD1 since the protein was detected in a proteome survey of mitochondrial membranes (Heazlewood et al. 2004).

The presence of another enzyme was suspected because the double knock out mutant *dgd1,dgd2* still contained some trace amounts of DGDG (Kelly et al. 2003). Indeed another DGDG-forming enzyme was recently identified (Moellering et al. 2010). This enzyme called SFR2 does not require UDP-galactose and processively transfers galactosyl residues from MGDG to different galactolipid acceptors forming oligogalactolipids (i.e. DGDG, TriGDG,

TetraGDG) and DAG. It is a GH1 glycosyltransferase that leads to the formation of a different structure of DGDG compared to DGDG formed by the previously described DGDG synthases. The second galactose is linked to the first one by a β-glycosidic bond instead of classical α-glycosidic bond in DGDG. The activity was previously described in isolated plastids as a galactolipid:galactolipid galactosyltransferase (van Besouw and Wintermans 1978) and localized on the outer surface of the chloroplast envelope (Dorne et al. 1982). Several results indicated that this enzyme was activated under some specific conditions. Its activation was, in particular, shown in isolated chloroplast envelopes (Wintermans et al. 1981; Dorne et al. 1982). The activation was also shown under ozone treatment (Sakaki et al. 1990), during protoplast formation (Klaus et al. 2002) and in *tgd* mutants (Xu et al. 2003). Characterization of the *sfr2* mutant now indicates that SFR2 plays a determinant role in freezing tolerance (Moellering et al. 2010). Formation of oligogalactolipids from MGDG corresponds to an increase in the ratio of lamellar versus hexagonal II forming lipids and therefore strengthening of the membrane bilayer structure. This could prevent inter-membrane fusion during dehydration by freezing. Additionally, formation of TAG by acylation of SFR2-produced DAG constitutes a lipid storage that helps the plant to restore after freezing.

D. Phosphatidylglycerol Synthesis

In plant cells, PG synthesis occurs in three different compartments: ER, mitochondria and chloroplast. However, as stated above, chloroplast PG has a unique prokaryotic structure that points out its origin from the prokaryotic PA synthesized in the chloroplast envelope. Furthermore, its production is restricted to the chloroplast envelope since the overproduction of eukaryotic PA, specifically in the envelope, artificially activates production of eukaryotic chloroplast PG (Fritz et al. 2007). Activities of CDP-DAG synthetase, phosphatidylglycerol-phosphate synthase and phosphatidylglycerol-phosphate phosphatase were detected in the inner envelope membrane (Andrews and Mudd 1985). Proteomics on *A. thaliana* confirmed the presence of one specific CDP-DAG synthetase in the chloroplast envelope (Joyard et al. 2010). Two related genes encode phosphatidylglycerol-phosphate synthases: *PGP1* and *PGP2* (Muller and Frentzen 2001; Frentzen 2004). Although proteomics identified both PGP1 and PGP2 in the chloroplast envelope (Joyard et al. 2010), it is considered that PGP2 is a microsomal phosphatidylglycerol-phosphate phosphatase while PGP1 can be imported into both mitochondria and chloroplast. The importance of PGP1 in synthesis of chloroplast PG was brought by analysis of *pgp1* mutants (Xu et al. 2002; Babiychuk et al. 2003). The *pgp1-1* mutant has an overall PG content reduced by 30% and shows 80% reduction in chloroplast phosphatidylglycerol-phosphate phosphatase activity.

E. Sulfoquinovosyldiacylglycerol Synthesis

The formation of UDP-sulfoquinovosyl is the first distinctive step in SQDG synthesis (Benning 1998). It is catalyzed by SQD1 (Sanda et al. 2001). The mechanism of the reaction has been described by Essigmann et al. (1999) and Mulichak et al. (1999). In a second step, SQD2 transfers sulfoquinovose from UDP-sulfoquinovosyl onto DAG (Yu et al. 2002). The SQD2 activity was localized in the inner envelope membrane (Tietje and Heinz 1998). It is noticed that the pool of DAG molecules present in the chloroplast envelope is shared by galactolipid and SQDG synthesis as was shown by experiments on isolated envelope membranes supplied with UDP-galactose and UDP-sulfoquinovose (Joyard et al. 1986; Seifert and Heinz 1992). The balance between MGDG and SQDG synthesis is dependent

on the relative level between UDP-galactose and UDP-sulfoquinovose and on the affinity of the enzymes for specific DAG molecular species. For instance, specific enrichment of SQDG in C16:0 fatty acid is presumably related to the high affinity of SQD2 for C16:0/C16:0 DAG.

F. Chloroplast Lipid Desaturation

In opposition to the first desaturation of (C18:0) stearic acid, which is achieved on stearoyl-ACP (see above), desaturation on (C16:0) palmitic acid and (*cis*-9-C18:1) oleic acid is introduced on fatty acids esterified to lipids. Desaturation results in incorporation of double bonds at a few preferential positions always in the same order. In PG, C16:0 is *trans*-desaturated on ω13 (ω indicates numbering from the fatty acid distal carbon) whereas in glycolipids, C16:0 is desaturated first on ω9, then on ω6 and finally on ω3. Double bonds are on *cis* configuration. In all glycerolipids, C18:1 is desaturated first on ω6 and then on ω3 with a *cis* configuration. Characterization of the desaturase genes was early achieved by a ground-breaking reverse genetics strategy on Arabidopsis (Ohlrogge et al. 1991). These desaturases are membrane-bound enzymes located for most of them in the chloroplast except for FAD2 that is present in the ER. FAD4 catalyzes the *trans*-desaturation of C16:0 on PG, FAD5 and FAD6 the ω9 and ω6 desaturation on MGDG, FAD7 and FAD8 the ω3 on all chloroplast glycerolipids. All the chloroplast desaturases, except FAD4 that is presumably lower-represented, were found in the chloroplast envelope by proteomics (Joyard et al. 2010). This corroborates with previous data that showed that it was possible to achieve desaturation of MGDG in vitro on isolated chloroplasts and isolated envelope membranes (Heinz and Roughan 1983; Schmidt and Heinz 1990, 1993). However, desaturation is presumably dependent on an active system of electron transport (Andrews et al. 1989; Schmidt and Heinz 1990). Using EPR spectroscopy on spinach chloroplast envelope membranes, Jager-Vottero et al. (1997) characterized EPR signals corresponding to putative components of an electron transfer chain but characterization of the electron transport chain accurately associated with fatty acid desaturation is still underway.

G. Transport of Lipids from Envelope to Thylakoids

Thylakoids do not contain the lipid synthetic machinery. Once synthesized in the envelope, chloroplast glycerolipids, i.e., MGDG, DGDG, PG, and also SQDG, are transported to the thylakoids (Siebertz et al. 1980). We don't yet know exactly what the mode of transport is. Vesicle budding from the envelope inner membrane suggests a bulk transfer of lipids through vesicular trafficking. In support of this hypothesis, vesicles were observed by electron microscopy in young chloroplasts, particularly at low temperature that should presumably slow down the process (Carde et al. 1982; Morre et al. 1991). Recent observations of an increase in number of invaginations developed from the inner envelope membrane in plants impaired in MGDG synthesis indicate indeed a link with a slowdown of lipid synthesis (Kobayashi et al. 2007; Botté et al. 2011). After preliminary attempts to analyze the biochemical characteristics of a chloroplast vesicular trafficking (Räntfors et al. 2000; Andersson et al. 2001), bioinformatic studies suggested that a system similar to the COPII vesicular pathway is present in plastids (Andersson and Sandelius 2004). Several proteins involved in this trafficking have been identified, such as an NSF homolog protein (Hugueney et al. 1995), a dynamin-like protein called ADL1 (Park et al. 1998), a putative regulator of vesicle coalescence called thylakoid formation 1 (THF 1) (Wang et al. 2004), and a vesicle-inducing protein called vesicle inducing protein in plastid 1 (VIPP1) (Kroll et al. 2001). VIPP1 is a hydrophilic protein found in both the inner envelope and the thylakoid membranes (Li et al. 1994).

In a mutant with reduced expression of *VIPP1,* a disturbed network of thylakoids was observed and vesicle budding from the inner envelope at low temperature was abolished (Kroll et al. 2001). Recent data demonstrated that VIPP1 forms a high molecular mass complex closely associated with the inner envelope membrane but with no apparent protein-protein interaction with lipid synthesizing enzymes. In addition, these results suggested that the C-terminus of the protein protrudes from the complex into the chloroplast stroma possibly for interaction with some other proteins (Aseeva et al. 2007), and indeed it has been shown that soluble VIPP1 interacts with the HSP70B/CDJ2 chaperone pair, a system that could favour a cycle of assembly/disassembly necessary for vesicle movement (Liu et al. 2005). Although there is no doubt that a vesicular trafficking exists inside the chloroplast and is important for the formation of thylakoid membranes, its relationship with lipid trafficking is uncertain. Even though the *vipp1* mutants have a distorted internal membrane network, they have normal lipid composition, likely because the inner envelope membrane and the thylakoids have the same lipid composition and, therefore, accumulation of an intermediary pool of membranes does not change the overall lipid composition. The observation of arrested vesicle/budding in plants impaired in MGDG synthesis is therefore puzzling and supports the existence of other proteins coordinating envelope invagination with galactolipid assembly.

IV. MGDG Synthase in Chloroplast Biogenesis: Molecular Mechanism and Regulation of the MGD1 Enzyme

A. MGD1 Structure

MGDG synthase is of key importance in chloroplast biogenesis. As reported above, there are two types of MGD enzymes. In most plants, MGD1 is the only type A enzyme. In Arabidopsis, MGD1 is essential for the biogenesis of the thylakoid membranes whereas the type B enzymes, MGD2/MGD3, can perhaps contribute to the biogenesis of the chloroplast envelope. Most of the enzymatic characterization on MGDG synthase was done on MGD1. In this chapter we will describe the functional characteristics of this enzyme in comparison with those of MGD2/MGD3. We will analyze the different regulatory mechanisms that control its activity and introduce how they contribute to the integration of environmental signals in plant development with specific reference to photosynthesis.

Since none of the MGDG synthase could be crystallized for obtaining structural details at atomic level, a computed model for the enzyme structure has been developed. The different MGD sequences share a strong similarity with MURG, a bacterial glycosyltransferase, and using the x-ray structure of *E. coli* MURG as a template, several features of the MGD structure have been established (Botté et al. 2005). These characteristics were compared with those previously deduced from enzymology analysis (Maréchal et al. 1995), and were further refined by site directed mutagenesis approaches of MGD1 in *Spinacia oleracea* and *Arabidopsis thaliana* (Botté et al. 2005). The predicted architecture of MGD consists of a double Rossmann fold of equivalent size. Each Rossmann fold corresponds to either the N- or the C-terminal half of the sequence and the overall structure is consolidated by two α-helices at the extreme C-terminal end of the sequence coming back over the N-domain. The catalytic site is predicted in the deep cleft separating the two Rossmann folds. From similarity with MURG it is supposed that, upon binding of the substrates, there is a change in the relative orientation of the twofolds on both sides of the cleft. Binding sites for UDP-galactose and DAG are spatially distinct since analysis of MGDG synthase activity from the spinach chloroplast envelope has shown that the reaction works as a sequential, either random or ordered, bireactant system, in which the

binding of one substrate does not significantly change the specificity for the cosubstrate. Moreover, inhibition by UDP is competitive in regard of UDP-galactose and non-competitive in regard of DAG. In the computed model, the binding sites were predicted in the C-domain for UDP-galactose and in the N-domain for DAG. The position of the UDP-galactose binding site was verified by site directed mutagenesis. The proline residue at position 422 in SoMGD1, conserved in all MGD sequences, is likely to be important for galactose specificity. On the other side, the recognition domain for DAG could not be exactly determined because MURG does not handle such a hydrophobic substrate and, secondly, there is a high discrepancy between MURG and MGD in this part of the sequence. Since the MGD1α2-β2 loop has no counterpart in MURG, and since mutation of the tryptophan residue at position 117 affects affinity for DAG, it is likely that the MGD1α2-β2 loop is involved in DAG handling (Botté et al. 2005).

MGD1 is a membrane protein and the way it is positioned in the membrane should be related to the way the protein interacts with lipids. MGD1 is a monotopic enzyme associated with one lipid leaflet of the inner envelope membrane (Block et al. 1983b; Miège et al. 1999; Awai et al. 2001). Using the computed model, membrane association was sought by surface analysis. Surface hydrophobic patches were evidenced on the N-domain as expected for interaction of the N-domain with the membrane and protruding of the C-domain over the membrane. Inactivation kinetics after γ-ray irradiation have shown that the enzyme is active as a dimer, which was later confirmed by cross-linking experiments (Miège et al. 1999; Nishiyama et al. 2003). The two monomers might therefore stand on the membrane, catalytic clefts most probably close to each other in order to pick up DAG at a single point in the membrane although a more distant location can not be excluded at this stage. Distribution of DAG in the membrane is presently unknown and will have to be considered to understand how MGDG synthase works. An additional feature that may be important in the overall structure of the enzyme is the possible presence of a metal. The sensitivity to the hydrophobic chelating agent *o*-phenanthroline, and the recovery of the activity after addition of bivalent metal cations such as Zn^{2+}, Mg^{2+} or Cu^{2+}, supported the existence of one or more metal associated with the enzyme (Maréchal et al. 1994a, b, 1995). This was further supported by the high sensitivity of MGD1 to nitric oxide (E. Maréchal, personal communication). Metal coordination might imply cysteine and/or histidine residues, which were indeed shown to be important for activity. It is, however, still not clear whether metal coordination might be directly involved in catalysis, stability of the overall structure, and/or MGD dimerization. Altogether, MGD1 is positioned at the interface between a membrane lipid substrate and an aqueous phase and its activity is likely constrained in a complex structure depending on how DAG is reachable.

B. MGD1 Regulation by Substrates and Products

MGD1 catalyzes the synthesis of both prokaryotic and eukaryotic MGDG molecular species. On a mixed micelle system including CHAPS detergent, using the surface dilution model, it has been shown that the MGDG synthase from spinach chloroplast envelopes is able to use several DAG molecular species, but with different affinities (Maréchal et al. 1994a). The prokaryotic DAG synthesized within the chloroplast, i.e., with C18:1/C16:0, was a better substrate (K_m C18:1/C16:0 = 2.9 mol%) than eukaryotic DAG with C16:0/C18:1 (K_m C16:0/C18:1 = 4.2 mol%). In the analysis, the highest affinity was observed for eukaryotic dilinoleoylglycerol (K_m C18:2/C18:2 = 0.89 mol%). The relevance of these results was further confirmed in envelope lipid vesicles and on recombinant Arabidopsis MGD1 (Maréchal et al. 1994b; Awai et al. 2001). Interestingly, the type B enzymes

were much less active than MGD1 with a five times lower Vmax and a 3–10 times higher Km, although they exhibited a higher affinity for the eukaryotic DAG with C18:2/C18:2 than for the prokaryotic DAG with C18:1/C16:0 (Awai et al. 2001). This difference of activity has to be considered with the difference of location of the enzymes, type B MGDs on the cytosolic surface of the chloroplast whereas MGD1 is in the inner envelope membrane facing the inter-membrane space (Awai et al. 2001; Xu et al. 2005; Vojta et al. 2007), and also with the manner DAG is supplied to the enzymes.

While the supply of DAG is critical, the supply of UDP-galactose is considered non-limited. This later point was not unambiguously proven. However, it is assumed that the UDP-galactose concentration in the envelope membrane inter-space is in equilibrium with the UDP-galactose concentration in the cytosol which is likely not limited since first, UDP-galactose results from epimerisation of UDP-glucose, second, UDP-glucose concentration in the cytosol is far above the Km of MGD1 for UDP-galactose, and finally epimerase expression does not correlate with galactolipid synthesis. Feed back control of the enzyme by its products does not appear either as a major control point. It is considered that MGDG does not inhibit the reaction and that UDP, although a competitive inhibitor of MGD for UDP-galactose, has a Ki (order of magnitude 10–20 μM) far above the UDP concentration in the vicinity of the envelope.

C. Activation of MGD1 by Phospholipids

If we consider that the envelope membrane is mainly composed of neutral galactolipids (MGDG and DGDG) and anionic lipids (PG and SQDG), MGDG synthesis activity increases the ratio of neutral versus anionic lipids in the membrane. In order to maintain lipid homeostasis in the membrane, coordination between the different lipid supplying systems must occur. A possible control relies on enzyme regulation by lipids. Several works indeed indicated that anionic lipids such as PG, PA and SQDG enhance MGDG synthase activity (Coves et al. 1988; Ohta et al. 1995; Kelly et al. 2007). We have seen above with the *ats2* and *lpat2* mutants that blockage of PA synthesis results in severe and early arrest of plant development. PA is a general precursor in the glycerolipid synthesis pathway and a signaling molecule. Regulation of MGD1 by PA might therefore represent a potential way of controlling chloroplast biogenesis during plant development. In this context, regulation of MGD1 by PA and PG was recently analyzed in detail on native enzymes in leaf extracts or on the recombinant AtΔ1-137 MGD1 enzyme (Dubots et al. 2010). No activity was measured when the enzyme was, except for the supply of its substrate DAG, totally depleted of lipids. However, addition of a very low concentration of PA was able to restore the activity. Restoration was also possible with PG but at much higher concentration. No activity was recorded with PA or PG in absence of DAG. These results therefore suggested an allosteric regulation of MGDG synthase by PA and PG that is to say that the conformation of the enzyme was modified by binding of PA and PG at another site than the substrate binding sites. Binding of PA and PG on MGD1 was indeed observed by lipid/protein overlay. Activation constants were 0.2 mol% for PA and 5 mol% for PG. PG concentration in the envelope is near 8 mol% and PA is not detected in the envelope. However, activity of the native MGDG synthase in the chloroplast envelope lipid background was drastically reduced after the envelope was incubated in optimum condition for PA phosphatase and was restored by addition of PA. This experiment therefore indicated the crucial importance of PA for the enzyme activity. $K_{0.5}$ of spinach chloroplast envelope MGDG synthase for PA was estimated to 0.6 mol%. Several molecular species of PA were able to activate the enzyme, some eukaryotic as well as some prokaryotic molecular species, suggesting a

connection with different sources of cellular PA including some non-chloroplast sources. On the other hand, PG is likely to be also important for MGDG synthase activity since PA and PG do not activate the enzyme by the same process. It was observed that the curve of enzyme velocity versus lipid activator concentration was sigmoid with PA but hyperbolic with PG, which is indicative of a simpler way of interaction of the enzyme with PG than with PA. Although a higher concentration of PG was required to reach apparent maximum velocity, PG activation allowed a higher apparent maximum velocity than PA activation, closer to the true maximum velocity of the enzyme. Different and complementary activations were confirmed by observation of the synergetic effects of PA and PG on the enzyme. Finally, a molecular analysis of the activation was attempted. Analysis of salt effects indicated hydrophobic interaction of PA with the enzyme, i.e. increased activation by PA when salt concentration was high, and electrostatic interaction of PG, i.e. decreased activation by PG when the salt concentration was high, and, lastly, point mutations of the enzyme allowed determination of two amino acids specifically important for activation by PG (Dubots et al. 2010). These two residues, proline at position 189 and tryptophan at position 287 in AtMGD1, are present in the N-domain of MGD1 which was previously proposed to interact with the membrane (Botté et al. 2005), but their mutation apparently did not affect binding of MGD1 to the membrane. Challenge is now to show that activation of MGD1 by PG plays indeed a role in coupling MGDG synthesis with PG synthesis thus leading to a synchronized formation of MGDG and PG, two essential components of photosynthetic membranes.

Activation of MGD1 by PA may play more diverse functions in the cell. It is likely that the PA level in the envelope is quickly turning over and is, therefore, connected with several different metabolic pathways. PA in leaves represents about 0.3 mol% of total glycerolipids with mainly molecular species containing a total of 34 carbons and 2 or 3 double bonds altogether in fatty acids (34:2 and 34:3) (Wang et al. 2006). In chloroplasts, PA level is very low, not detected in the envelope, and around 0.08 mol% in thylakoids, mainly prokaryotic type of PA with C18:1 at *sn-1* position of glycerol and C16:0 at *sn-2* as reported for tobacco by Fritz et al. (2007). This molecular species of PA is synthesized in the envelope; therefore we can assume that it is transiently present in the envelope. Very likely, the eukaryotic type of PA is imported from non-chloroplast membranes and/or resulting from PLD activity on the envelope. PC is also present in the envelope (Fig. 7.2a). In plants under cold stress, detection of molecular species of PA rich in C16:3 (Welti et al. 2002), a fatty acid typical of MGDG, indicates that it is possible that PA is also formed from MGDG. Its relationship with DAG formed from MGDG by activation of SFR2 under cold stress (Moellering et al. 2010) is not known. The PA level can moreover decrease in the envelope by the activity of several enzymes such as PG synthesizing enzymes, PAPs, and probably phospholipases A and acylhydrolases, each pathway with its own specificity for PA molecular species. Connection with PG synthesis suggests that activation by prokaryotic PA could play the same role as suggested above for activation by PG. Since PAP activity in the envelope is highly inhibited by Mg^{2+}, whose level in chloroplast is increased during the light phase of photosynthesis, activation of MGDG synthase by PA may alternatively suggest a control depending on the stage of photosynthetic activity. Inhibition of PAP by DAG may at this step favour supply to MGDG synthase of DAG coming from outside the chloroplast. At last, PA issued from PLD is a mediator in numerous stress responses in plants (Wang et al. 2006; Munnik and Testerink 2009).

Twelve PLDs have already been reported in Arabidopsis. They can specifically hydrolyze a diverse array of phospholipids from PC, PE, PS, PG to N-acyl PE with possible effectors such as Ca^{2+}, oleate or phosphoinositides.

Altogether they are present in all cellular membranes and are activated in a large panel of stresses, from cold, saline, defense to nutritional stresses. Analysis of Arabidopsis mutants indicated that PLDζs, particularly PLDζ2, plays a role in galactolipid synthesis under phosphate deprivation (Cruz-Ramirez et al. 2006; Li et al. 2006b). In leaves, it has been observed that PLDζ2 is a potent source of PA for the activation of MGDG synthase since MGDG synthase activity is weaker in the *pldζ2* knock out mutant unless it is activated by an exogenous source of PA (Fig. 7.2b) whereas DGDG synthase activity is stable and independent of PA addition (Fig. 7.2c). Since PLDζ2 is located on the membrane of vacuoles where phosphate is stored (Yamaryo et al. 2008), it has been proposed that PA produced by PLDζ2 can tune MGDG synthesis as a function of phosphate availability (Dubots et al. 2010). Finally, activation of MGD1 by PA suggests many different lines of control of MGDG synthesis, which stress the importance of this enzyme in chloroplast biogenesis.

V. Conclusions and Perspectives

Chloroplast membranes have a very well-maintained lipid composition characterized by a high level of galactolipids, MGDG and DGDG, with a moderate level of phospholipids, mostly PG, a finely compartmentalized amount of PC and a very low and transitory level of PA. Mutant analysis coupled to biochemical and structural investigations indicate that galactolipid and PG syntheses are essential for building up and functioning of photosynthetic membranes. However, much still remains to understand how the lipid synthesizing enzymes work.

These enzymes are constrained to work on the bilayer with their substrates and they deeply interact with a variety of lipid components present in the membrane. Studies on the MGDG synthase indicated that this enzyme is not only dependent on the nature and the flux of lipid substrates, i.e. DAG molecular species, that flows through the membrane but that its active conformation is also affected by other lipids, noteworthy PG and PA. This suggests that the homeostatic control of the membrane composition is driven by such fine regulation of lipid synthesizing enzymes. This additionally suggests that the integration of chloroplast membrane biogenesis according to the physiological status of the plant is dependent on these regulations. The challenge is now to show that these interactions indeed affect the final lipid composition of the membrane *in planta* and to determine precisely the physiological conditions modulating MGDG synthesis.

Galactolipid and PG synthesis occur in the envelope membranes and are necessary for thylakoid development. Different works suggest that these lipids are transferred from the envelope to thylakoids by vesicular trafficking. On the other hand, membrane invaginations connected with the inner envelope have been observed in plants if MGDG synthesis is blocked. Altogether this suggests that formation of a membrane with a correct lipid composition is necessary to allow disconnection of what we would call pre-thylakoid membranes from the inner envelope before final insertion of protein components characteristic of photosynthetic membrane. It will be interesting to determine if there is a specific location in the envelope of these sites of membrane generation and how they get organized.

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Chapter 8

Chloroplast Contact to the Endoplasmic Reticulum and Lipid Trafficking

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Summary

The higher plant chloroplast membranes are mainly composed of galactolipids assembled from diacylglycerol backbones in the chloroplast envelope. All plants depend on import of diacylglycerol backbones from the endoplasmic reticulum (ER). During phosphate limitation digalactosyl diacylglycerol synthesized in the plastid envelope is exported to other extra-plastid membranes. In addition, use of fatty acid desaturase mutants demonstrates lipid export from the chloroplast under normal growth conditions as well. Isoprenoid compounds such as plastoquinone are also most likely transported from the ER to the chloroplast. Thus, there must be one or several mechanisms present in the plant cell to mediate directional transport of highly hydrophobic molecules between the ER and the plastid. The molecular details of the transport system(s) remain to be determined. However, there is evidence to suggest that a specialized domain of the ER is associated with chloroplasts and thus likely to be directly involved in transfer of hydrophobic compounds between the compartments. The identification of the TGD-transporter complex represents a significant advance towards understanding the transport of precursors for membrane lipid synthesis in the plastid. The recently described molecular details of contact sites between ER and mitochondria in yeast and mammalian cells should inspire similar studies to uncover the same details in ER chloroplast contacts in plants.

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I. The Need for Exchange of Compounds Between the Chloroplast and the Endoplasmic Reticulum (ER)

The chloroplast membrane lipids as outlined in the chapter by Block (Chap. 7) consist mainly of the galactolipids mono- (MGDG) and digalactosyl diacylglycerol (DGDG). The backbones for the galactolipids are assembled by two distinct biosynthetic pathways. A portion is assembled directly in the plastid envelope from glycerol phosphate and acyl-ACP. This pathway is directly descendent from the plastids prokaryotic origin and the lipids produced in this pathway are sometimes referred to as "prokaryotic". The rest of the diacylglycerol backbones needed in the plastid are assembled in the ER from acyl-CoA acyl donors, these lipids are consequently sometimes referred to as "eukaryotic" plastid lipids. It is however, important to realize that all the fatty acids used in both pathways are derived from fatty acid synthesis inside the plastid. All higher plants depend on the "eukaryotic" ER-pathway for supply of diacylglycerol backbones for synthesis of the major plastid galactolipids in the plastid envelope. In a minority (Mongrand et al. 1998) of all investigated plants, the prokaryotic pathway contributes a portion varying from a few percent to about half of the diacylglycerol backbones to galactolipid synthesis. The latter are referred to as 16:3 plants, since the prokaryotic pathway always introduces a C16 acyl group on the *sn-2* position, whereas the ER-localized acyl transferases introduce C18 acyl groups at this position. Importantly, the model plant *Arabidopsis thaliana* is a 16:3 plant and thus amenable for genetic manipulation of both pathways. Finally, the prokaryotic pathway is used in all plants to supply the plastid with the anionic phospholipid phosphatidyl glycerol (PG). For a full description of acyl lipid metabolism in the plastid envelope the reader is referred to one of several extensive reviews on the topic (Block, Chap. 7; Dörmann and Benning 2002; Andersson and Dörmann 2008; Li-Beisson et al. 2010).[1] Since all plants depend on the ER localized lipid biosynthesis pathway, there is a need for transfer of diacylglycerol or a precursor for this molecule from the ER to the plastid envelope during normal plastid development. Under some circumstances there also seems to be export of intact acyl lipids from the plastid to other membranes in the plant cell. Finally in addition to acyl lipids, there are other hydrophobic compounds, which appear to be transported between the ER and the plastid. Thus, highly hydrophobic compounds are exchanged between the plastid envelope and the ER. Molecular details for the trafficking mechanism(s) are scarce. As I will outline below, several lines of evidence suggest that hydrophobic compounds are transported by specialized transporter complexes localized at specific domains of close physical association between the plastid and the ER membrane.

Abbreviations: ACP – Acyl carrier protein; CoA – Coenzyme A; DAG – Diacylglycerol; DGDG – Digalactosyl diacylglycerol; ER – Endoplasmic reticulum; LACS – Long chain acyl coenzyme A synthase; LTP – Lipid transfer protein; MAM – Mitochondria associated membranes; MGDG – Monogalactosyl diacylglycerol; PA – Phosphatidic acid; PAP – Phosphatidic acid phosphatase; PC – Phosphatidylcholine; PG – Phosphatidylglycerol; PLAM – Plastid associated membranes; PLD – Phospholipase D; TGD – Trigalactosyl diacylglycerol

II. Lipids Transported from the ER to the Envelope and the Identity of the Transported Galactolipid Precursor

Before setting out to discuss the possible modes of transfer between the ER and the plastid and evidence for physical connections, I will pause for a moment on the topic of which membrane lipid precursors are actually transported between the ER and the plastid.

[1]For a specialized review on acyl lipid metabolism in Arabidopsis including clickable pathway maps see Li-Beisson et al. in The Arabidopsis Book 8: e0133. 2010 and the accompanying web site found at: http://aralip.plantbiology.msu.edu/

Galactolipids in the plastid are synthesized from diacylglycerol (DAG) in the inner envelope and there is a clear substrate product relationship between the plastid galactolipids and ER synthesized phospholipids, particularly phosphatidylcholine (PC) (Ohlrogge and Browse 1995; Dörmann and Benning 2002). However, the exact identity of the precursor transported from the ER to the plastid has not been fully established. Several candidates have been proposed, but remarkably little direct experimental evidence for actual transport between the ER and the plastid envelope has been presented. I will briefly outline the available evidence for each suggested transported intermediate. After this, I will also suggest a few other hydrophobic compounds which might be transported between the compartments.

Since the chloroplast envelope contains a rather large proportion of the phospholipid PC (Cline et al. 1981; Block et al. 1983; Dorne et al. 1985) but is devoid of PC synthesis activity (Joyard and Douce 1976; Bessoule et al. 1995), this lipid must somehow be transferred from the ER to the envelope. There is also evidence for a role for outer envelope PC as a precursor for plastid galactolipids (Andersson et al. 2004). There are not many examples of in vitro reconstitutions of lipid transfer from ER to chloroplasts (or other plastids) published. However, of the published studies one directly shows transfer of PC (Andersson et al. 2004) and one infers that PC is probably the transferred lipid (Xu et al. 2008). The latter study shows that acyl radiolabel in PC can be transferred in vitro to MGDG in coincubation of labelled ER fractions with unlabelled chloroplasts. Both these studies also show that a cytosolic fraction enables conversion of labelled PC to MGDG in the chloroplast envelope. One of the papers also directly demonstrates that cytosolic phospholipase D (PLD) and phosphatidic acid phosphatase (PAP) acitivity acting on radiolabelled PC in the chloroplast envelope can support formation of labelled MGDG in isolated pea chloroplasts (Andersson et al. 2004). The level of PAP activity in the inner envelope membrane has been suggested to be the cause of the difference between 16:3 and 18:3 plants (Heinz and Roughan 1983; Gardiner et al. 1984). This activity is much lower in envelopes prepared from 18:3 plants than 16:3 plants. It would thus be a straight forward explanation that eukaryotic DAG is formed directly in the outer envelope. This would also avoid the problem of feeding eukaryotic PA into PG synthesis in the inner envelope (see further below). On the other hand, DAG formation in the outer envelope would require transport of this lipid to the inner envelope MGDG synthase. Exchange of lipids between the two envelope membranes is over all poorly understood and most likely extremely hard to study experimentally. Clever use of transgenics using lipid modifying enzymes with different localization might help, but actually measuring transport between the envelopes is probably very difficult.

PC and other diacyl lipids have miniscule solubility in water. A lyso lipid on the other hand, has a comparatively high solubility in water. Likewise, acyl-CoA has high water solubility. If the ER released lyso-PC and there was an acyl-CoA dependent lyso-PC acyltransferase in the chloroplast envelope, this could sustain vectorial transfer of PC from the ER to the envelope provided that the envelope PC is further metabolized into galactolipids (Bessoule et al. 1995). This suggestion is supported by the demonstration of a highly active acyl-CoA dependent lyso-PC acyl transferase in the chloroplast envelope (Bessoule et al. 1995; Kjellberg et al. 2000; Mongrand et al. 2000). Additionally, an isolated ER fraction containing radiolabelled lyso-PC could supply substrate for acyl-CoA dependent acylation in isolated chloroplasts (Bessoule et al. 1995). Isotope pulse chase studies in leek seedlings also seems to support this notion (Mongrand et al. 1997, 2000). Label associated with the *sn-1* position in extraplastid PC was transferred together with glycerol label to galactolipids, whereas label associated with the *sn-2* position was not. However, the recent reports on the important role of exchange of acyl groups between the

acyl-CoA pool and the *sn-2* position of PC in *de novo* ER lipid synthesis (Bates et al. 2007, 2009) complicates the interpretation of earlier studies. The lack of product substrate relationship between the *sn-2* position of extraplastidial PC and chloroplast galactolipids might be explained by acyl-editing in the ER rather than lyso-PC transfer to the envelope. Nevertheless, all the necessary prerequisites for lyso-PC exchange between the ER and the chloroplast do seem to be there. Further studies are needed to determine whether it really is a bulk pathway for the synthesis of eukaryotic chloroplast galactolipids.

In 16:3-plants the activity of the envelope localized phosphatidic acid phosphatase is high and thus ER localized PA would be a reasonable substrate for this and could serve as precursor for galactolipid synthesis in the envelope. A few papers proposed that PA is the transported intermediate (Xu et al. 2005; Awai et al. 2006). This proposal was mainly spurred by the discovery that the TGD2 protein binds PA and that the *tgd1* and *tgd2* mutants accumulated PA. However, the TGD1 and 2 proteins are localized to the inner rather than the outer envelope (Xu et al. 2005; Awai et al. 2006) and the PA binding site is thus probably not close to the ER. Yet another complication with the proposal of PA as the transported intermediate is that this requires a mechanism for the exclusion of PA derived from the ER from synthesis of PG in the plastid envelope. PA formed by overexpression of a bacterial DAG kinase in the plastid envelope resulted in the formation of PG with a typical "eukaryotic" fatty acid configuration (Fritz et al. 2007). This might otherwise be explained by tight metabolic channelling of the imported PA into galactolipid synthesis. A TGD3 protein complex isolated from Arabidopsis chloroplasts exhibits a larger molecular weight than expected from a complex containing only TGD1, 2 and 3 (Roston et al. 2011). The additional components may well be involved in PA channelling or other previously not described metabolic processes. Provided that the PA binding property of TGD2 is of a more regulatory role, it might suggest a link between "eukaryotic" and "prokaryotic" lipid synthesis in the plastid though. Finally, the notion that PC rather than PA is the principal product of *de novo* phospholipid synthesis (Bates et al. 2007, 2009) also makes PA seem like a less likely candidate.

Diacylglycerol (DAG) produced in the ER has been suggested to be the direct precursor of chloroplast eukaryotic galactolipid species (Williams et al. 2000). However, no actual evidence for substrate product relationship between ER localized DAG and chloroplast galactolipids has been presented. There is also a lack of evidence showing transport of DAG in any in vitro system. Thus, even though it seems like a reasonable suggestion that DAG might be transported from the ER to the plastid envelope, there is to date no actual evidence for this.

Ambiguity as to the actual identity of transported precursor(s) for eukaryotic galactolipids remains. This is probably due to a few different factors. Firstly, the interest has been rather limited and not very many studies addressed the problem directly. Secondly, the dynamic nature of lipid metabolism makes interpretation of labelling studies difficult.

Apart from the precursor(s) of the chloroplast galactolipids, a number of other hydrophobic compounds are also probably trafficked between the ER and the plastid. Most reports on the lipid composition of the chloroplast envelope include a minor portion of phosphatidylinositol (Cline et al. 1981; Block et al. 1983), and since the envelope seems to lack a synthesis pathway for this lipid just like PC it seems likely that it is also transported there from the ER to the envelope. Direct transfer of PI from an isolated ER fraction to isolated chloroplasts has been shown with membrane fractions isolated from pea (Andersson et al. 2004). In addition to membrane lipids there is also evidence for transport of plastoquinone from isolated microsomes to chloroplasts (Wanke et al. 2001). There may be many other candidates for hydrophobic compounds transported between the plastid and the ER; in particular terpenoids might be interesting to explore in

this respect as this pathway is shared between the ER and the plastid and contains many potential hydrophobic intermediates and products (Tholl and Lee 2011).

III. Lipid Export from the Plastid

There is one clear instance when intact glycerolipids are exported from plastids to other membranes in the plant cell. This occurs during phosphate limited growth. When phosphate is limited, the plant saves phosphate by replacing phospholipids with the galactolipid DGDG in extraplastid membranes (Härtel and Benning 2000; Härtel et al. 2000, 2001). This replacement has been shown to occur in the plasma membrane (Andersson et al. 2003, 2005), mitochondria (Jouhet et al. 2004) and the tonoplast (Andersson et al. 2005). In addition, the peribacteroid membrane in nitrogen fixating root nodules contains a high proportion of DGDG (Gaude et al. 2004). The DGDG synthesis induced by phosphate deprivation is localized to the outer envelope membrane and dependent on the DGD2 protein (Kelly and Dörmann 2002). Thus, DGDG is exported from the plastid envelope during phosphate limited growth. Actual evidence for the exact route taken by DGDG from the plastid envelope to the plasma membrane, tonoplast and peribacteroid membrane is so far missing. However, given the role of the ER as an otherwise central hub in lipid supply for the extraplastidial membranes, it seems likely that the DGDG would pass the ER en route to the other membranes. The finding that plastids in some tissues exhibit long tubular extensions of the envelope, so called stromules, to constitute something similar to a plastid network in the plant cell (Kwok and Hanson 2003, 2004a, b; Hanson and Sattarzadeh 2008, 2011; Gray, Chap. 9) hints on that the plastid envelope itself might make contact with other organelles as well. In the case of DGDG transport to mitochondria the evidence does point to a direct route of transfer from plastids to mitochondria. In this case there is an increase in the number of contact sites between mitochondria and plastids during the phospholipid replacement (Jouhet et al. 2004).

Analysis of Arabidopsis fatty acid desaturase mutants reveals that di- and trienoic fatty acids formed while bound to plastid lipids can be exported from the envelope to the ER (Miquel and Browse 1992; Browse et al. 1993). This is evident since plants harbouring loss of function mutations in the genes encoding the ER localized lipid desaturases FAD2 and FAD3 still retain a high proportion of di- and trienoic fatty acids in their extraplastidial phospholipids. The extraplastidial lipids must thus have received unsaturated fatty acids from the plastid. However, it is not clear whether the exchange between the plastid and ER in this case is based on free fatty acids, intact diacyl lipids, lyso lipids or a combination of all three.

All the fatty acids fed into *de novo* acyl lipid biosynthesis in the ER of course also exported from the plastid to the ER. This likely constitutes one of the largest single fluxes in the plant cells lipid metabolism. Labelling studies with ^{18}O demonstrate that the nascent acyl-ACP synthesized in the plastid stroma goes through one round of hydrolysis before incorporation into phospholipids in the ER (Pollard and Ohlrogge 1999). This is demonstrated as fatty acids lose almost exactly half of ^{18}O label associated with the carboxyl group as the fatty acids are transferred from Acyl-ACP to eukaryotic lipids. The acyl-ACPs are probably cleaved by an inner envelope acyl-ACP esterase and the free fatty acid transported to the outer envelope. In the outer envelope, the fatty acids are activated to acyl-CoA by long chain acyl CoA synthase (LACS) (Joyard and Stumpf 1981; Andrews and Keegstra 1983). The mechanism of transfer of fatty acids from the inner envelope to the outer envelope is not well understood. Evidence in favour of specific channelling of fatty acids to the outer envelope LACS is the lack of competition from exogenously added BSA to isolated chloroplasts (Koo et al. 2004). The fast turnover of this very small pool of free fatty acids also hints on

channelling of the fatty acids to the outer envelope LACS. There are nine *LACS* genes found in the Arabidopsis genome (Shockey et al. 2002). Knock out of *LACS9* results in a 90% loss of fatty acid activation in isolated chloroplasts (Schnurr et al. 2002). This, however, does not appear to translate into any apparent phenotype of the *lacs9* mutant (Schnurr et al. 2002). This could be interpreted as that there is remaining plastid LACS activity from redundant LACS enzymes in plastid envelope. Alternatively, it could be suggested that free fatty acids are transported from the plastid envelope to the ER, where they are activated by ER localized LACS. A recent study supports the latter view as LACS1 was found to be ER localized, but nevertheless functionally overlaps with LACS9 in triacylglycerol synthesis (Zhao et al. 2010). Further studies on fatty acid export kinetics in chloroplasts isolated from relevant *lacs* mutants might help shed more light on the export process.

IV. Possible Modes of Transfer Between the ER and the Plastid Envelope

In theory, there are three possible mechanisms for the transfer of lipids between two separated membrane compartments: (1) Vesicular trafficking between the compartments, (2) Monomer diffusion and (3) Exchange at sites of close physical contact. Again, the number of studies actually addressing transfer of lipids between the ER and the plastid envelope is small and the amount of direct biochemical evidence is limited. However, analyzing the little data there is, applying a dose of educated guesswork and inspiration from other more well characterized systems does allow some conclusions to be drawn.

Vesicular trafficking seems like an unlikely explanation since the plastids (and mitochondria) are generally not considered part of the secretory apparatus. At least there seems to be no bulk exchange of proteins between the ER lumen and the plastid. One report, however, complicates the picture somewhat (Villarejo et al. 2005; Ling et al. Chap. 12). A small subset of plastid localized proteins does in fact seem to be trafficked through the ER and Golgi before final targeting to the plastid. A bulk flow of vesicles between a secretory compartment and the plastid would, however, be difficult to reconcile with the very strong sorting of lipids and proteins between the compartments. In vitro reconstitution of transfer of phospholipids between mitochondria and ER isolated from yeast and mammalian cells seems to demonstrate a lack of dependence on nucleotides and cytosolic factors (Vance 1990, 1991; Achleitner et al. 1999; Voelker 2000, 2003). Reconstituted transfer of PC from isolated plant microsomes and chloroplasts also seems to be independent of nucleotides and cytosolic proteins (Andersson et al. 2004; Xu et al. 2008). This strongly argues against a vesicular pathway as these generally depend on soluble factors and nucleotides.

Unassisted monomer diffusion of intact membrane lipids seems extremely unlikely as these compounds exhibit very low solubility in water. The whole point of having a membrane would be lost if the constituents were to easily drift off into the aqueous surrounding. The only possible exceptions to this would be lyso-lipids, which demonstrate much stronger propensity to diffuse in and out of membranes (McLean and Phillips 1984; Bai and Pagano 1997). Lyso-PC labelled with the fluorescent reporter BODIPY could be exchanged between model membranes with half time of ~2 min, about 2,000 times faster than the analogs of PC (Bai and Pagano 1997). So called non specific lipid transfer proteins (nsLTPs) could aid in a monomer diffusion based exchange mechanism, and this was proposed early on following the discovery of nsLTPs in plants. Just like lyso-PC exchange, vectorial transport could be maintained if the lipid was continuously metabolized in the receiving membrane compartment. In reconstituted systems, purified nsLTPs could supply phospholipids from microsomes to chloroplasts (Dubacq et al. 1984). However, this hypothesis was mostly

abandoned after it was concluded that all plant nsLTPs seem to be extracellular proteins (for a review see Kader 1996, 1997). While the nsLTP protein family certainly warrants interest in many other aspects, they are most likely not involved in the bulk transfer of lipids between the cellular organelles. On the other hand, there are many more intracellular proteins which harbour various lipid binding domains and thus could aid in lipid transport (Holthuis and Levine 2005; Jouhet et al. 2007). Several proteins with lipid binding domains have been implicated in lipid transport in between membranes in mammalian and yeast cells. The best characterized ones are the CERT proteins which transfers ceramide between the ER and the Golgi in mammalian cells (Hanada 2004), and the oxysterol binding protein Osh4p which transfers sterols between the ER and the plasma membrane (Raychaudhuri et al. 2006). Both these proteins contain dual membrane targeting domains which would allow docking to both acceptor and donor membranes (Holthuis and Levine 2005). The Arabidopsis genome contains a number of genes with sequence similarity to CERT and Osh4p, all of which except one remain uncharacterized to date (Jouhet et al. 2007). The Arabidopsis oxysterol binding protein ORP3a does, in fact, display dual targeting to the ER and the Golgi and binds a plant sterol (Saravanan et al. 2009). However, the actual in vivo function of the protein remains unknown.

Close contacts between membranes would facilitate transfer of lipids by lipid binding proteins. Furthermore, close enough contacts could allow for specialized transporters anchored to the donor or acceptor membrane to carry lipids from one membrane to the other. The biochemical data available which show a lack of dependence on soluble proteins for reconstituted lipid transfer from ER to chloroplasts favours the latter scenario. Again, the Arabidopsis genome does contain a large number of membrane anchored putative lipid transporter proteins and only a very small subset of these are actually characterized (Jouhet et al. 2007). Components of one ABC-transporter complex have been identified to contribute to transport of lipid precursors between the ER and the plastid envelope and a specialized domain of the ER that has been found to be in close association to chloroplasts in higher plants. To conclude, it seems likely that the exchange of lipids between the ER and the plastid envelope is a monomeric exchange mechanism aided by protein components present in the envelope and/or the ER. This process would be facilitated by the physical proximity of the two membranes, the possibility of which will be discussed in the next section.

V. ER Plastid Contact Sites: Plastid Associated Membranes (PLAM)

Close associations between the ER and plastid envelope have been known from ultrastructural studies for a long time (Wooding and Northcot 1965; McLean et al. 1988; Whatley et al. 1991; Kaneko and Keegstra 1996; Kunst and Samuels 2003). The first biochemical evidence of physical associations between chloroplasts and ER was presented in 2000 (Kjellberg et al. 2000). Chloroplast preparations obtained from young pea seedlings apparently contained a significant amount of the otherwise ER localized lipid metabolising enzymes PC-synthase, PI myo-inositol exchange and the palmitoyl-CoA dependent acylation of PG at the *sn-1* position (C16 fatty acids at the *sn-1* position is associated with non plastid phospholipids). The amount of ER co-purified with the chloroplasts apparently decreased in more developed leaf tissue. This was interpreted as that a specialized domain of the ER was co-isolated with chloroplasts. This would be analogous to the report of mitochondria associated microsomes found in yeast (Gaigg et al. 1995) and mammalian liver cells (Vance 1990). The concept of ER membranes attached to mitochondria was originally described as early as 1977 (Shore and Tata 1977). Only recently, the molecular details of such contact sites were established in yeast (Kornmann et al. 2009; Kornmann and Walter 2010) and mammalian cells

(Csordas et al. 2006; Szabadkai et al. 2006; de Brito and Scorrano 2008; Hayashi et al. 2009; Csordas et al. 2010). In particular, molecular complexes tethering the two compartments together have been described in great detail. Interestingly, so far three different ER-mitochondrial tethering complexes have been described. Two different tethering complexes are found in mammalian cells (Hayashi et al. 2009) and a completely different one is found in yeast cells (Kornmann and Walter 2010). In yeast the tethering complex is formed by the four different proteins Mmm1, Mdm10, Mdm12 and Mdm34, these proteins appear not to be conserved in other organisms. However, more sophisticated similarity searches reveal that the SMP domain found in Mdm12 and Mmm1 is conserved in proteins in several other organisms including plants (Lee and Hong 2006). In mammalian cells, the tethering between mitochondria and ER seems to depend on chaperones and ion channels in both organelles (Hayashi et al. 2009). A different tethering complex seems to be formed by the homo- and heterotypic interactions between mitofusin proteins (de Brito and Scorrano 2008). This implies that the tethering between the compartments is evolutionarily plastic and the components making up an ER plastid contact site may not be very related to those found in the tethering between ER and mitochondria in mammalian and yeast cells. The disruption of the tethering complex in yeast cells does decrease phospholipid transfer, but it does not completely abrogate it (Kornmann et al. 2009). Thus the lipid transfer is apparently not completely dependent on the contact site, but nevertheless facilitated by the physical closeness of the membranes.

A microsomal fraction was recovered from chloroplasts isolated from young pea seedlings (Andersson et al. 2007). This fraction was found to have a polypeptide composition distinct from bulk microsomes as well as purified chloroplast envelope. The fraction was clearly microsomal in nature as the phospholipids PC and PE made up the bulk of its membrane lipids and it was enriched in ER marker enzyme activities. Use of transgenic Arabidopsis stably expressing ER localized GFP and an ER specific dye (DiOC6) provided direct microscopic evidence for associations between the ER and chloroplasts in pea and in the Arabidopsis leaf (Andersson et al. 2007). Confocal microscopy on protoplasts isolated from Arabidopsis expressing ER localized GFP revealed that the ER network was in close proximity to many chloroplasts in the cell. Furthermore, when the protoplasts were ruptured by means of an optical scalpel and chloroplasts were pulled out with optical tweezers, strands of ER remained attached to the chloroplasts. The results could be replicated using a chemical dye and pea protoplasts. Finally, chloroplasts isolated by percoll gradient centrifugation were also shown to exhibit small fluorescent bodies of ER attached to them. The attached ER could be pulled with a force exceeding 400 pN without breaking off from the chloroplast. The number of attached ER bodies seems to decrease during leaf development (Tjellström and Sandelius, personal communication). Taken together, this clearly demonstrates the presence of an ER domain physically associated with chloroplasts. The molecular details of this ER domain remain to be characterized in detail. Drawing from what is known about ER mitochondrial contact sites in mammalian and yeast cells, it seems likely that these domains are involved in channelling of membrane lipids and other metabolites between the ER and the plastid.

VI. The Trigalactosyldiacylglycerol (TGD) Transport System

It is well known that chloroplast isolation or tissue disruption triggers the accumulation of unusual oligogalactolipids in several different plants (van Besouw and Wintermans 1978; Heemskerk et al. 1990; Sakaki et al. 1990). It was reasoned that this might in fact be caused by the disruption of ER to chloroplast connections and that this might be used as a tool to identify genes involved in the transport of lipids between the organelles (Xu et al. 2003). Activation of the processive galactosyl transferase could potentially

rescue the phenotype of the *dgd1* mutant which lacks normal DGDG synthesis in the plastid envelope. Following this rationale, the lab of Christoph Benning performed a screen for suppressor mutants of the *dgd1* mutation. This revealed a whole series of components of what appears to constitute a transport machinery for lipids between the plastid and the ER (Xu et al. 2003, 2005, 2008; Awai et al. 2006; Lu et al. 2007). The TGD1 protein is a permease like protein localized to the inner envelope membrane (Xu et al. 2003, 2005), the TGD2 protein is an inner envelope localized protein with a PA binding domain (Awai et al. 2006) which is capable of disrupting model membranes during PA binding (Roston et al. 2011) and the TGD3 protein is an ATPase localized to the stromal side of the chloroplast inner envelope (Lu et al. 2007). These proteins show similarity to bacterial systems which typically form a complex, however, to date there is no direct evidence for their formation of an envelope localized complex. The TGD4 protein is localized to the ER and lacks identifiable domains (Xu et al. 2008).[2] All the *tgd* mutants identified are weak alleles and complete loss of function insertion mutants seem to be invariably embryo lethal. The *tgd* mutants all demonstrate constitutively active processive synthesis of oligogalactolipids, and the substrate product relationship between extraplastidial phospholipids and chloroplast galactolipids is affected. Combination of *tgd* mutations with the *ats1*-mutant, which has a severely decreased flux through the prokaryotic chloroplast lipid pathway, leads to a much more severe phenotype, illustrating that the *tgd* mutants are impaired in flux from ER lipid synthesis to the plastid (Xu et al. 2005, 2008). Chloroplasts isolated from *tgd1* plants are less efficient than wild-type chloroplasts in incorporating radiolabelled diacylglycerol backbones into MGDG from exogenously fed PA and PC (Xu et al. 2005). On the other hand, microsomes isolated from *tgd4* mutant plants are less efficient than wild-type microsomes as donor for radiolabelled PC for production of labelled MGDG in isolated chloroplasts (Xu et al. 2008). This taken together demonstrates that the TGD4 protein is involved in transferring a galactolipid precursor from the ER to the chloroplast and that the TGD1, 2 and 3 proteins are somehow responsible for presenting the diacylglycerol backbone from this lipid to the MGDG synthase in the inner envelope membrane. Originally it was thought that the TGD1 permease and thus likely the other components of the TGD complex were localized to the outer envelope membrane (Xu et al. 2003). However, later studies revealed that the TGD1 2 and 3 protein are in fact localized to the inner envelope membrane (Xu et al. 2005; Awai et al. 2006; Lu et al. 2007). A hypothetical scheme for the transport of lipids into the chloroplast could be that PC is transported from the ER to the plastid envelope by TGD4 and possibly other unknown factors. On the outside of the plastid envelope the PC is accessible for degradation by PLD and/or PAP. The resulting PA or DAG could then be the substrate for transport by the TGD1,2,3-complex to the inner envelope. Alternatively, the TGD1,2,3-complex could transfer lipids from the ER rather than between the envelopes. The latter is, however, hard to reconcile with the inner envelope localization of the TDG1,2,3-complex. It should also be noted that actual transport of PA through the complex has not been shown. PA transport is based on the observation that TGD2 binds specifically to PA (Awai et al. 2006). The details of this remain to be worked out. However, there seems to be some redundancy in the PAPs required for eukaryotic lipid synthesis (Nakamura et al. 2007, 2009). Since the soluble PAP can be knocked out without total loss of flux through the eukaryotic pathway, this would support that PA of eukaryotic origin can be used by the inner envelope PAP for MGDG synthesis. Clever use of fatty acid desaturase mutations

[2]Since the original preparation of the manuscript, the TGD4 protein has been shown to be localized to the outer chloroplast envelope rather than the ER (Wang et al. 2012) and the TGD1, 2 and 3 components reported to indeed form an ABC transporter complex (Roston et al. 2012).

in combination with the *tgd1* and *tgd4* mutants and phosphate deprivation (see further above) demonstrates that the two TGD proteins seem to support unidirectional lipid traffic from the ER to the plastid only (Xu et al. 2010).

VII. Conclusions and Suggestions for Future Research

Although there are substantial gaps in our understanding of many central points of ER plastid contacts and lipid transport between the organelles, some conclusions can be drawn and progress has been made. A specialized domain of the ER is physically associated with the chloroplast envelope. The involvement of this domain in lipid metabolism and transfer to the plastid, however, remains to be demonstrated. A number of different membrane lipids and possibly other hydrophobic compounds are probably transferred between the ER and the plastid envelope. The phospholipid PC seems to be the most likely precursor of galactolipids transferred from the ER to the plastid envelope. The transport of PC from the ER to the plastid is most likely somehow mediated by the ER localized protein TGD4. Finally, the presentation of the DAG backbone to the inner envelope localized MGDG synthase depends on the inner envelope localized TGD1, 2 and 3 proteins. During phosphate limited growth, DGDG is exported from the plastid envelope, likely through the ER. Several other hydrophobic compounds are probably also transported between the ER and the plastid envelope. The molecular details of the tethering of the ER to the plastid as well as actual mechanistic understanding of the lipid transfer between the compartments are still missing. In order to elucidate this, more carefully designed studies on the proteomics of the compartments and in vitro reconstitution assays are needed. Finally, clever use of genetic screens may well help in the elucidation of more components.

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Chapter 9

Stromule Formation

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Summary

Stromules are highly dynamic stroma-filled tubules extending from the surface of plastids in all multicellular plants. Although stromules may interconnect two, or more, plastids and allow the transfer of stromal proteins as large as Rubisco (~550 kDa) between plastids, their function is still largely a matter of conjecture. They may increase the plastid surface area to facilitate movement of materials into or out of plastids, be involved in sensing the cellular environment, and/or have signaling functions due to close apposition of stromules and nuclei, plasma membrane and other cell organelles. Stromule formation appears unrelated to chloroplast division or to light-intensity-dependent chloroplast movement. Stromules are most easily observed by confocal microscopy of cells expressing plastid-targeted fluorescent proteins, and the definition of stromules is based on such observations. Identification of stromules in electron microscope images is problematic, and ideally requires examination of thin serial sections. In leaves of both monocots and dicots, stromules are most abundant in epidermal cell-types, such as trichomes, guard cells and pavement cells, and are more difficult to observe in mesophyll cells containing large closely packed chloroplasts.

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Stromule formation and movement depends on the actin cytoskeleton and requires the ATPase activity of myosin XI proteins. A 42-amino-acid-residue region of myosin XI that directs myosin XI to the chloroplast periphery offers prospects for identification of stromule components required for stromule mobility. Stromules are affected by various environmental factors and by biotic stresses. Stromules are induced by water stress, acting via abscisic acid signaling pathways, and by viral infection. Stromule abundance is also affected by light and temperature.

I. Introduction

Stromules are stroma-filled tubules extending from the surface of plastids in all multicellular plants examined so far. Although stromules have been recognized as a feature of plastid morphology for over 100 years (Gray et al. 2001; Kwok and Hanson 2004c), the name 'stromule' was introduced only in 2000 (Köhler and Hanson 2000; Hanson and Sattarzadeh 2011) following the visualization of stroma-filled tubules in transgenic tobacco (*Nicotiana tabacum*) and petunia (*Petunia hybrida*) plants expressing plastid-targeted green fluorescent protein (GFP) (Köhler et al. 1997). The expression of fluorescent proteins in plastids, coupled with the use of confocal laser-scanning microscopy, has enormously facilitated the observation of stromules in different plant cell types and tissues. Stromules are usually 0.35–0.85 μm in diameter and of extremely variable length, ranging from short beak–like extensions of less than 1 μm to 220-μm-long tubules in tomato (*Solanum lycopersicum*) fruit mesocarp tissue (Waters et al. 2004). They are surrounded by the inner and outer plastid envelope membranes (Gray et al. 2001; see images in Fig. 9.1 showing fluorescent proteins targeted to each of the envelope membranes) and contain only stromal components; thylakoid membranes are excluded, based on the absence of chlorophyll fluorescence from stromules (Wildman et al. 1962; Köhler et al. 1997; Tirlapur et al. 1999). Stromules can be highly dynamic, rapidly extending and retracting, occasionally branching and rarely releasing vesicles from the end of the stromule, a process known as tip-shedding (Wildman et al. 1962; Gunning 2005; Schattat et al. 2011). Stromules may occasionally interconnect two, or more, plastids and allow the transfer of stromal proteins between plastids (Köhler et al. 1997, 2000; Tirlapur et al. 1999). Proteins as large as Rubisco (~550 kDa) can move between plastids via stromules (Kwok and Hanson 2004a). The exchange of materials between interconnected plastids is one possible function of stromules, although most stromules appear not to interconnect plastids, so the functions of stromules are still largely a matter of conjecture (Gray et al. 2001; Kwok and Hanson 2004c; Natesan et al. 2005; Hanson and Sattarzadeh 2008, 2011). These include: the increased surface area generated by stromule formation for movement of materials into or out of plastids, improved sensing of the cellular environment by long stromules (Pyke and Howells 2002), and signaling functions by close apposition of stromules to nuclei, plasma membrane and other cell organelles (Tirlapur et al. 1999; Kwok and Hanson 2004b; Huang et al. 2006). In addition, stromules have been implicated in the initiation of small starch granule formation in the developing endosperm of wheat

Abbreviations: ABA – Abscisic acid; ACC – 1-aminocyclopropane-1-carboxylic acid; APM – Amiprophosmethyl; BDM – 2,3-butanedione 2-monoxime; BiFC – Bimolecular fluorescence complementation; CFP – Cyan fluorescent protein; DIC – Differential interference contrast; GFP – Green fluorescent protein; OEP14 – Outer envelope protein of 14 kDa; PEG – Polyethylene glycol; TIC – Translocon of the inner chloroplast envelope membrane; TMV – Tobacco mosaic virus; TOC – Translocon of the outer chloroplast envelope membrane; TPT – Triose phosphate–phosphate translocator; VIGS – Virus-induced gene silencing; YFP – Yellow fluorescent protein

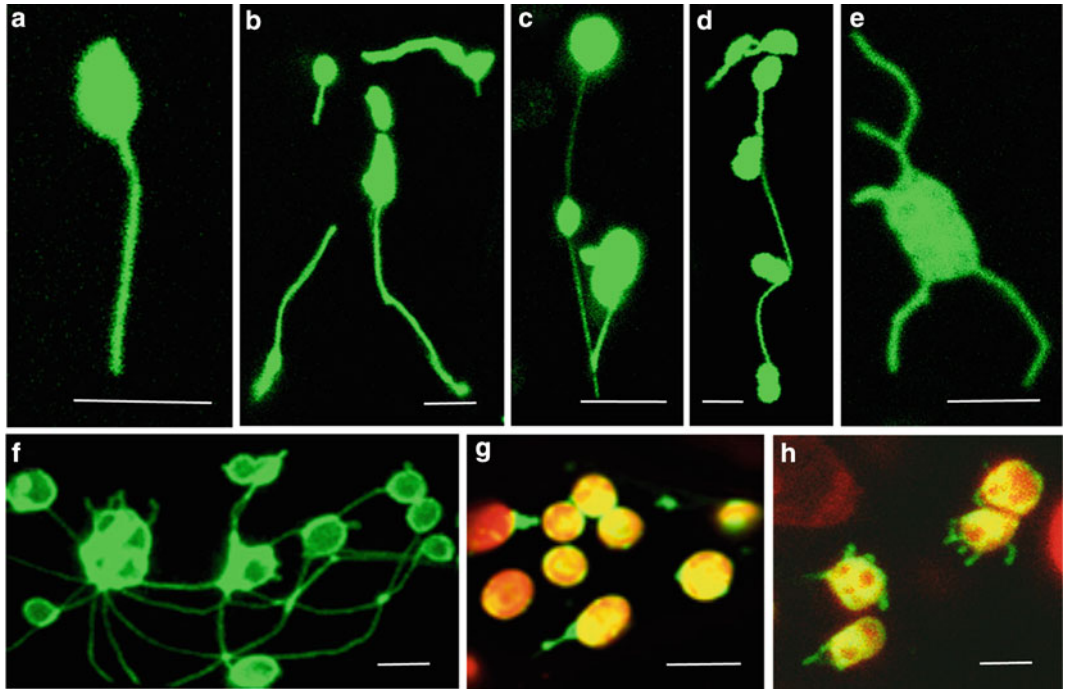

Fig. 9.1. Confocal images of stromules in cells expressing plastid-targeted fluorescent proteins. (**a**, **b**) Plastids in hypocotyl epidermal cells of transgenic tobacco seedlings expressing a fusion protein of the triose phosphate translocator inner envelope targeting sequence (TPT) and YFP; (**c**) plastids in a leaf epidermal cell of transgenic Arabidopsis plants expressing an RbcS-GFP fusion protein; (**d**, **e**) plastids in hypocotyl epidermal cells of transgenic tobacco seedlings expressing an TPT-YFP fusion protein; (**f**) plastids in a leaf trichome of transgenic tobacco plants expressing an OEP14-YFP fusion protein; (**g**) plastids in a leaf cell of *Physcomitrella patens* transiently expressing a TPT-YFP fusion protein following particle bombardment; (**h**) plastids in a leaf epidermal cell of *Nicotiana benthamiana* transiently expressing a TPT-YFP fusion protein following particle bombardment. Images (**a**)–(**f**) show fluorescence collected in the FITC (*green*) channel of a Leica-TCS SP2 confocal system; images (**g**) and (**h**) show merged images of the FITC (*green*) and TRITC (*red*) channels. All *scale bars* are 5 μm.

(*Triticum aestivum*) and barley (*Hordeum vulgare*) (Buttrose 1960, 1963; Parker 1985; Langeveld et al. 2000; Bechtel and Wilson 2003). However, it appears that stromule formation is unrelated to chloroplast division or to light-intensity-dependent chloroplast movement. Despite several mutant screens, Arabidopsis (*Arabidopsis thaliana*) mutants lacking stromules have not been identified, suggesting stromules fulfill a vital function within the plant. However, even in the absence of a clearly defined functional role, there is no doubt stromules are a major feature of plastid morphology. The structure and occurrence of stromules has been extensively reviewed (Gray et al. 2001; Kwok and Hanson 2004c; Natesan et al. 2005; Hanson and Sattarzadeh 2008). The aim of this review is to examine the mechanisms of formation and movement of stromules within the context of a higher plant leaf. However, this will be informed by studies on roots and seedling hypocotyls that are more amenable to observation of stromule behaviour.

II. Stromules Defined

Our understanding of the structure and behaviour of stromules is based very largely on light microscope images, obtained using phase contrast or differential interference contrast (DIC or Normarski) optics (Wildman et al. 1962; Gunning 2005) or confocal laser-scanning

microscopy of plastids containing fluorescent proteins, such as GFP, YFP or CFP (Köhler et al. 1997). The definition of a stromule as a stroma-filled tubule extending from the surface of a plastid seems clear-cut, and can be easily applied where a long stromule extends from a wider plastid body, as in Fig. 9.1a–f. However, there are several situations where the use of the term stromule may not be wholly satisfactory. For plastids examined by light microscopy, these include protrusions that are shorter and wider than most of those shown in Fig. 9.1a–f (as in Fig. 9.1g, h), and structures where the whole plastid resembles a tubule without a clearly demarcated plastid body (for example the plastid in the top right corner of Fig. 9.1b). For images obtained by electron microscopy, there can be very serious concerns about the proper identification of stromules, particularly with respect to very thin plastid extensions observed on amoeboid plastids.

A. Light Microscopy

Köhler and Hanson (2000) proposed that stromules are defined as extensions of the plastid envelope that are less than 0.80 μm wide, to distinguish them from protrusions on irregularly shaped plastids. The diameter of stromules was initially given as 0.35–0.85 μm, on the basis of the original observations of petunia and tobacco plants containing plastid-targeted GFP (Köhler et al. 1997), and this agreed with earlier light microscopy observations of stomules in leaf tissues (Esau 1944; Wildman et al. 1962). However, wider protrusions from chloroplasts have been observed for over 100 years. In 1908, Senn described the spontaneous movement of pseudopodia that form from the 'peristromium,' the mobile jacket of colourless material surrounding the chlorophyll-containing region of chloroplasts in several lower plants. His drawings of large (~30 μm) chloroplasts in the alga *Bryopsis* show pseudopodia ~10 μm long and ~5 μm wide (Senn 1908; figure reproduced in Gray et al. 2001). The presence of a mobile jacket of chlorophyll-free stromal material around a central stationary chlorophyll-containing region is a feature of chloroplasts examined by phase contrast microscopy (Spencer and Wildman 1962; Wildman et al. 1962; Wildman 1967). This 'mobile phase' constantly changes shape, producing a variety of protrusions, some of which would now be called stromules. A distinction between stromules and other protrusions was made by Holzinger et al. (2007a) in their study of heat effects on chloroplasts in mesophyll cells of Arabidopsis. They calculated a shape index (effectively the ratio of the length and the radius) for stromules and other protrusions observed on chloroplasts at various temperatures up to 45°C. The shape index for stromules was 7.0 ± 1.3, with lengths up to 20 μm and diameters of 0.4–0.6 μm, whereas the shape index for other protrusions was 0.8 ± 0.3, with lengths up to 3–5 μm and widths up to 3–5 μm. These wider protrusions are more abundant at higher temperatures in leaves of Arabidopsis (Holzinger et al. 2007a) and various high alpine plants, including *Ranunculus glacialis* and *Oxyria digyna* (Buchner et al. 2007a, b). However, apparently similar protrusions can be seen on chloroplasts in leaves of *Physcomitrella patens* and *Nicotiana benthamiana* plants grown at 20°C (Fig. 9.1g, h) and these can develop into longer protrusions that would be described as stromules.

Although stromules are normally 0.35–0.85 μm in diameter (Köhler et al. 1997), much thinner plastid tubules have been observed in tomato leaf trichomes (Pyke and Howells 2002; Gunning 2005). In transgenic tomato plants expressing plastid-targeted GFP, thin plastid tubules (~0.1 μm diameter) and beads (~0.15 μm) of GFP fluorescence along a barely visible strand have been observed (Pyke and Howells 2002). A similar beaded appearance on very thin stromules was also observed in tomato leaf trichomes by light microscopy with DIC optics (Gunning 2005). This has been explained by a redistribution of stroma contents when stromules are stretched, producing beads that are interconnected by membrane regions

with minimal stromal contents (Pyke and Howells 2002; Gunning 2005). These structures are undoubtedly stromules, indicating that the lower limit for the diameter of a stromule is at least ~0.1 μm.

The identification of stromules in cell types containing non-green plastids can occasionally be problematic. Plastid morphology can be extremely variable and long thin plastids without an obvious plastid body may resemble stromules extending from the plastid body of other plastids. Many plastids in epidermal cells undergo rapid morphological changes resulting in elongation and loss of the plastid body (Guilliermond 1934; Nishibayashi and Kuroiwa 1982). Freehand sketches of the morphological changes of leucoplasts in onion (*Allium cepa*) bulb epidermal cells (Guilliermond 1934; reproduced in Gray et al. 2001) and in parenchyma cells of beet (*Beta vulgaris*) hypocotyls (Esau 1944) illustrate the potential problems of identification and definition of stromules in images obtained at a single time-point. However, because of the rapidity of the morphological changes, it can be relatively straightforward to observe in real time which of the plastids produce stromules from a plastid body. This may not be possible from a single static image. For example, it may be difficult to decide from Fig. 9.1b whether the plastid in the top right corner has a stromule or is an extended plastid body. However, continued observation of the original cell would usually resolve whether the plastid could form a stromule or not.

B. Electron Microscopy

The identification of stromules by transmission electron microscopy is not straightforward, and ideally serial sections are needed to determine whether the chloroplast extension (or proliferation, protrusion or protuberance) is a tubule or a section through a sheet of membranous material. It is particularly important to establish whether thin (~0.1 μm diameter or less) extensions from plastids are tubular, or not. Such extensions have been regularly described in the literature. Images of thin extensions from chloroplasts have been obtained from *Nicotiana rustica* (Weier and Thomson 1962), tomato (Shalla 1964), spinach (*Spinacia oleracea*) (Vesk et al. 1965), *Phaseolus vulgaris* (Whatley 1974), soybean (*Glycine max*) (Musser et al. 1984), *Ranunculus glacialis* (Lütz 1977; Lütz and Moser 1977; Lütz and Engel 2007), *Pinus ponderosa* (Dvorák and Stokrová 1993), *Oxyria digyna* (Lütz and Engel 2007; Holzinger et al. 2007b), the moss *Polytrichum formosum* (Proctor et al. 2007), Arabidopsis (Holzinger at al. 2008; Albrecht et al. 2010) and *Cassiope tetragona* (Lütz 2010), but only the *Ranunculus glacialis* and *Polytrichum formosum* chloroplasts have been examined in serial sections (Lütz and Moser 1977; Lütz and Engel 2007; Proctor et al. 2007). These sections clearly indicate that, in these plants, the structures are not tubules, and therefore cannot be described as stromules. This probably also applies to most of the structures observed in the other plants listed above.

Images of plastid extensions of similar dimensions to those of stromules observed by light microscopy have been obtained for several plants. Bourett et al. (1999) used rapid high-pressure freezing to prepare samples of rice (*Oryza sativa*) leaves for electron microscopy and detected extensions of ~0.4 μm diameter and up to 4 μm long. Similar extensions have also been observed on chloroplasts of rice mesophyll cells without the need for rapid high-pressure freezing (Sage and Sage 2009). It seems highly likely that these images show stromules as defined by light microscopy methods. This conclusion also applies to images of Arabidopsis chloroplasts that have been interpreted to show longitudinal and transverse sections of stromules ~2 μm long with a diameter of 0.4–0.5 μm (Holzinger et al. 2007a). However, there are many more published electron microscope images that remain equivocal. This particularly applies to many of the extensions (or proliferations or protrusions) on chloroplasts of plants with a high alpine or polar distribution (Lütz 2010).

These plants show broad extended stroma-filled areas of chloroplasts when visualized by transmission electron microscopy (Lütz 2010). In *Oxyria digyna*, these chloroplast protrusions detected by electron microscopy can apparently be distinguished from stromules by the effect of latrunculin B, which disrupts the actin-based cytoskeleton (Holzinger et al. 2007b). Long stromules detected by DIC light microscopy disappeared on latrunculin B treatment, whereas thylakoid-free protrusions were still detectable by transmission electron microscopy (Holzinger et al. 2007b). These protrusions probably represent regions of the highly mobile stromal jacket observed by light microscopy (Senn 1908; Wildman et al. 1962; Wildman 1967) and therefore should not be called stromules unless they can be shown to be tube-like. The experiment with latrunculin B (Holzinger et al. 2007b) suggests that mobility of the stromal jacket is independent of the actin cytoskeleton, whereas the actin cytoskeleton is involved in the formation of stromules (see Sect. IV below).

III. Stromules and Leaf Development

There have been no comprehensive studies of stromules during leaf development, although various images showing stromules in different cell types at different developmental stages have been published. From these images, it is possible to make suggestions about when and where stromules are most likely to be present. However, it is clear that the occurrence of stromules is not just determined by internal developmental signals, but is strongly influenced by biotic and abiotic environmental factors (see Sect. V below). The following account will consider stromules in leaves of angiosperms. There is very little information on stromules in leaves of other vascular plants, such as ferns and gymnosperms. Dvorak and Stokrova (1993) showed electron micrographs of amoeboid plastids with thin extensions in needles of *Pinus ponderosa*, but it is not clear if they represent stromules (see Sect. II.B). The occurrence of stromules in leaves of monocotyledonous plants will be considered first, because of the relatively simple developmental sequence along the length of the leaves.

A. Stromules in Monocot Leaves

Monocot leaves develop from a basal meristem and present a developmental series from the base (youngest) to the tip (oldest) of the leaf. However, so far, there has been no systematic analysis of stromules in different cell types at different developmental stages along the leaves of any monocot. This should now be possible with the creation of transgenic wheat and maize (*Zea mays*) lines expressing plastid-targeted fluorescent proteins (Primavesi et al. 2008; Sattarzadeh et al. 2010; Shaw and Gray 2011). The initial characterization of these lines examined the use of different promoter elements, plastid-targeting sequences and fluorescent proteins to mark the plastids in as many tissues and cell types as possible (Primavesi et al. 2008; Sattarzadeh et al. 2010). Although the description of stromules was not the primary aim of these publications, several images show stromules in different cell types (described below).

Stromules have been observed in monocot leaves by light microscopy (Faull 1935; Heitz 1937) and, with the necessary caveats concerning the proper identification of stromules (see Sect. II.B above), by electron microscopy (von Wettstein 1957; Vesk et al. 1965; Laetsch and Price 1969; Mittelheuser and van Steveninck 1971; Freeman and Duysen 1975; Hashimoto et al. 1989; Bourett et al. 1999; Scepánková and Hudák 2004; Gielwanowska et al. 2005; Voznesenskaya et al. 2005; Sage and Sage 2009). In addition, stromules have been observed in monocot leaves or leaf cells transiently expressing plastid-targeted fluorescent proteins following particle bombardment (Arimura et al. 2001) or PEG-mediated transfection of mesophyll protoplasts (Rokov-Plavec et al. 2008).

A single image of a proplastid with a stromule extending about 50% of the length of

the plastid body was obtained by transmission electron microscopy of thin sections of barley leaf meristematic tissue (von Wettstein 1957) indicating that stromules can form on proplastids. However, there have been no other images of stromules on proplastids in monocot leaves, indicating either that stromule formation on proplastids is a very rare event or, possibly more likely, that there have been insufficient attempts to detect stromules in meristematic tissue. A small (1 × 0.7 μm) plastid with a short (0.4 μm long and 0.2 μm wide) protrusion was detected by transmission electron microscopy in the basal meristematic region of a leaf as part of a study of chloroplast development in sugar cane (*Saccharum officinarum*) (Laetsch and Price 1969). However, the plastid contained stacked thylakoid membranes indicating it had started to develop as a chloroplast. All of the other plastids in the same cell contained either thylakoid membranes, described as immature grana, or prolamellar bodies (Laetsch and Price 1969). If the protrusion represents a short stromule, it indicates that stromules can form on plastids in the earliest stages of chloroplast development.

Stromules have been observed in mature leaf epidermal and mesophyll cells of several different monocot species, although rarely have several cell types been examined in the same study. The transgenic lines of wheat and maize expressing plastid-targeted fluorescent proteins offer the best prospects for examination and detection of stromules in multiple cell types of the same leaf. Stromules were observed in leaf trichomes, epidermal pavement cells and mesophyll cells of maize (Sattarzadeh et al. 2010) and in trichomes, stomatal guard cells, epidermal pavement cells and mesophyll cells of wheat (Shaw and Gray 2011). In both of these studies, stromules were more abundant and longer in epidermal cell types than in mesophyll cells. In trichomes and epidermal pavement cells, the plastids are morphologically very variable and rapidly change shape and position. However, images of long (15–40 μm) stromules extending from a distinct plastid body have been obtained from epidermal pavement cells of maize and wheat (Sattarzadeh et al. 2010; Shaw and Gray 2011). Stromules, 5–10 μm long have been observed in epidermal cells of rice and dayflower (*Commelina communis*) leaves after particle bombardment with a plasmid encoding plastid-targeted GFP (Arimura et al. 2001) and in epidermal cells of *Iris versicolor* leaves (Faull 1935).

The stromules on mesophyll chloroplasts are usually much shorter (<4 μm) and are observed on a lower proportion of the plastids in the cell, compared to leaf epidermal cells. Short stromules (<2 μm), visualized by plastid-targeted fluorescent proteins, were observed on ~10% of the chloroplasts in maize and wheat mesophyll cells (Sattarzadeh et al. 2010; Shaw and Gray 2011). Short protrusions of comparable dimensions have been observed by electron microscopy of mesophyll cells of maize, wheat and rice (Vesk et al. 1965; Mittelheuser and van Steveninck 1971; Freeman and Duysen 1975; Hashimoto et al. 1989; Bourett et al. 1999; Sage and Sage 2009). Similar, but thinner, protuberances have been observed by electron microscopy of mesophyll cells of snowdrop (*Galanthus nivalis* L.) and snowflake (*Leucojum aestivum* L.) (Scepánková and Hudák 2004), of the antarctic grass *Deschampsia antarctica* Desv. (Gielwanowska et al. 2005) and of the C4 grass *Aristida purpurea* Nutt. (Voznesenskaya et al. 2005).

C4 plants usually contain morphologically distinct chloroplasts in their bundle sheath and mesophyll cells, but it is not clear what influence, if any, this has on the occurrence of stromules in these cell types. Thin protrusions extending from both bundle sheath and mesophyll chloroplasts were detected by electron microscopy of *Aristida* leaves (Voznesenskaya et al. 2005) and images possibly representing stromules in both bundle sheath and mesophyll cells were obtained by electron microscopy of dark-grown leaves of sugar cane (Laetsch and Price 1969). Further examination of stable transgenic lines of

maize expressing plastid-targeted fluorescent proteins (Sattarzadeh et al. 2010) should provide a definitive answer to the relative occurrence of stromules in bundle sheath and mesophyll cells. In rice, a C3 plant without such a well-developed bundle sheath, stromules were rarely observed in bundle sheath cells (Sage and Sage 2009).

B. Stromules in Dicot Leaves

As with monocots, there has been no comprehensive study of the formation and occurrence of stromules during leaf development in dicots, although there are many images of stromules in leaf cells. Chloroplasts in mesophyll cells usually have fewer, shorter stromules than the plastids in other cell types in a leaf. An inverse correlation between the number of stromules and plastid size and density within the cell has been noted previously (Köhler and Hanson 2000; Pyke and Howells 2002; Waters et al. 2004), and mesophyll cells usually contain large closely packed chloroplasts to maximize the photosynthetic activity of the leaves. The observation of long stromules, some longer than 10 μm, on chloroplasts of spinach mesophyll cells by phase-contrast microscopy (Spencer and Wildman 1962; Wildman et al. 1962) led to the remarkable cinephotomicrographic images showing in real time the highly dynamic nature of changes in chloroplast morphology (Hongladarom et al. 1964). Stromules of 5 μm, or more, have also been observed on mesophyll chloroplasts of spinach and *Oxyria digyna* by phase-contrast or DIC light microscopy (Köhler et al. 1997; Holzinger et al. 2007b). However, stromules on most mesophyll chloroplasts are much shorter and are more clearly seen in confocal images of cells expressing plastid-targeted fluorescent proteins, as first introduced by Köhler et al. (1997). Mesophyll chloroplast stromules have been observed in stable transgenic lines of petunia (Köhler et al. 1997), tobacco (Köhler et al. 1997; Shiina et al. 2000; Köhler and Hanson 2000; Natesan et al. 2005; Hanson and Sattarzadeh 2008), poplar (*Populus alba*) (Okumura et al. 2006) and Arabidopsis (Holzinger et al. 2007a). They have also been observed in mesophyll cells of tobacco and tomato transiently expressing chimeric genes encoding plastid-targeted fluorescent proteins introduced by transfection with a *tobacco mosaic virus* (TMV) vector (Escobar et al. 2003) or by PEG-mediated transfection of protoplasts (Kandel-Kfir et al. 2006; Sapir-Mir et al. 2008). Short stromules ~2 μm in length have been observed in living Arabidopsis mesophyll cells using femtosecond near-infrared laser scanning microscopy (Tirlapur and König 2001).

Stromules have also been detected in bundle sheath cells in leaves of Arabidopsis and tobacco (Tirlapur and König 2001; Natesan et al. 2005). The chloroplasts in bundle sheath cells of Arabidopsis are smaller and further apart than mesophyll chloroplasts and contain longer stromules. Stromules up to 15 μm long and shorter stromules interconnecting two chloroplasts have been visualized in living Arabidopsis by femtosecond near-infrared laser scanning microscopy (Tirlapur and König 2001). Stromules have also been detected in non-green parenchyma and vascular cells of leaves. Esau (1944) drew freehand sketches of the rapid changes in the structure of a single leucoplast in a beet leaf parenchyma cell over a period of 15 min, clearly illustrating the presence of a long stromule. A plastid with a branched stromule of over 10 μm long was observed in a vascular cortical cell of a mature leaf of a transgenic tobacco plant expressing GFP from a gene integrated in the plastid genome (Natesan et al. 2005).

In leaves, stromules are usually most abundant in epidermal cells. They have been observed in epidermal pavement cells, stomatal guard cells and subsidiary cells, and in trichomes. Trichomes of tobacco and tomato leaves have been a favored cell type for observation of stromules by light microscopy (Wildman et al. 1962; Hongladarom et al. 1964; Gunning 2005, 2009) and the behaviour of stromules up to 45 μm long has been recorded on film or as video images (Hongladarom et al. 1964; Gunning 2009).

The stromules may interconnect two, or more, plastids and display rapid extension, retraction, branching and tip shedding (Gunning 2005). Long stromules have also been visualized in trichomes of tobacco leaves and tomato petioles expressing plastid-targeted fluorescent proteins (Köhler et al. 1997; Köhler and Hanson 2000; Pyke and Howells 2002; Kwok and Hanson 2004c; Natesan et al. 2005; Hanson and Sattarzadeh 2008). Long stromules have also been observed in epidermal pavement cells of petunia (Köhler et al. 1997), tobacco (Gray et al. 1999; Arimura et al. 2001; Natesan et al. 2005, 2009; Hanson and Sattarzadeh 2008), Arabidopsis (Gray et al. 2001; Vitha et al. 2001; Haswell and Meyerowitz 2006), lettuce (*Lactuca sativa*) and turnip (*Brassica rapa*) (Gnanasambandam et al. 2007) and *Nicotiana benthamiana* (Caplan et al. 2008; Krenz et al. 2010). In all cases, the stromules were visualized by the presence of plastid-targeted fluorescent proteins, in stable transgenic lines (Köhler et al. 1997; Gray et al. 1999; Vitha et al. 2001; Natesan et al. 2005; Haswell and Meyerowitz 2006), by particle bombardment (Gray et al. 2001; Gnanasambandam et al. 2007; Natesan et al. 2009), or by agroinfection (Caplan et al. 2008; Natesan et al. 2009; Krenz et al. 2010).

Stromules are easily detected in stomatal guard cells expressing plastid-targeted fluorescent proteins and may reach lengths of 9–14 μm (Tirlapur et al. 1999; Vitha et al. 2001; Natesan et al. 2005). They have been observed in guard cells in leaves of Arabidopsis (Tirlapur et al. 1999; Vitha et al. 2001; Raab et al. 2006), tobacco (Köhler and Hanson 2000; Shiina et al. 2000; Fujiwara and Yoshida 2001; Natesan et al. 2005), tomato (Forth and Pyke 2006) and cabbage (*Brassica oleracea*) (Gnanasambandam et al. 2007). They have also been detected in guard cells and subsidiary cells of Arabidopsis leaves using femtosecond near-infrared laser scanning microscopy (Tirlapur and König 2001). Branched stromules and stromules interconnecting two chloroplasts were detected in the subsidiary cells using this method (Tirlapur and König 2001).

There have been few reports on the presence of stromules in cells of cotyledons, most probably because they have been rarely examined. However, stromules were detected in guard cells of cotyledons of green and etiolated Arabidopsis seedlings, and in seedlings treated with the bleaching herbicide norflurazon (Tirlapur et al. 1999). Stromules up to 40 μm long have been detected in cotyledon epidermal cells of the Arabidopsis chloroplast division mutant *atminE1* (Kojo et al. 2009). Extremely long thin stromules have also been observed in epidermal cells of leaves and hypocotyls of other Arabidopsis chloroplast division mutants (Gray et al. 2001; Holzinger et al. 2008). Cells of the cotyledon petioles of tobacco seedlings contain long stromules, up to 20 μm, extending from the plastids clustered around the nucleus to the cell periphery (Kwok and Hanson 2004c, d). Some of these long stromules connect to plastids adjacent to the plasma membrane, but others appear to make contact with the plasma membrane and then run parallel to it (Kwok and Hanson 2004d). In several cells, the GFP fluorescence in the stromules appears to cross the propidium iodide-stained cell wall, and in some instances connect with stromules in the adjacent cell (Kwok and Hanson 2004c, d). There is currently no evidence for the transfer of chloroplast material from one cell to another, but these images suggest that cotyledon petiole cells may provide suitable material for photobleaching experiments (Köhler et al. 1997; Tirlapur et al. 1999) or for microinjection (Knoblauch et al. 1999). Knoblauch et al. (1999) injected individual chloroplasts in *Nicotiana rustica* mesophyll cells with a plasmid gene construct encoding GFP and detected GFP in the injected chloroplasts 24 h later. After 3 days, GFP was detected in at least 12 additional uninjected chloroplasts in the same cell, and it was suggested that the GFP had spread from chloroplast to chloroplast via stromules (Knoblauch et al. 1999). On several occasions in subsequent experiments, GFP fluorescence spread to chloroplasts in adjacent cells (Gray et al. 2012). Unfortunately, the phenomenon was insufficiently reproducible for further

study, but it raises the intriguing possibility that chloroplast material can move from cell-to-cell via stromules.

IV. The Formation and Movement of Stromules

The rapid movement of stromules has been a feature of the descriptions of stromules for over 70 years (Guilliermond 1934; Heitz 1937; Esau 1944; Wildman et al. 1962; Kwok and Hanson 2004b; Gunning 2005). The earlier descriptions relied on series of freehand sketches (Guilliermond 1934; Esau 1944), but Hongladarom et al. (1964) produced a movie of the stromules in basal cells of trichomes, and in palisade and mesophyll cells of tobacco, tomato and spinach leaves using phase-contrast microscopy. The movements observed were described in Wildman et al. (1962). A superb set of video time-lapse images of stromules in tomato trichomes and sub-epidermal cells of petals of *Iris unguicularis* observed with DIC optics has been produced by Gunning (2009) and described in detail in Gunning (2005). Stromules extend from the plastid body along tracks marked by linear streaming of other cell organelles, with stops and starts and transient retractions. Stromules usually extend in the direct of the cytoplasmic streaming, but have been observed to extend upstream against the flow (Gunning 2005, 2009). The rates of stromule extension are very variable, with rates of 0.05–0.23 μm/s reported by Gunning (2005). The rates of retraction may be considerably greater; a rate of 1.16 μm/s was measured for a stromule in iris petal tissue (Gunning 2005). This retraction has been called 'recoil' by Hongladarom et al. (1964), suggesting a degree of elasticity in the stromule. Gunning (2005) observed that fully extended stromules were thinner than they had been previously, as if they had been attenuated by stretching, and Holzinger et al. (2008) showed electron micrographs of thin highly convoluted chloroplast extensions that were interpreted to be recoiled stromules. However, stromules may be completely static for long periods, and no movement may be detected in some cells despite prolonged observation.

Stromules can be seen to be associated with the actin microfilament network in Arabidopsis, tobacco and tomato epidermal cells (Kwok and Hanson 2004b; Gunning 2005). Kwok and Hanson (2004b) visualized microfilaments by expressing the human actin-binding protein talin fused to GFP and detected stromules and plastid bodies by DIC microscopy. The stromules appeared to be in direct contact with both fine and thick microfilaments, and rearrangements of the microfilaments in close contact with stromules resulted in altered morphology of the stromules. The stromule-associated actin cables support cytoplasmic streaming and the movement of other organelles, including mitochondria (Kwok and Hanson 2004b), suggesting that there is no unique cytoplasmic trackway for stromule movement. A direct role for the actin cytoskeleton in stromule movement was indicated by the use of the actin inhibitors cytochalasin D and latrunculin B, which rapidly prevented stromule extension and led to stromule shortening and eventual disappearance (Gray et al. 2001; Kwok and Hanson 2003; Holzinger et al. 2007b; Natesan et al. 2009). Similar results have been obtained with actin inhibitors in epidermal cells of tobacco seedling hypocotyls (Kwok and Hanson 2003), epidermal peels of tobacco leaves (Gray et al. 2001; Natesan et al. 2009) and mesophyll cells of *Oxyria digyna* (Holzinger et al. 2007b), suggesting a common role for the actin cytoskeleton in stromule movement. This is contrary to the results obtained with tubulin inhibitors, such as oryzalin and amiprophosmethyl (APM). Kwok and Hanson (2003) reported that APM decreased the frequency of stromules and reduced the average stromule length to 75% of the initial length in tobacco hypocotyl epidermal cells. However, no effect of the tubulin inhibitors oryzalin and APM on stromule length or frequency was observed in tobacco leaf epidermal cells (Gray et al. 2001; Natesan

et al. 2009) or in mesophyll cells of *Oxyria digyna* (Holzinger et al. 2007b). This suggests that microtubules are not directly involved in stromule movement, but that interactions of microfilaments and microtubules that shape the cytoskeleton may influence stromule movement in some cell types.

A role for myosin in the movement of stromules was indicated by the effects of 2,3-butanedione 2-monoxime (BDM), an inhibitor of myosin ATPase activity (Gray et al. 2001; Natesan et al. 2005, 2009). Application of BDM to lower epidermal peels of tobacco leaves expressing a plastid-targeted YFP resulted in decreased numbers of plastids with stromules and decreased length of the few remaining stromules (Natesan et al. 2009). BDM also caused the apparent fragmentation of long stromules into vesicles of diameter 0.4–0.8 μm in onion bulb epidermal cells (Natesan et al. 2005), suggesting that myosin ATPase activity may be responsible for stromule integrity, as well as stromule movement. Transient RNA interference in leaves of *Nicotiana benthamiana* indicated a role for myosin XI in stromule formation and movement (Natesan et al. 2009). Agroinfiltration was used to introduce a construct containing two copies of the coding region of part of the C-terminal tail domain of *N. benthamiana* myosin XI-2 in an inverted repeat configuration into leaf cells of *N. benthamiana* and the effect on stromules and transcripts of myosin XI, myosin VIII and actin was monitored over 8 days (Natesan et al. 2009). A decrease in myosin XI transcripts, but not those for myosin VIII or actin, was observed 5 days after infiltration and this was accompanied by a decreased proportion of chloroplasts in leaf epidermal cells with stromules. Stromules had completely disappeared from leaf epidermal cells 8 days after infiltration (Natesan et al. 2009). A similar approach using virus-induced gene silencing (VIGS) with a *tobacco rattle virus* vector containing a highly conserved region encoding part of the motor domain of Arabidopsis myosin XI-K was unsuccessful because transcripts for myosin VIII were also decreased (Sattarzadeh et al. 2009). However, a fusion of part of the tail domain of *N. benthamiana* myosin XI-F to YFP localized to chloroplasts and stromules in leaf epidermal cells (Sattarzadeh et al. 2009). Similar experiments with a fusion of the *N. benthamiana* myosin XI-2 tail domain with GFP showed localization to chloroplasts, but not to stromules, and it was suggested that the fusion protein was acting as a dominant negative inhibitor, replacing the endogenous myosin molecules (Natesan et al. 2009). The absence of the motor domain in the fusion proteins should preclude association with the actin cytoskeleton and therefore might be expected to prevent stromule movement. Such dominant negative effects on organelle movement have been observed with myosin tail-domain fluorescent protein fusions in plants (Avisar et al. 2008, 2009). However, the efficacy of the fusion proteins in inhibiting organelle movement and the specificity of organelle binding appear to depend critically on the region of the myosin tail domain included in the fusion protein. Reisen and Hanson (2007) had previously failed to detect binding of several YFP-myosin XI tail fusions to chloroplasts, including fusions of YFP to several Arabidopsis myosins that are closely related to the *N. benthamiana* myosin XI-2 used by Natesan et al. (2009). To detect binding of the GFP-myosin XI-F fusion protein to chloroplasts, Sattarzadeh et al. (2009) used a 42-amino-acid-residue region of the myosin XI-F tail domain that showed similarity to the vacuole-binding domain of yeast myosin myo2p. This region is highly conserved in myosin XI proteins from plants, including the *N. benthamiana* myosin XI-2 (Fig. 9.2). The identification of a small region of the myosin XI tail domain required for interaction with chloroplasts raises the prospect of identifying potential interacting proteins, leading to greater understanding of the mechanisms of stromule formation and movement.

Co-immunoprecipitation of chloroplast envelope proteins with actin filaments has identified components of the TOC-TIC complex for chloroplast protein import (Jouhet and

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At XI-F      VEAKYPALLFKQHLAAYVEKTYGMIRDKLKKEINPLLNLCIH
Nb XI-F      IEAKYPALLPKQHLTACVEKIYGMIRDNLKKEISPFLNQCIH
Nb XI-2      VEAKYPALLFKQQLTAYVEKIYGIIRDNLKKELGSLLSLCIQ
At Mya2      VEAKYPALLFKQQLAAYVEKMFGMVRDNLKRELSTLLSLCIQ
              ******** ** * * ***  *  ** ** *    *  **
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Fig. 9.2. Amino acid sequence comparison of a region of myosin XI proteins that directs a myosin XI-fusion protein to the chloroplast envelope. The amino acid sequences of the 42-amino-acid-residue regions of myosin XI-F proteins from Arabidopsis (At XI-F) and *N. benthamiana* (Nb XI-F) that directed YFP to the chloroplast and stromule membranes in *N. benthamiana* (Sattarzadeh et al. 2009) have been aligned with the sequence of a comparable region of the *N. benthamiana* myosin XI-2 (Nb XI-2) tail region that also directed YFP to chloroplast membranes (Natesan et al. 2009). The comparable region of the orthologous Arabidopsis protein (At Mya2) is also shown. Identical residues in all four sequences are identified by *asterisks.*

Gray 2009). The TOC-TIC complex formed on translocation of unfolded precursor proteins across the chloroplast envelope membranes is currently the only known structure that links the outer and inner envelope membranes. Some linkage of the outer and inner envelope membranes would appear to be a prerequisite for movement of stromules if the principal driving force is the ATPase activity of myosin located outside the chloroplast (Gray et al. 2001). However, more work is needed to establish how the two chloroplast envelope membranes remain closely appressed during stromule extension, and whether there is any form of internal 'plastoskeleton' involved in stromule formation and movement.

V. Environmental and Stress Effects on Stromule Formation

Various environmental and biotic stresses have been suggested to affect stromule formation, although in many cases the experimental evidence is equivocal, either due to doubts on the identification of stromules or to lack of quantification of suitably controlled experiments (Kwok and Hanson 2004c; Hanson and Sattarzadeh 2008). However, there is a growing body of evidence to suggest that stromule formation is affected by environmental factors such as temperature, light and water availability and is induced by virus infection.

A. Temperature

Musser et al. (1984) reported long thin extensions emanating from the chloroplast envelopes in soybean plants chilled to 10°C for 24 h and examined by electron microscopy. However, in the absence of serial sections (see Sect. II.B) there is no evidence that they represent stromules. Holzinger et al. (2007a) have shown, using a thermostatically controlled microscope stage, that the number of protrusions on Arabidopsis mesophyll chloroplasts increased with increasing temperature up to 45°C. At 20°C, long stromules up to 20 μm in length were observed in mesophyll cells showing large spaces between the chloroplasts, although at higher temperatures (35°C and 45°C) the protrusions were much shorter and 'beaklike' and did not have the same 'shape index' as stromules. These short protrusions increased in number and in length as the temperature was increased. Increased numbers of short, broad protrusions were also observed on mesophyll chloroplasts of several high alpine plants as the temperature was increased (Buchner et al. 2007a, b). At low temperatures (5–15°C) very few stromules or other protrusions were visible on Arabidopsis mesophyll chloroplasts (Holzinger at al. 2007a). Fewer chloroplasts with stromules were observed in hypocotyl epidermal cells of dark-grown tobacco seedlings subjected to lower temperatures for eight hours (Gray et al. 2012). The proportion of plastids with stromules fell from 97% at 23°C,

to 39% at 15°C and 26% at 10°C. These studies show there are clear temperature effects on stromule formation and morphology, although as yet the underlying mechanisms of temperature perception and stromule formation have not been addressed.

B. Light

Light has many effects on plastid form and function, and there are indications that light negatively affects stromule formation. Tirlapur et al. (1999) observed that a much higher proportion of plastids in guard cells of etiolated Arabidopsis plants had stromules, compared to guard cells in light-grown plants. In etiolated leaves and cotyledons, almost all guard cell plastids had stromules and the majority of the plastids were interconnected. However, they also noted that guard cell plastids were smaller and more numerous (up to 8–10 plastids) in etiolated tissue, compared to light-grown material. The hypocotyl epidermal cells of dark-grown tobacco seedlings have a high frequency of plastids with long stromules (see Fig. 9.1) and Kwok and Hanson (2003) noted that most of the stromules were lost on exposure to light over a period of 24 h. They ascribed this loss of stromules to be due to the differentiation of the plastids into chloroplasts. However, the transfer of light-grown tobacco seedlings into the dark results in the formation of stromules on virtually all plastids in hypocotyl epidermal cells within 8–10 h (Gray et al. 2012). These plastids retain chlorophyll during this time and can therefore still be regarded as chloroplasts. This suggests that light may be having a more direct effect on stromule formation than solely through plastid differentiation. Red and far-red light were effective in decreasing the number of stromules in hypocotyl epidermal cells of dark-grown tobacco seedlings, whereas the transfer of light-grown seedlings into blue light was as effective as transferring seedlings into the dark in increasing the numbers of stromules in hypocotyl epidermal cells (Gray et al. 2012). This suggests that the light receptor affecting stromule formation is sensitive to red and far-red light, but is insensitive to blue light. This indicates that stromule formation is unrelated to light-intensity-dependent plastid movement, which is blue-light sensitive (Kadota et al. 2009).

A variety of effects of light on short, broad protrusions from mesophyll chloroplasts of different high alpine plants has been described (Buchner et al. 2007a), but it is not clear how relevant these observations are to stromule formation. They used DIC light microscopy to examine sections of leaves from nine different high alpine plants exposed to darkness or to two different light intensities (500 and 2,000 μmol photons m^{-2} s^{-1}) in a temperature-controlled chamber at two different temperatures (10°C and 30°C). Some species (*Poa alpina*, *Silene acaulis* and *Ranunculus glacialis*) showed increased numbers of protrusions in the light compared to the dark at 10°C, whereas *Carex curvula* and *Oxyria digyna* showed a significant decrease in protrusions in the light compared to the dark at 30°C (Buchner et al. 2007a). Most species did not show any significant effect of light intensity on the numbers of protrusions, although *Poa alpina*, *Silene acaulis* and *Ranunculus glacialis* showed decreased protrusions with increased light intensity at 10°C (Buchner et al. 2007a).

C. Water Stress

Freeman and Duysen (1975) reported that wheat seedlings subjected to water stress due to watering with polyethylene glycol (PEG) developed plastid extensions visualized by transmission electron microscopy. Increased concentrations of NaCl also resulted in the appearance of amoeboid plastids in meristematic cells of barley roots (Huang and van Steveninck 1990; Yan 1995). For all of these studies, there are questions concerning the identification of the plastid extensions as stromules and the proportion of the plastids affected in treated and control tissues. However, there can be no doubt that PEG and salt treatments affect plastid morphology. There are also clear differences in

plastid morphology between hydrated and desiccated mosses (Pressel and Duckett 2010). After 2 h of rehydration of desiccated *Polytrichum formosum*, prominent stroma-filled extensions of the chloroplast envelope were observed by transmission electron microscopy of leaf lamella cells (Proctor et al. 2007). However, confirmation that these structures are stromules is required, and this would probably be best obtained with the use of confocal microscopy on cells expressing plastid-targeted fluorescent proteins. An image of stromules on chloroplasts in leaves of *Physcomitrella patens* following particle bombardment with a gene construct encoding plastid inner envelope-targeted YFP is shown in Fig. 9.1g.

In transgenic plants expressing plastid-targeted fluorescent proteins, the proportion of plastids with stromules in hypocotyl epidermal cells of tobacco seedlings and in wheat root hairs was increased by water stress by treatment with PEG, mannitol, NaCl and KCl (Gray et al. 2012). Stromule induction by water stress in these cell types appears to operate via abscisic acid (ABA) because the induction was prevented by ABA synthesis inhibitors such as abamine, naproxen and norflurazon (Gray et al. 2012). Treatment of tobacco and wheat seedlings with ABA resulted in induction of stromules on virtually all plastids in the hypocotyl epidermal cells and root hairs within 8 h (Gray et al. 2012). This is contrary to the report of Raab et al. (2006), who did not succeed in reliably observing differences in stromule density after ABA or mannitol treatments of tissue expressing an AtCSP41D-CFP fusion protein. However, it is not clear if they examined the effects of ABA and mannitol on stable transgenic lines of Arabidopsis expressing AtCSP41D-CFP, or on tobacco leaves subjected to particle bombardment (Raab et al. 2006). It may be more difficult to detect stress effects on tissues subjected to particle bombardment because the tissue damage caused by the bombardment may itself produce a stress response.

The proportion of plastids with stromules in tobacco hypocotyl epidermal cells and wheat root hairs was also increased by treatment with H_2O_2, ACC (an ethylene synthesis precursor) and jasmonic acid, but was decreased by treatment with silver nitrate (an inhibitor of ethylene action) and salicylic acid (Gray et al. 2012), suggesting that stromules are induced by a variety of stress signaling pathways. The application of auxin, cytokinins or gibberellins had no discernable effect on stromule formation in tobacco hypocotyl epidermal cells.

D. Viral Infection

Stromules have been observed in several virus-infected plant tissues (Esau 1944; Caplan et al. 2008; Krenz et al. 2010), raising the possibility that virus-induced stresses may affect stromule formation. Esau (1944) produced freehand sections of mesophyll cells of yellow and green areas of beet leaves infected with a mosaic virus clearly showing protrusions (up to ~1.5 μm long and ~0.6 μm wide) on plastids in the yellow, but not the green, areas of the leaf. Shalla (1964) reported that chloroplasts in tomato leaves infected with TMV were severely distorted and showed the "formation of projections which rapidly extended and contracted". Unfortunately, he did not show any images of these projections, which were apparently observed by light microscopy; instead, he showed an electron microscope image of a very thin looping extension from a chloroplast (Shalla 1964). However, as discussed in Sect. II.B, without images from serial sections, it is impossible to determine whether this represents a tubular structure.

Much clearer images of stromules induced in plants undergoing a defense response have been obtained by Caplan et al. (2008) using confocal imaging of leaf epidermal cells of *N. benthamiana* expressing an NRIP1-Cerulean fusion protein. NRIP1 (N-receptor interacting protein1) is required for N-mediated resistance to TMV and interacts with the 50-kDa helicase (p50) of TMV.

Transgenic plants expressing an NRIP1-Cerulean fusion protein showed long (>10 μm) stromules, and there was a large increase in stromules in leaf epidermal cells of when the NRIP1-Cerulean fusion protein was transiently expressed in plants containing both N and p50 (Caplan et al. 2008). Long stromules are also induced in leaf epidermal cells of *N. benthamiana* infected with *Abutilon mosaic virus* (Krenz et al. 2010). Stromules were visualized by confocal microscopy following co-expression of constructs encoding chloroplast HSC70-1 fused separately to the N- and C-terminal halves of YFP. Oligomerization of HSC70-1 fusion proteins allows bimolecular fluorescence complementation (BiFC) and the production of YFP fluorescence. Infection with *Abutilon mosaic virus* resulted in the production of long stromules (up to ~10 μm) containing spots of high YFP fluorescence resembling 'strings of pearls' (Krenz et al. 2010). The stromules interconnected chloroplasts, and some extended to the cell periphery (Krenz et al. 2010). The relevance of stromule production in virus-infected cells is not yet clear; it may indicate a general stress response of the cell, although it may be a more specific response to viral infection resulting in increased communication between chloroplasts and other cell compartments.

VI. Conclusions

Stromules are an important feature of plastid structure in all cell types in multicellular plants, although the frequency, morphology and mobility of stromules vary enormously between different cell types. A reasonable body of information on the distribution and form of stromules in different plants and cell-types has been accumulated with the aid of confocal microscopy on tissues expressing plastid-targeted fluorescent proteins. Our understanding of the processes and mechanisms regulating stromule formation during plant development and in response to environmental factors is increasing slowly, although there is much to learn. Similarly, our understanding of stromule function remains in its infancy, with more speculation than experimentally established functional roles. The identification of a region of myosin XI involved in chloroplast association should provide a handle for more molecular insights into the mobility of stromules, possibly leading to methods for the specific manipulation of stromule frequency. That, in turn, may provide a means of testing some of the ideas on stromule function.

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Part III

The Plastid Genome and Its Expression During Chloroplast Development

Chapter 10

Dynamic Features of Plastid Genome and Its Transcriptional Control in Plastid Development

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Summary

Land plants usually have different types of plastids, e.g. etioplasts, chloroplasts, amyloplasts, and chromoplasts. Although identical copies of the plastid genome are present in all plastid types, the level and pattern of accumulation of plastid transcripts varies largely among the different plastid types and during plastid differentiation and development. Plastid genomes possess many promoters of widely differing strength, and genes often have multiple initiation sites. Two distinct plastid RNA polymerases, plastid-encoded RNA polymerase (PEP) and nuclear-encoded RNA polymerase (NEP), direct such a complex transcription of plastid genes. Photosynthetic genes, e.g. *psbA*, *psbD* and *rbcL* are mainly transcribed by PEP. Some non-photosynthetic genes such as *rpoB* and *accD* are exclusively transcribed by NEP, and *rrn* and *clpP* genes are mutually transcribed by both PEP and NEP. The interplay of PEP and NEP results in a highly complex transcript pattern in plastids. PEP controls chloroplast development in leaves and its functional maintenance is primarily mediated by the variation of sigma factors. *Arabidopsis thaliana* has six nuclear-encoded plastid sigma factors (AtSIG1 to 6). In addition to the temporal dynamics of sigma factors during chloroplast development, the expression profile of each plant sigma factor in organs and cell types is diverse and probably correlated with the major function of each sigma factor. Extensive forward and reverse genetics studies revealed the role and specificity of respective sigma factors in transcription of different plastid genes involved in the biosynthesis and maintenance of the photosynthetic apparatus, during chloroplast development or under various environmental conditions such as light, salt and cold/heat stresses. Thus, transcriptional regulation in plastids, particularly in chloroplasts, is important for fine-tuned plastid gene expression.

Abbreviations: asRNA – Antisense RNA; BLRP – Blue light responsive promoter; BS cell – Bundle sheath cell; CK – Caseine kinase; cpCK2 – Nuclear-coded plastid-targeted casein kinase 2; CSK – Chloroplast sensor kinase; DAPI – 4′,6-diamidino-2-phenylindole; IR – Inverted repeat sequence; LSC – Large single copy region; M cell – Mesophyll cell; ncRNA – Non-coding RNA; NEP – Nuclear-encoded (plastid) RNA polymerase; NIP – NEP interacting protein; ORF – Open reading frame; PCR – Polymerase chain reaction; PEP – Plastid-encoded (plastid) RNA polymerase; PPR – Pentatricopeptide repeat; PS I – Photosystem I; PS II – Photosystem II; pTAC – Plastid transcriptionally active chromosome; ptDNA – Plastid DNA; qRT-PCR – Quantitative reverse transcription-PCR; RPOT – T3/T7 phage-type single-subunit RNA polymerase; SIB1 – Sigma factor-binding protein 1; SIG – Sigma factor; snmRNA – Small non-messenger RNA; SPP – Stromal processing peptidase; spRNAP-IV – Single-polypeptide nuclear RNA polymerase; SSC – Small single copy region; UTR – Untranslated region; *Ycf* – The conserved hypothetical open reading frame

I. Introduction

All plants and algae possess plastids that contain their own genome and a transcription-translation machinery distinct from that of the nucleus-cytoplasm. Plastids evolved from an ancestral cyanobacterium engulfed by a host cell, and therefore the transcription-translation machinery of plastids has a number of prokaryote-like features (Sugiura 1992). For example, plastid ribosomes are 70S in size, plastid rRNAs and tRNAs are very similar in sequence to their *Escherichia coli* and cyanobacterial counterparts, and plastid mRNAs contain triphosphates at their 5′ ends and lack long poly(A) tails. In addition, many plastid genes of higher plants are co-transcribed as polycistronic pre-RNAs, which are then extensively processed into shorter RNA species. Most components of

the plastid genetic system are not encoded by the plastid DNA but are instead nuclear-encoded. Thereby, formation of the plastid genetic system requires the coordinated expression of nuclear and plastid genes (Woodson and Chrory 2008).

Plastids of multicellular plants usually differentiate into different types of plastids: undifferentiated proplastids (in meristematic tissues or embryos), etioplasts (in dark-grown seedlings), photosynthesis-performing chloroplasts (in leaves), starch-storing amyloplasts (in roots), carotenoid-accumulating colored chromoplasts (in flowers and fruits) and gerontoplasts (Biswal et al. 2003; Pyke 2007). In the photosynthesis-active chloroplasts, photosynthetic genes are actively transcribed and their transcripts accumulate to substantial levels. Although identical copies of the plastid genome are present in all plastid types, the levels and patterns of accumulation of plastid transcripts vary largely among the different plastid types and under different environmental conditions.

Transcription rates and steady-state RNA levels of plastid genes are generally not coincident, and many plastid genes are known to be constitutively transcribed, suggesting that post-transcriptional RNA processing of primary transcripts represents an important step in the control of plastid gene expression (Gruissem et al. 1988; Mullet 1988). Post-transcriptional RNA processing steps include endonucleolytic cleavage, 3′-end trimming, *cis*/*trans*-splicing and RNA editing (Sugita and Sugiura 1996; Monde et al. 2000; Herrin and Nickelsen 2004). Most regulatory factors required for plastid gene expression are encoded by nuclear genes. To understand the molecular basis of plastid gene expression, many studies have focused on the posttranscriptional regulation in plastids and identified nuclear-encoded regulatory proteins (Nakamura et al. 2004; Schmitz-Linneweber and Small 2008; Falcon de Longevialle et al. 2010). In addition to this research trend, the importance of transcriptional regulation in plastids has been reconsidered and many outstanding findings on transcription have accumulated since the existence of nuclear encoded RNA polymerases were proposed (Falk et al. 1993; Hess et al. 1993).

The first part of this chapter describes the overall structure and gene contents of plastid genomes in higher plants. The second part summarizes the characteristics of plastid gene expression in different types of plastids. Finally, we review our current knowledge of transcriptional regulation in plastid gene expression of higher plants. Transcriptional and posttranscriptional regulations of gene expression in plastids have been discussed in recently published review articles (Maier et al. 2008; del Campo 2009; Tillich et al. 2010; Stern et al. 2010; Lerbs-Mache 2011).

II. Plastid Genome

A. Genetic Information of Plastid DNA in Higher Plants

1. Overall Structure and Gene Content of the Plastid Genome

Plastid DNA (ptDNA) was first extracted by CsCl density gradient centrifugation from the isolated chloroplasts of the green alga *Chlamydomonas reinhardtii* (Sager and Ishida 1963). The circular molecules of ptDNA were observed in *Euglena gracilis* (Manning et al. 1971) and then also in higher plants (Herrmann et al. 1975). The first entire nucleotide sequences of plastid genomes were determined from tobacco (Shinozaki et al. 1986) and liverwort (Ohyama et al. 1986). In the past 25 years, 200 plastid genomes were fully sequenced at the end of 2010 (the NCBI organelle genome resources database (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid)). Extensive genomic changes have been revealed in the plastid genomes, such as a 30-kb or short inversions, gene losses and/or gains, which are common events throughout evolution of the plastid genome. Recent plastid genome phylogenomics studies have provided additional evidence for deep-level phylogenetic

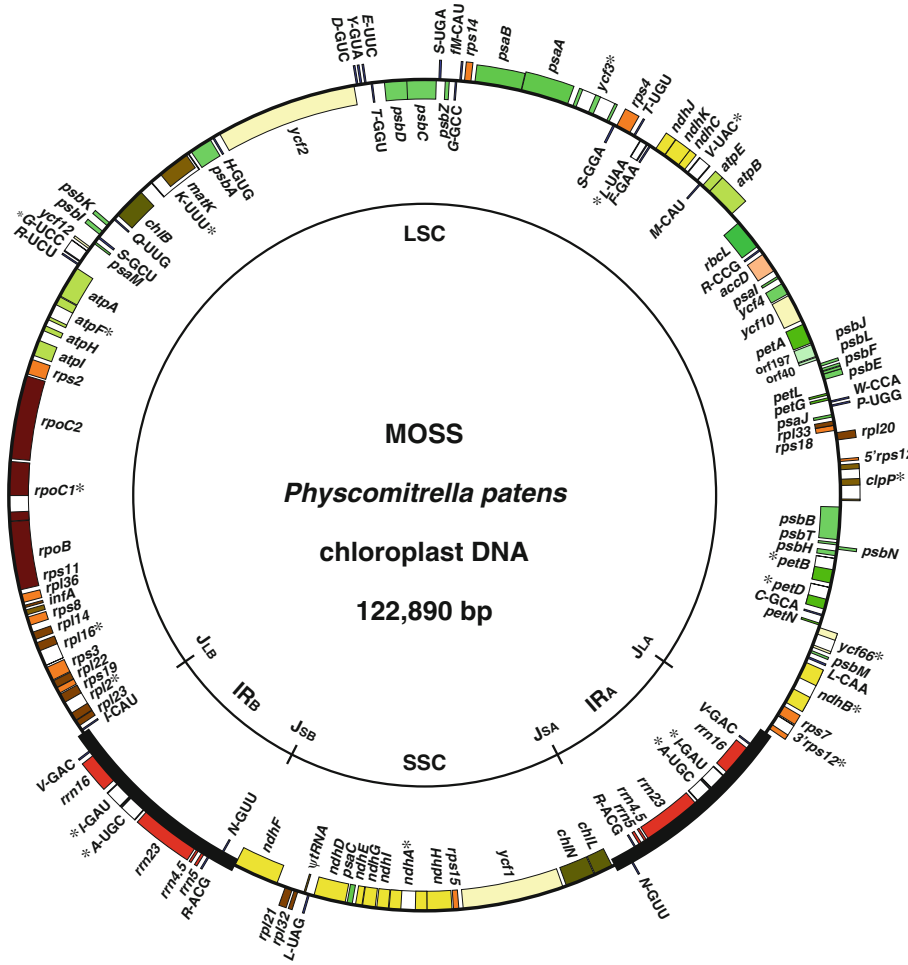

Fig. 10.1. Physical map of the *Physcomitrella patens* plastid genome. The large inverted repeat sequences (IR_A and IR_B) are separated by large single copy (LSC) and small single copy (SSC) regions. Genes inside the *circle* are transcribed clockwise and genes outside the circle are transcribed counterclockwise. Genes with related functions are shown in the same color. *Asterisks* indicate intron-containing genes; introns are depicted as open boxes. For gene products and their functions, compare with Table 10.1. (This figure was modified from Sugiura et al. (2003).)

relationships as well as increased phylogenetic resolution at low-taxonomic levels (Bock 2007; Ravi et al. 2008; Gao et al. 2010).

In land plants, most plastid genomes display a tetrapartite genome organization with a large single copy region (LSC) and a small copy region (SSC) separating two inverted repeat sequences (IR_A and IR_B). The two IR sequences are identical in their nucleotide sequence containing the ribosomal DNA gene cluster (*rrn16-rrn23-rrn4.5-rrn5*), several tRNA and protein-coding genes (Fig. 10.1). Pea (*Pisum sativum*) and black pine (*Pinus thunbergii*) plastid genomes lack such long IR sequences. Overall, the GC content of ptDNA is 36–42% in vascular plants and 28–37% in non-vascular plants (bryophytes). The plastid genomes of land plants contain 120–160 genes (Sugiura 1992; Bock 2007). In the case of *A. thaliana* plastids (Sato et al. 1999), the genome contains 79 distinct protein-coding genes including the conserved hypothetical open reading frames (*ycf*), and 34 RNA-coding genes. Among them 6 protein

genes and 11 RNA genes are present in the IR sequences. Plastid-encoded proteins include 35 photosynthesis-related, 11 NAD(P)H oxidoreductase subunits, 21 ribosomal proteins, 4 RNA polymerase subunits, and 8 other proteins including AccD, ClpP, and Ycfs. RNA-coding genes include 4 rRNA genes and 30 tRNA genes. In addition to the structural RNA genes, unidentified small RNA-coding genes can be present in the plastid genome. Eighteen genes for 6 tRNAs and 12 genes encoding proteins contain introns as shown in Fig. 10.1. Genes and their products described in this chapter are listed in Table 10.1. The *rps12* gene for ribosomal protein S12 is divided into 5′-*rps12* (in LSC) and 3′-*rps12* (in IRs), and each gene segment can be transcribed independently and the transcripts *trans*-spliced. Several small open reading frames (ORFs) also are present in the plastid genome. The lack of evolutionary conservation, even among closely related species, is interpreted as evidence for these ORFs that have no functional significance.

As shown in Fig. 10.1, most plastid genes of land plants are organized in clusters and are co-transcribed as polycistronic pre-RNAs, which are then extensively processed into shorter RNA species (Sugita and Sugiura 1996). Transcription rates and steady-state RNA levels of plastid genes are generally not coincident as described below, and all plastid genes are transcribed by either one or two distinct plastid RNA polymerases, depending on the different stages of plastid development or fluctuation of environmental conditions.

2. Non-coding RNAs in Plastids

In bacteria, regulatory non-coding RNAs (ncRNAs) are widespread. For example, to date, over 80 regulatory ncRNAs have been identified in *E. coli* (Gottesman 2005) and several ncRNAs in cyanobacteria (Axmann et al. 2005; Dühring et al. 2006; Nakamura et al. 2007; Ionescu et al. 2010). In plastids, tscA RNA (430 nt) was first identified as ncRNA required for *trans*-splicing of exons 1 and 2 of *psaA* pre-mRNAs in the green alga *C. reinhardtii* (Goldschmitz-Clermont et al. 1991). Thereafter, SpRNA (218 nt) was found as an ncRNA in tobacco plastids (Vera and Sugiura 1994). Disruption of the SpRNA gene (*sprA*) revealed that this RNA is dispensable for cell viability under normal growth conditions and its function is still unknown (Sugita et al. 1997). Furthermore, several small ncRNAs were identified in tobacco and Arabidopsis chloroplasts.

At least 7 small non-messenger RNA (snmRNA) ranging from 50 to 290 nt are encoded by the Arabidopsis ptDNA (Marker et al. 2002). Among these, five snmRNAs are located to the intergenic regions, one is located to the intron of the *petD* gene, and the remaining one is located to the 3′ end of the *rbcL* gene. All are encoded by the same strand of their neighboring genes. Likewise, Lung et al. (2006) identified 12 ncRNAs (20–500 nt) from tobacco chloroplast cDNA libraries. Most plastid ncRNAs are located to intergenic regions and one ncRNA is encoded by the antisense strand of neighbouring genes. Another ncRNA, Ntc-5 RNA, is transcribed in opposite (antisense) orientation to the 3′-untranslated region (UTR) of the *atpE* gene (located on the complementary strand). This might function in analogy to an antisense RNA or miRNA.

Recently, a long antisense RNA (asRNA, 650 nt) was identified in the chloroplasts in Arabidopsis, tobacco and poplar (Georg et al. 2010). asRNA maps in an antisense orientation to *ndhB* and is predominantly accumulated in young leaves and at physiological growth temperatures. Interestingly, the correlation between the accumulation of asRNA and RNA editing of the *ndhB* transcript appeared weak in a temperature shift experiment, suggesting involvement of this RNA in RNA maturation or in the control of RNA stability. Posttranscriptional regulation via ncRNAs would provide a fast and efficient mechanism for the rapid adjustment of organellar gene expression to changing environmental conditions and/or metabolic demands of the cell. Further studies need to address their function in plastid biogenesis.

Table 10.1. Plastid-encoded genes described in this chapter

Genes	Gene product	Functions and remarks
Photosynthesis-related genes (35)		
psaA	PS I P700 apoprotein A1	Reaction center subunit, essential for PS I function
psaB	PS I P700 apoprotein A2	Reaction center subunit, essential for PS I function
psaJ	PS I J-protein	Small subunit, not essential for PS I function
psbA	PS II D1-protein	Reaction center, essential for PS II function
psbC	PS II CP43-protein	Inner antenna protein, essential for PS II function
psbD	PS II D2-protein	Reaction center, essential for PS II function
psbK	PS II K-protein	Presumably involved in PS II assembly/stability
psbN	PS II N-protein	Function unknown, assignment as PS II subunit uncertain
petA	Cytocrome *f*	Core subunit of cyt b_6f complex, essential for cyt b_6f function
**petD*	Subunit IV of cytocrome b_6/f complex	Essential for cyt b_6f function
atpA	α subunit of ATP synthase	CF_1, nucleotide-binding site
atpB	β subunit of ATP synthase	CF_1, catalytic site
atpE	ε subunit of ATP synthase	CF_1, regulation of CF_1CF_o activation, required for proton gating
**atpF*	b subunit of ATP synthase	CF_o, binding of CF1
atpH	c subunit of ATP synthase	CF_o, proton translocation
atpI	a subunit of ATP synthase	CF_o, proton translocation
rbcL	Rubisco large subunit	CO_2 fixation
NADH oxidoreductase genes (11)		
**ndhB*	ND2 subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
ndhC	ND3 subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
ndhD	ND4 subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
ndhF	ND5 subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
ndhG	ND6 subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
ndhK	27 kD subunit of NADH dehydrogenase	Chlororespiration, cyclic electron transfer
Other protein genes and conserved open reading frames (8)		
accD	Acetyl-CoA carboxylase subunit	Fatty acid biosynthesis, essential for cell survival
**clpP*	Catalytic subunit of the protease Clp	ATP-dependent protein degradation, essential for cell survival
matK	Maturase within *trnK* intron	Splicing factor for group II introns
ycf	Conserved hypothetical open reading frame	
RNA polymerase subunit genes (4)		
rpoA	α subunit of RNA polymerase	Transcription, plastid-encoded RNA polymerase (PEP)
rpoB	β subunit of RNA polymerase	Transcription, plastid-encoded RNA polymerase (PEP)
**rpoC1*	β′ subunit of RNA polymerase	Transcription, plastid-encoded RNA polymerase (PEP)
rpoC2	β″ subunit of RNA polymerase	Transcription, plastid-encoded RNA polymerase (PEP)
Ribosomal protein genes (21)		
rps2	Ribosomal protein S2	Translation, small ribosomal subunit
**rps12*	Ribosomal protein S12	Translation, small ribosomal subunit, *trans*-splicing
rps15	Ribosomal protein S15	Translation, small ribosomal subunit
**rpl16*	Ribosomal protein L16	Translation, large ribosomal subunit

(continued)

Table 10.1. (continued)

Genes	Gene product	Functions and remarks
Ribosomal RNA genes (4)		
rrn16	16S ribosomal RNA	Translation, small ribosomal subunit
rrn23	23S ribosomal RNA	Translation, large ribosomal subunit
rrn4.5	4.5S ribosomal RNA	Translation, large ribosomal subunit
rrn5	5S ribosomal RNA	Translation, large ribosomal subunit
Transfer RNA genes (30)		
trnD-GUC	tRNA-Asp (GUC)	Translation
trnE-UUC	tRNA-Glu (UUC)	Translation, tetrapyrrole biosynthesis
trnV-GAC	tRNA-Val (GAC)	Translation
**trnV-UAC*	tRNA-Val (UAC)	Translation
trnY-GUA	tRNA-Tyr (GUA)	Translation
Other RNA genes		
asRNA_ndhB	Long antisense RNA to *ndhB* transcript	Involvement in RNA maturation or the control of RNA stability
sprA	Small plastid non-coding RNA	Function unknown, present in tobacco and tomato

Asterisks indicate the intron-containing genes
The numbers in parenthesis are the number of genes present in *Arabidopsis thaliana* plastid genome

B. Conformation and Copy Number of Plastid DNA in Plastids

1. Conformation of ptDNA and Plastid Nucleoids

Plastid DNA (ptDNA) is usually depicted as a circular molecule. However, conformation of ptDNA seems to be rather complex because some fractions of ptDNAs were observed as circular dimer or multimers (Herrmann et al. 1975; Deng et al. 1989) as well as in linear molecules (Bendich 1991; Lilly et al. 2001). Herrmann et al. (1975), observed using an electron microscopy, that a large fraction (80%) of the ptDNA extracted from isolated chloroplasts from young leaves of four plant species (*Antirrhinum majus*, spinach, *Oenothera hookeri*, and *Beta vulgaris*) was circular including supertwisted circles and circular dimers. Thereafter, in-gel procedures by pulsed-field gel electrophoresis revealed that at least four distinct forms of monomers to tetramers exist in isolated spinach leaf chloroplasts (Deng et al. 1989). In contrast, 25% (in pea) to 45% (in tobacco) of the ptDNA within developing leaf tissue was observed as circular forms and 22–35% are linear forms using DNA fiber-based fluorescence in situ hybridization (fiber-FISH) (Lilly et al. 2001). Linear molecules are presumably produced by breakage of the circles during extraction of ptDNA or are intermediates of replicating DNA molecules. Even now, the functional relevance of most of the different conformations of the ptDNA is still unclear. Nevertheless, we cannot exclude the possibility that conformational changes of the ptDNA are involved in the regulation of plastid gene expression. Of consequence, fluctuations of ptDNA topology are tightly correlated with changes in transcriptional activity of *C. reinhardtii* plastid *psaB*, *rbcL*, *atpA* and *atpB* genes (Salvador et al. 1998).

ptDNAs are organized into so-called plastid nuclei or nucleoids that are composed of DNA, proteins and RNAs (Kuroiwa 1991; see also Liere and Börner, Chap. 11). Number, shape, and size of the nucleoids vary depending on the plant species and the stage of plastid development. Likewise, ptDNA copy number per nucleoid is variable between plant species and independent of plastid differentiation (Kuroiwa 1991). Proplastids often contain only one small

nucleoid whereas mature chloroplasts contain several or even dozens of nucleoids. The plastid nucleoids are localized in the inner membrane in young leaves but are localized in the matrix between thylakoid membrane and/or grana stacks in mature leaves (Kuroiwa 1991). Various enzymatic activities for DNA synthesis, transcription, DNA condensing, and Rubisco degradation are retained in the plastid nucleoids (Sakai 2001; Sekine et al. 2002; Kato et al. 2004). Proteomic analysis of a Triton X-100-insoluble 30,000 × *g* pellet (equivalent to plastid nuclei) from purified pea chloroplasts identified over 30 proteins including DNA gyrases and proteins required for transcription and translation (Phinney and Thelen 2005). Likewise, a proteomic analysis of Arabidopsis and mustard plastid transcriptionally active chromosome (pTAC) identified 35 components involved in transcription, DNA replication, DNA topology, DNA binding, detoxification, or protein modification (Pfalz et al. 2006). These analyses will provide a clue in the understanding of how ptDNAs are organized in a higher ordered configuration and how plastid nucleoids contribute to the regulation of plastid gene expression in plastid development.

2. Copy Number of ptDNA

A single leaf mesophyll cell contains 50–100 chloroplasts, each of which harbors 100 or more ptDNA copies (Bendich 1987). Accordingly, the ploidy of leaf cells reaches 10,000 ptDNA. The copy number might change during plastid differentiation or leaf development. The multiplicity of ptDNA can be explained by genome partition during plastid division, plastid partition during cell division, and increased gene dosage (Bendich 1987).

DNA reassociation kinetics measurements showed that the percentage of pea ptDNA in total cellular DNA ranges from 0.4% in roots, 1.4% in embryos and etiolated tissues to 12% in fully greened leaves (Lamppa and Bendich 1979). This accounts for 244 copies per plastid in young leaves and 174 in fully greened leaves. An early study using the DNA-specific 4′,6-diamidino-2-phenylindole (DAPI) fluorochrome showed that the level of ptDNA increases 26-fold (500–13,000 copies per cell) from the basal to mature regions of spinach leaves (Lawrence and Possingham 1985). Quantification by Southern dot blot hybridization indicated that plastids isolated from the basal primary barley leaves of 4-days-old seedlings contain 130 copies of ptDNA in plastids and that the DNA copy number increased to 210 in plastids of the developing leaf, declining gradually with increasing cell age, reaching 50 copies per plastid in the oldest cells of leaves (Baumgartner et al. 1989). In contrast, Southern blot analysis of the amount of ptDNA in Arabidopsis and tobacco plants shows that ptDNA copy number remains remarkably constant during leaf development and even in the senescent leaf (Li et al. 2006). This indicates that during leaf development, plastid gene expression in higher plants is not significantly regulated at the level of genome copy number. A similar result was reported by Zoschke et al. (2007). They showed that ptDNA copy numbers varied from only 1,000 to 1,700 per cell during development from young to old rosette leaves of Arabidopsis plants. In contrast, the transcriptional activity and steady-state transcript levels of plastid genes were significantly reduced in older rosette leaves (Zoschke et al. 2007). Thus, once plastid differentiation is complete, ptDNA copy number remains constant and does not vary significantly with leaf age or the plant's developmental stage. This topic is addressed in detail by Liere and Börner (Chap. 11).

III. Plastid Gene Expression in Different Types of Plastids

A. Dynamics of Gene Expression During Plastid Development

Proplastids, etioplasts, and amyloplasts retain the ability to develop into photosynthetic chloroplasts when exposed to light. Although identical copies of the plastid genome are present in all plastid types, plastid genes are differentially expressed depending on plastid types. In the photosynthetically active chloro-

plasts, photosynthesis genes are actively transcribed and their transcripts accumulate at higher levels. In contrast, photosynthesis genes are rarely or not transcribed while non-photosynthesis genes are constantly or actively transcribed in non-photosynthetic plastids.

1. Plastid Gene Expression During Amyloplast Formation

Root cells contain starch-storing amyloplasts. The steady-state transcript levels of photosynthetic genes *psbA*, *psaA* and *rbcL* are 200–900-fold lower in roots than in leaves, whereas they are constitutively transcribed at rates similar to those in root amyloplasts and leaf chloroplasts (Deng and Gruissem 1988). When cultured tobacco BY-2 cells in the stationary phase were transferred to auxin-depleted but cytokinin-supplemented culture medium, leucoplast-like plastids were converted to amyloplasts within two days. The number of plastids and the relative amount of ptDNA per cell remained nearly constant throughout conversion of leucoplasts to amyloplasts while overall transcriptional activity of the isolated plastid-nuclei rapidly decreased (Sakai et al. 1999). This suggests that the transcriptional activity of plastid genes is downregulated during plastid conversion.

2. Plastid Gene Expression During Chloroplast Development

In monocots such as barley and wheat, leaves are composed of cells containing different types of plastids in a developmental gradient ranging from proplastids in the meristematic cells at the base of the leaf to either fully mature chloroplasts or etioplasts in the cells at the tip. Baumgartner et al. (1989) showed that transcriptional activity is low in proplastids near the base of 4-day-old dark-grown or illuminated seedlings and increases tenfold in the region just above the base of the leaf. By contrast, transcriptional activity rapidly declines in older apical cells during light dependent differentiation of etioplasts to chloroplasts. Krupinska and Apel (1989) showed that the relative transcription rates of the various plastid DNA fragments were almost identical in etioplasts and chloroplasts. This suggests that etioplasts are already well prepared to differentiate into photosynthetically active chloroplasts.

DNA copy number per plastid increased only 1.6-fold during early leaf development and remained constant with increasing cell age of seedlings. A similar result was observed in spinach chloroplasts (Deng and Gruissem 1987). Thus, the general concept being accepted is that the plastid transcriptional activity per plastid and DNA copy number increase early in chloroplast development and that transcriptional activity per DNA template varies during leaf biogenesis (Mullet 1993).

3. Plastid Gene Expression During Chromoplast Formation and Maturation

Chromoplasts are carotenoid-accumulating plastids conferring color to many flowers and fruits. Chromoplast differentiation proceeds from preexisting plastids, most often chloroplasts (Egea et al. 2010). During the differentiation process the plastid genome is essentially stable and transcriptional activity is limited (Kahlau and Bock 2008; Egea et al. 2010). Piechulla et al. (1985) first analyzed the transcript levels of several plastid photosynthesis-related genes during tomato fruit ripening. Their transcript levels relative to cytoplasmic rRNA dramatically decreased during fruit ripening. A similar observation was reported in chromoplasts from bell pepper (Gounaris and Price 1987). Thus, plastid gene expression can be generalized to be down-regulated in nonphotosynthetic cells and tissues. To further address this issue, Kahlau and Bock (2008) carried out a systematic transcriptomics and translatomics analysis of the tomato plastid genome during fruit development and chloroplast-to-chromoplast conversion. The data showed that photosynthesis genes are much more strongly downregulated than the genetic system genes, including *matK* and the genes encoding ribosomal proteins, the subunits of plastid-encoded plastid RNA poly-

merase (PEP). Interestingly, the transcript abundance of *accD* encoding a subunit of the acetyl-CoA carboxylase increased during chloroplast-to-chromoplast conversion (Kahlau and Bock 2008). Such a global decrease of transcript levels during tomato fruit development may be attributed to a dramatic down-regulation of transcription by PEP that directs the transcription of photosynthesis-related genes. RbcL and PsbD protein levels dramatically decreased while the AccD protein accumulated to substantial levels in the plastids during this process. AccD is involved in fatty acid biosynthesis required for fruit development to provide the membrane lipids that accommodate the storage carotenoids in ripening fruits. In addition, splicing and RNA editing of most gene transcripts investigated did not significantly change during ripening whereas splicing of *ndhB* transcripts and RNA editing of *ndhB*, *ndhD*, and *ndhF* transcripts was significantly reduced or absent from chromoplasts. Thus, expression of many plastid genes is negatively controlled at transcriptional and post-transcriptional levels in chromoplast formation and maturation. This study provides new evidence that NEP-dependent genes (*accD*, *matK*, *clpP*, etc.) are actively transcribed in chromoplasts.

B. Cell Type-Specific Expression of Plastid Genes in C_4 Plant Leaves

In maize, a C_4 plant, the leaves are mostly composed of two types of cells, bundle sheath (BS) and mesophyll (M) cells. M cells are responsible for the linear light reactions of photosynthesis and BS cells complete the Calvin cycle and perform cyclic Photosystem I (PS I) reactions (Kanai and Edwards 1999). M and BS cells contain anatomically and biochemically distinct chloroplasts, M and BS chloroplasts, respectively. Both chloroplast types have the same proplastid origin. M chloroplasts develop thick granal stacks and BS chloroplasts develop mostly parallel lamellae with diffuse grana and accumulated starch. Rubisco activity is detected in BS cells but not in M cells (Kanai and Edwards 1973). The absence of Rubisco from M cells is accounted for by the absence of *rbcL* mRNA (Link et al. 1978). Thereafter, many studies on the control of C_4 photosynthesis gene expression have been described (Sheen 1999).

Cahoon et al. (2008) performed DNA microarray analysis to quantify the levels of 62 plastid transcripts in the yellow base region (etioplasts) and the green tip (mature BS and M chloroplasts) of maize leaves. They showed that 51 plastid genes are expressed >twofold higher in the leaf tip than the leaf base and 10 genes, mostly ribosomal protein genes, showed less than a twofold difference between the base and the tip. Only *clpP* was more than twofold higher in the base. However, this analysis could not reveal differences in the transcript levels of respective plastid genes between BS and M chloroplasts.

Sharpe et al. (2011) carried out qRT-PCR to compare the transcript abundance of 18 plastid genes between young and mature BS and M cells of maize leaves. The middle region contained young BS and M while the tip section had mature BS and M chloroplasts. Among the 18 transcripts, the following 10 changed abundance in significant cell type-specific patterns. Photosystem II (PS II) transcripts (*psbA*, *C*, *D*, and *K*) accumulated at 8–19-fold higher in M cells than BS cells in the middle section, but these differences were diminished in the tip section due to an increase of transcript abundance in BS. *rbcL* mRNA was 17-fold higher in BS in middle tissue and 240-fold higher in BS in mature tip tissue. Expression of PS I (*psaA* and *B*) and cytochrome b_6f complex (*petA*) genes was enhanced in the BS of the leaf tip. Although NAD(P)H dehydrogenase complex mRNAs (*ndhA* and *ndhC*) had a similar expression pattern as *rbcL*, the abundance of *ndhA* and *ndhC* mRNA in BS increased only 2- and 28-fold, respectively as the leaf matured. Thus, changes in transcript abundance followed no single pattern, suggesting that transcription and/or transcript turnover for plastid genes is tissue-, cell-, and gene-specific. Further, quantitative analyses are needed to obtain general conclusions on this issue.

IV. Transcription by Two RNA Polymerases in Plastids

During the endosymbiotic evolution of mitochondria from proteobacteria and of plastids from cyanobacteria-like prokaryotes, the two organelles established a unique internal gene expression system. In consequence, genes on the ptDNA are, therefore, semi-autonomously expressed with well-defined machineries consisting of a combination of nuclear encoded and plastid encoded gene products.

Until the 1980s, the importance of transcriptional regulation of plastid genes had not been emphasized that much, because meaningful transcriptional differences between etioplasts and chloroplasts or between dark-adapted and light-illuminated chloroplasts in seedlings had not been found due to limited observations (Deng and Gruissem 1987; Mullet and Klein 1987). However, the next two decades clarified the dynamics of plastid transcription in response to environmental stresses and in the process of chloroplast development (Allison 2000). It is now widely recognized that transcriptional regulation in plastids, particularly sigma factor-dependent transcription in chloroplasts, is primarily important (Lysenko 2007) and that subsequent post-transcriptional controls, including RNA editing and splicing, are also critical for correct and full gene expression from ptDNA (del Campo 2009).

Plastids have two different types of DNA-dependent RNA polymerases from lower to higher plants. One is PEP (*p*lastid-*e*ncoded RNA *p*olymerase) and the other is NEP (*n*uclear-*e*ncoded RNA *p*olymerase) (Maliga 1998). Genes on the ptDNA are classified into three groups based on the transcriptional dependency on PEP and NEP in chloroplasts. Class-I genes such as photosynthetic *psbA*, *psbD* and *rbcL* are mainly transcribed by PEP. Class-III genes such as *rpoB* and *accD* are exclusively transcribed by NEP. Class-II genes such as *rrn* and *clpP* are mutually transcribed by both PEP and NEP (Hajdukiewicz et al. 1997).

A. Plastid-Encoded RNA Polymerase (PEP)

1. Transcriptional Activity and Physiological Roles of PEP

PEP is a bacterial type multisubunit enzyme consisting of catalytic core subunits and a promoter recognition sigma factor (Allison et al. 1996; Serino and Maliga 1998). Most ptDNAs, even in non-photosynthetic plastids of parasitic plants, have four *rpo* genes (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*) encoding the PEP core subunits (αββ′ and β″, respectively). However, it should be noted that the *rpoA* gene in the moss *Physcomitrella patens* had been deleted from its ptDNA, and two paralog genes exist on the nuclear DNA (Kabeya et al. 2007). PEP is indispensable for chloroplast development because disruptants of each plastid-encoded *rpo* gene no longer develop chloroplasts (Krause et al. 2000). However, the autonomy of PEP activity is on the side of nuclear DNA because the promoter recognition sigma factors of PEP had been deprived from ptDNA and relocated to the nuclear DNA during symbiotic evolution. In chloroplasts, PEP exists as a much larger protein complex with more than 13 accessory proteins (Bülow and Link 1987; Loschelder et al. 2004; Pfalz et al. 2006; Bollenbach et al. 2009), although the physiological roles of these accessory proteins have been only elucidated in part (Schröter et al. 2010).

Most of the Class-I and -III genes have characteristic upstream sequences similar to bacterial sigma70-type promoter consisting of −35 (TTGACA) and −10 (TATAAT) consensus sequences (Harley and Reynolds 1987; Weihe and Börner 1999). In bacteria, sigma factors recognize the promoter sequences and initiate transcription with the core subunits (Gruber and Gross 2003). Thus the bacterial promoter-like sequences on the ptDNA should be similarly recognized by plant sigma factors to initiate the PEP-dependent transcription. It is noteworthy that additional *cis* elements such as a bacterial extended −10 region (Sato et al. 1999) and a eukaryotic TATA box-like TATATAAgt

element between the −35 and −10 regions are also found in some plastid genes (Eisermann et al. 1990).

2. Structure and Multiplicity of Sigma Factors

The nuclear-encoded sigma factors are synthesized as precursor proteins having an N-terminal transit peptide that consists of 32–80 amino acids in length. These precursors are transported into chloroplasts and then the N-terminal domain is cleaved by the stromal processing peptidase (SPP). The C-terminal region of plant sigma factors is well conserved and contains the regions 1.2–4.2 as well as those assigned in bacterial sigma factors (Hakimi et al. 2000). In contrast, the N-terminal halves of these mature sigma factors do not share significant homology with the bacterial orthologs (Schweer 2010).

Plant sigma factor genes were cloned from unicellular red algae for the first time (Liu et al. 1996; Tanaka et al. 1996). So far, sigma factors were also identified and characterized in a wide variety of photosynthetic organisms, including a moss *P. patens* (Hara et al. 2001), monocotyledonous rice (Tozawa et al. 1998; Kasai et al. 2004; Kubota et al. 2007), maize (Tan and Troxler 1999; Lahiri and Allison 2000), dicotyledonous mustard (Kestermann et al. 1998), tobacco (Oikawa et al. 2000), and *A. thaliana* in which a set of six sigma factor genes named *AtSIG1* to *6* are encoded by the nuclear genome (Tanaka et al. 1997; Isono et al. 1997; Kanamaru et al. 1999; Fujiwara et al. 2000).

From a comparison of the conserved C-terminal domain, AtSIG2 and its orthologs in other plant species are most closely related to bacterial sigma70. Interestingly, AtSIG4 seems to be unique because no ortholog of this sigma factor has been identified so far (Schweer 2010).

3. Expression and Gene Specificity of Sigma Factors

PEP controls chloroplast development and its functional maintenance is primarily mediated by variation of sigma factors (i.e. sigma heterogeneity). In the last decade, the expression profile of Arabidopsis sigma factors (AtSIGs) and their gene specificity have been extensively studied. The latter topic has been mainly promoted by the use of T-DNA insertion (knockout) mutants of each sigma factor gene, except for *AtSIG1* (Fig. 10.2).

Although very little is known about the function of AtSIG1, it appears as if the transcript is induced by blue and red light (Onda et al. 2008). An in vitro transcription assay using the *psbA* or *rbcL* promoter showed that AtSIG1 was less active than AtSIG2 or AtSIG3 (Privat et al. 2003). It is noteworthy that a disruptant of the rice *SIG1* homolog (*OsSIG1*), whose gene products accumulate at a late stage during leaf development, was obtained (Tozawa et al. 2007). The mature leaves of the *OsSIG1* mutant accumulated only a third of the chlorophyll content compared to the wild type. Since the level of the transcripts of the *psaA* operon was markedly reduced in the mutant, OsSIG1 seems to play an important role in the maintenance of PS I components in rice, and this is possibly similar in Arabidopsis.

The *AtSIG2* knockout mutant was first reported by Shirano et al. (2000). The mutant showed reduced chlorophyll and aberrant organization of thylakoid membranes even in the mature leaves. AtSIG2 primarily regulates transcription of a part of housekeeping tRNA genes, including the *trnEYD* operon and two *trnV* genes (Kanamaru et al. 2001; Hanaoka et al. 2003). *trnE*, encoding tRNA-Glu, is particularly important because tRNA-Glu has at least three physiological functions indispensable for plastidial translation, tetrapyrrole synthesis, and the switch from NEP to PEP during chloroplast development (Hanaoka et al. 2005) as described below. AtSIG2 is also involved in the transcription of *psbD* initiated from one of multiple promoters (Hanaoka et al. 2003), *psaJ* (Nagashima et al. 2004a) in vivo, and rRNA operons initiated from the P2 promoter in vitro (Privat et al. 2003).

An *AtSIG3* knockout mutant did not show a significant visual phenotype, but the expression of *psbN* was selectively decreased and indeed photosynthetic electron transfer was

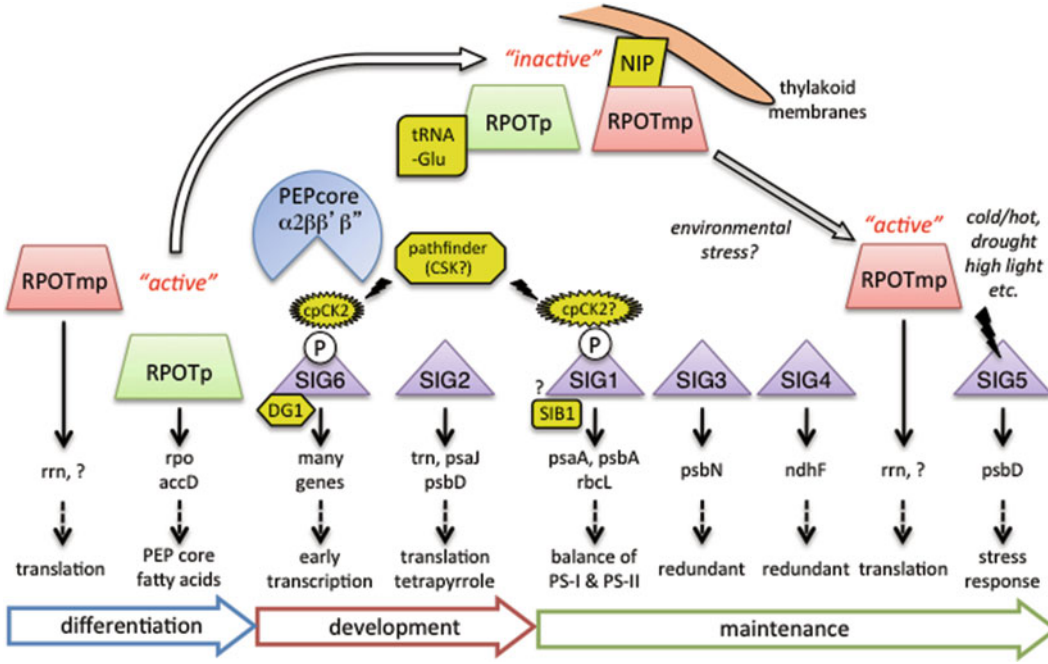

Fig. 10.2. Transcriptional control in chloroplasts. NEP (RPOTp and RPOTmp) transcribes some primary genes, including *rpo* genes for plastid-encoded PEP core subunits, at the beginning of plastid differentiation into chloroplast. The PEP core enzyme binds to a promoter recognition sigma factor (SIG1 to 6) and transcribes proper genes for the establishment and maintenance of chloroplast function. Switching from NEP to PEP during the chloroplast development is achieved by NEP inactivation. Plastidial tRNA-Glu and thylakoid membrane-localized NIPs directly bind to RPOTp and RPOTmp, respectively, and repress their transcriptional activity. Among sigma factors, at least SIG1 and SIG6 are phosphorylated by kinase(s) such as cpCK2. The phosphorylated sigma factor would change its transcriptional activity and/or promoter specificity. Chloroplast-localized DG1 and SIB1 have been identified as sigma factor-binding proteins.

affected (Zghidi et al. 2007). It is notable that the AtSIG3 protein has a membrane-bound form (Privat et al. 2003).

An *AtSIG4* knockout mutant also did not show any visual phenotype but the transcription of *ndhF* and possibly *ndhG* was significantly decreased (Favory et al. 2005). The functions of AtSIG3 and AtSIG4 may be redundant or minor compared to those of other sigma factors, otherwise their transcriptional activity is weaker than other sigma factors and consequently they rather function as a competitive "low gear" for plastid gene expression.

The *AtSIG5* gene was preferentially activated under various environmental conditions such as light, salt and cold/heat stresses and its knockout mutant was surely less resistant under salt stress and strong light (Nagashima et al. 2004b). Since AtSIG5 was involved in the transcription of *psbDC* from the blue light responsive promoter (BLRP) that is dependent on the upstream AAG-box (Baba et al. 2001; Nagashima et al. 2004b; Tsunoyama et al. 2004), this stress-responsive sigma factor is very likely to be required for rapid reconstruction of the photosynthetic reaction center (Kanamaru and Tanaka 2004).

Two *AtSIG6* knockout lines have been isolated from different mutant pools. One has pale-green cotyledons at the beginning of germination (Ishizaki et al. 2005), while another allele showed a more severe "almost white" phenotype in cotyledons (Loschelder et al. 2006). However, both mutants recovered the early-stage deficiency and exhibited normal green color and growth after a week. AtSIG6 is a specific sigma factor functioning at the early-stage of chloroplast development

in seedlings. Thus, AtSIG6 as well as AtSIG2 is a major sigma factor in chloroplast development. Furthermore, AtSIG6 may have an additional role in the transcription of two operons, *atpBE* and *ndhC-psbG-ndhJ*, even after the early stage (Schweer et al. 2006).

In addition to the temporal dynamics of sigma factors during chloroplast development, the expression profile of each plant sigma factor gene in organs and cell types is also diverse, and is probably correlated with the major function of each sigma factor (Homann and Link 2003). AtSIG5 proteins exist almost equally in both cotyledons and true leaves. AtSIG1 and 2 were shown to be more abundant in cotyledons, whereas AtSIG3 was abundant in true leaves (Privat et al. 2003). Any sigma factor gene significantly expressed in roots has not been reported yet. In monocotyledonous wheat leaves, PEP in the young cells along the expanding leaf axis showed different activity and specificity than in the mature cells at the leaf tip (Satoh et al. 1999). Maize has six sigma factors, ZmSIG1AB, 2AB, 3, and 6. ZmSIG1A and 1B are abundant in mature chloroplasts at the leaf tip, while the other four factors primarily accumulated in immature plastids at the leaf base. In vitro observation indicated that ZmSIG1A and SIG1B recognized characteristic promoters containing an extended −10 region raising the idea that transcription from such promoters is more active in leaf tips but less active at the leaf base (Lahiri and Allison 2000).

4. Regulation of Sigma Factors by Phosphorylation and Binding Proteins

In 2010 several exciting papers related to Arabidopsis sigma factors were published (Schweer et al. 2010; Shimizu et al. 2010; Chi et al. 2010). They suggest that PEP-dependent gene expression is controlled not only by the variation of sigma factors (i.e. sigma heterogeneity) but also by the phosphorylation of sigma factors and by direct binding of proteins with sigma factors (Fig. 10.2).

Mature sigma factors without a transit peptide have a unique variable extra region at the N-terminal portion that is not found in bacterial sigma factors. This plant specific sigma domain would confer functional differences not observed with bacterial sigma factors (Schweer 2010). In addition, it has been demonstrated that nuclear encoded plastid-targeted casein kinase 2 (cpCK2) is closely related to eukaryotic kinase rather than to the bacterial kinase and can phosphorylate plant sigma factors in vitro (Baginsky et al. 1999; Ogrzewalla et al. 2002; Jeong et al. 2004; Schliebner et al. 2008).

Schweer et al. (2010) transformed an *AtSIG6* knockout mutant with a series of mutated *AtSIG6* genes in order to prove the in vivo importance of phosphorylation of the sigma factor. Although they have not shown direct evidence of the AtSIG6 phosphorylation in vivo yet, their data indicate that the state of multiple phosphorylation of AtSIG6 by cpCK2 (encoded by At2g23070) affects the plastidial transcription profile and the visible phenotype. Furthermore, they propose that prephosphorylation of the Ser-177 residue of AtSIG6 by an unidentified "pathfinder" kinase is critical for generating a functional cpCK2 substrate site. The kinase may make the cpCK2 possible to phosphorylate the Ser residue of AtSIG6 located at the highly variable region of plastid sigma factors.

On the other hand, Shimizu et al. (2010) showed in vivo phosphorylation of AtSIG1, the most abundant factor among the AtSIGs, and proposed a novel molecular mechanism by which the phosphorylation of AtSIG1 would cause a change of its promoter specificity and adjust gene expression of PS I and PS II components. Since the stoichiometry of PS I and II is important for photosynthetic electron transfer that depends on the redox state of chloroplasts, its adjustment should enhance photosynthetic efficiency (Link 2003; Steiner et al. 2009). They suggest that phosphorylation of AtSIG1 was stimulated when the plastoquinone pool was oxidized and inhibited. Phosphorylation of AtSIG1 inhibited the transcription of the *psaA* gene encoding a major PS I protein. In addition, results from

transgenic Arabidopsis plants expressing *AtSIG1* with or without the putative phosphorylation site indicate the involvement of the Thr-170 residue. By use of the yeast two hybrid system Puthiyaveeti et al. (2010) showed a possibility that Arabidopsis chloroplast sensor kinase (CSK encoded by At1g67840), a bacterial-type sensor histidine kinase, could directly interact with both AtSIG1 and cpCK2 (alternatively named PTK). They also showed a direct interaction between AtSIG1 and cpCK2 in yeast.

Piecing together these findings indicate that the "pathfinder" kinase proposed by Schweer may be CSK, if it can phosphorylate not only unidentified site(s) in cpCK2 but also the initial site in AtSIG6 (Ser-177) and possibly AtSIG1 under oxidative conditions of plastoquinone; in this case, phosphorylated-cpCK2 would give rise to further phosphorylation of these AtSIGs, and this phospho-relay should cause the transcriptional repression of PS I. It seems to be true that at least one of the plant sigma factors, if not all, are phosphorylated and a consequent conformational change should alter their transcriptional activity or specificity in response to developmental states and/or environmental conditions.

Furthermore, Chi et al. (2010) reported a pentatricopeptide repeat (PPR) protein named DELAYED GREENING 1 (DG1 encoded by At5g67570) directly bound to AtSIG6. PPR proteins are known as site-specific RNA-binding proteins in various enzyme complexes involved in RNA editing, processing, splicing and translation (del Campo 2009). Chi et al. (2010) suggest that direct binding occurs between the C-terminal region of DG1 and the N-terminal unconserved region of AtSIG6. The *sig6 dg1* double mutant showed a more severe chlorotic phenotype and a greater decrease of PEP-dependent transcription than each single mutant. In contrast, overexpression of AtSIG6 complemented the chlorophyll deficiency in *dg1* cotyledons. Interestingly, the genetic defect of *dg1* caused an "increase" of AtSIG6-dependent gene transcripts in vivo.

DG1 is not the first example of a sigma factor-binding protein, because a chloroplast-localized sigma factor-binding protein 1 (SIB1) had already been reported as an AtSIG1 binding protein in vitro; however, the physiological function of SIB1 in plastidial transcription has not been shown (Morikawa et al. 2002). Concerning this point, the expression of SIB1 appeared to be induced by infection with a pathogenic bacterium. Furthermore, a *SIB1* knockout mutant caused an apparent decrease in the expression of some nuclear-encoded defense genes, triggered by pathogen infection, salicylic acid and jasmonic acid. Plastid gene transcription by PEP was, however, not altered under these conditions (Narusaka et al. 2008; Xie et al. 2010). Therefore, DG1 is likely to be the first protein whose binding to a plant sigma factor and its physiological involvement in the regulation of plastid gene expression has been shown.

B. Nuclear-Encoded RNA Polymerase (NEP)

1. Transcriptional Activity and Physiological Roles of NEP

During extensive studies of the plastidial transcription machinery, it came to light that a part of plastid-encoded genes were still transcribed in PEP-deficient mutants or under PEP-inactive conditions; for example, the *iojap* mutant of maize (Han et al. 1992), plastid ribosome-deficient mutants of barley (Falk et al. 1993; Hess et al. 1993), a deletion mutant of *rpoA* in tobacco (Allison et al. 1996), and plants treated with PEP-specific inhibitors like tagetin and rifampicin (Kapoor et al. 1997; Liere and Maliga 1999; Bligny et al. 2000). These facts suggest the existence of a second RNA polymerase in plastids (Bünger and Feierabend 1980). This enzyme was expected to be a T3/T7 phage-type single-subunit RNA polymerase (RPOT) for two reasons. First, T3/T7 phage-type promoter sequences were found just upstream of the PEP-independent

transcription initiation sites (Allison et al. 1996; Hajdukiewicz et al. 1997). Second, a single-subunit 110 kD RNA polymerase purified from spinach initiated transcription from the T7 promoter but not from a typical PEP promoter of *rbcL* gene in vitro (Lerbs-Mache 1993). The nuclear-encoded T7 phage-type plastidial RNA polymerase named NEP has been isolated from Arabidopsis, tobacco, spinach, rice, barley, *Nuphar advena*, and the moss *P. patens* (Hedtke et al. 1997, 2000, 2002; Kabeya et al. 2002; Richter et al. 2002; Emanuel et al. 2004; Kusumi et al. 2004; Liere et al. 2004; Azevedo et al. 2006; Yin et al. 2010). Dicotyledonous plants have two NEP proteins, chloroplast-targeted RPOTp and chloroplast/mitochondria-targeted RPOTmp (Kobayashi et al. 2001), whereas monocotyledonous plants have only chloroplast-targeted NEP (Chang et al. 1999; Kusumi et al. 2004). Both dicotyledonous and monocotyledonous plants have another T7 phage-type RNA polymerase named RPOTm targeted only to mitochondria (Tan et al. 2010).

NEP promoters have been identified from various genes such as *accD*, *atpB* operon, *atpI*, *clpP*, *rpoB* operon, rRNA operon, *rps15* and *rpl16*. These promoters were classified into Type-I and -II. Type-I was sub-classified into Ia and Ib (Liere et al. 2004). Most NEP promoters contain a core YRTA motif (Type-Ia) that is also found in mitochondria promoters (Allison et al. 1996; Liere and Maliga 1999). Some of the conserved NEP promoters have an additional GAA-box motif upstream of the core motif (Type-Ib). Type-II promoters have been identified upstream of *clpP*, *atpB*, and the *rps2-atpIHFA* operon in tobacco, and the rRNA operon (Kapoor and Sugiura 1999). They have neither the YRTA-motif nor any other consensus motif. A Type-II promoter in the *clpP* gene overlapping with the transcribed region (−5 to +25) is highly conserved even in moss and is active in mature chloroplasts (Sriraman et al. 1998). The plastid rRNA operon (*rrn*) in Arabidopsis and mustard has three promoters. The P1 promoter depends on PEP, whereas P2 and PC promoters are Type-I and -II, respectively (Pfannschmidt and Link 1997). Some tRNA genes have another nonconsensus-type NEP promoter in the coding sequence (Wu et al. 1997). It is also noteworthy that all plastid genes, including the photosynthesis Class-I genes, are transcribed by NEP in PEP-deficient tobacco, although the transcriptional level was very low (Legen et al. 2002). Thus, the structure of NEP promoters is diverse; however, it is still unknown how higher plant plastids established these NEP promoters in the process of symbiotic evolution.

Both *RpoTp* and *RpoTmp* are expressed in young leaf cells, although the tissue specificity of these NEP proteins differs: *RpoTp* is dominantly accumulating in primary cortex cells of the stem and sepals. On the other hand, *RpoTmp* is apparently present in meristematic cells, leaf veins, companion cells around the phloem, stipules and root distal cells. Expression of *RpoTmp* precedes that of *RpoTp* in developing seedlings (Emanuel et al. 2004).

T-DNA insertion mutants of both *RpoTp* and *RpoTmp* as well as of sigma factor genes shed light on their functional difference and relevance. *RpoTp* T-DNA insertion mutants were isolated as “leaf development” mutants. The *SCABRA3* mutants exhibit severe defects in chlorophyll biosynthesis and plant growth (Hricová et al. 2006). Despite having almost normal epidermal cells, the mesophyll cells were found to be irregular and expanded in the mutant. The transcripts of the NEP-dependent genes, including *accD*, *rpoB*, *rpoC1*, and *clpP*, were markedly reduced in the mutant. This is consistent with the observation that overexpression of *RPOTp* in tobacco enhanced transcription from a distinct subset of Type-I NEP promoters (Liere et al. 2004). The transcriptional compensation system of RpoTp by RpoTmp is unlikely because nuclear *RpoTm* and *RpoTmp* expression in the mutant were not altered at an early developmental stage. However, functional compensation of RPOTp by RPOTmp is likely because the *RpoTp RpoTmp* double mutant showed a severe developmental arrest of germination (Swiatecka-Hagenbruch et al. 2008). Thus, RPOTp-mediated gene expression is critical

for an early stage of chloroplast development, and it affects not only mesophyll cell proliferation but also leaf morphogenesis.

In contrast to the *RpoTp* mutant, the *RpoTmp* T-DNA insertion mutant was isolated as a "short root" mutant (Baba et al. 2004). The mutant also showed delayed growth of young seedlings and slow greening of etiolated seedlings that almost disappeared over time. The *RpoTmp* mutants did not result in a drastic change in plastidial transcription; however, there was specific and transient disordered transcription from the Type-II PC promoter of the *rrn* operon during seed imbibition and germination (Courtois et al. 2007). In addition, the mutants showed reduced transcription of specific mitochondrial genes, but no change in promoter utilization (Kühn et al. 2009).

An in vitro transcription assay using three Arabidopsis T7 phage-type RNA polymerases overexpressed in *E. coli* approached the nature of their transcriptional activities and promoter specificities (Kühn et al. 2007). The recombinant RPOTm protein recognized precisely most of mitochondrial promoters as expected. However, the recombinant RPOTmp that exerted high activity when supercoiled DNA was used as a template did not show the ability to recognize promoters correctly, except for mitochondrial *atp6* promoters. The RPOTp protein also could not initiate precise transcription from any well-characterized plastid NEP-dependent promoters in *rpoB*, *accD*, and *clpP* genes, although it recognized some mitochondrial promoters precisely. The inconsistency of in vivo and in vitro transcriptional specificity of RpoTmp and RpoTp may be due to the requirement of some additional factors for correct and full function of NEP in vivo.

2. Molecular Mechanisms of Switching from NEP to PEP

In an early stage of chloroplast development, the amount of NEP-dependent transcripts is promptly elevated for the construction of PEP core enzyme and initiation of fatty acid synthesis (Hess and Börner 1999). PEP binding to an adequate sigma factor accelerates transcription of photosynthesis genes including tRNAs; meanwhile, the NEP-dependent transcripts are progressively reduced to a minimum level. This NEP to PEP switch is likely to be critical for correct progression of the development of chloroplasts (Fig. 10.2), although a low level of PEP has already been stored in cells from the start of germination (Demarsy et al. 2006).

Plastid-encoded tRNA-Glu preferentially transcribed by PEP in a SIG2- and SIG6-dependent manner is very likely one of the switching molecules. The absence of functional AtSIG2 or SIG6 causes derepressed and continuous accumulation of NEP-dependent gene transcripts in vivo (Kanamaru et al. 2001; Loschelder et al. 2006). Then purified tRNA-Glu, but not other plastid tRNAs, directly bind to recombinant RPOTp and repress RPOTp-dependent *accD* transcription in vitro (Hanaoka et al. 2003). Increasing tRNA-Glu molecules in a SIG2- and SIG6-dependent manner at the early-to-middle stage of chloroplast development should cause enzymatic inhibition of the major NEP, RPOTp. The plastid tRNA-Glu has a unique third function as an inhibitor of RPOTp-dependent transcription in addition to its well-known fundamental functions in translation and as indispensable cofactor for 5-aminolevulinate synthesis (Schön et al. 1986).

The difference in turnover between NEP- and PEP-dependent mRNAs was proposed to contribute to the developmental switch (Cahoon et al. 2004). The amount of NEP-dependent mRNAs in maize was almost constant between leaf base and tip, corresponding to the early and late stages of chloroplast development, respectively. This constancy is probably caused by a decreased stability and increased transcription of the NEP-dependent mRNAs. tRNA-Glu bound RPOTp may not only be inactive but also unstable in nature, because the amount of intraplastidial RPOTp decreases as chloroplast development proceeds despite an increase in its transcriptional activity. On the other hand, the level of PEP-dependent mRNAs

increased as leaves/chloroplasts mature due to both their increased transcription and constant (or increased) stability.

In addition to the switching of RPOTp to PEP, RPOTmp is also developmentally regulated at the level of its sub-plastidial localization and activity. RPOTmp proteins in spinach and Arabidopsis are apparently associated with thylakoid membranes because intrinsic thylakoid membrane proteins fix RPOTmp on the stromal side of the membrane. The NEP interacting proteins (NIPs) have three N-terminal transmembrane domains and a C-terminal ring finger domain. Light-dependent expression of NIPs at an early developmental stage may determine the membrane association of RPOTmp and down-regulate plastid *rrn* transcription, consequently functioning as an "inactivate" switch of RPOTmp (Azevedo et al. 2006, 2008).

3. Possible Accessory Proteins and Extended Function of NEP

In mammals, some accessory proteins of mitochondrial T7 phage-type RNA polymerase POLRMT have been isolated and characterized (Sologub et al. 2009). Although no homologous proteins have been isolated in plants, nuclear-encoded factors having similar function may be translocated into plastids to fully activate or modulate NEP-dependent transcription. It is also noteworthy that human POLRMT itself contains two PPR motifs at its N-terminus (Asin-Cayuela and Gustafsson 2007). The N-terminal sequences of NEP proteins are different from that of POLRMT, but there might be some specific PPR proteins that bind directly to NEP and modify its activity. At least, it has been already shown that plastids utilize a PPR, DG1, in concert with PEP (Chi et al. 2010).

In humans and rodents, the mitochondrial POLRMT has an alternative transcript producing a single-polypeptide nuclear RNA polymerase (spRNAP-IV). The shorter gene product loses the N-terminal domain necessary for targeting mitochondria. The nuclear-localized protein recognizes some promoters that differ from those for RNA polymerase II (Kravchenko et al. 2005). However, any similar phenomenon has not been found in plants thus far.

During the last decade, genetic information and physiological features of both PEP and NEP have been clarified from lower to higher plants. However, the molecular and biochemical details of the transcription system in plastids are still unclear. How do PEP and NEP mechanically recognize a specific sequence and initiate transcription? How many proteins, including PPRs, function closely with PEP and NEP? What are their functions? How is NEP activity linked to high-order cellular dynamics, for example, morphological control or environmental responses? How is transcription controlled in other plastids besides chloroplasts? What is the true transcriptional feature of wild type expressing NEP and sigma factors in just the adequate proportion? In the next decade, these questions will surely be clarified.

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Chapter 11

Development-Dependent Changes in the Amount and Structural Organization of Plastid DNA

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Summary

Plastids usually contain numerous copies of their genome. The reason of maintaining high copy numbers of the plastome and their changes resulting from developmental, cellular (e.g., nuclear ploidy levels), and environmental cues remains elusive. The DNA is contained in certain regions of the plastids, the nucleoids. Number and shape of nucleoids change during leaf and chloroplast development. Generally, a substantial increase of nucleoids and therefore plastome copy numbers per organelle and cell occurs very early in leaf development, which later on provides not only enough plastomes for the distribution during plastid division, but may also meet the increasing demand for plastid gene products during chloroplast biogenesis. Later in leaf development the fate of chloroplast DNA seems to be regulated in a species-specific manner. While some species further increase the amount of plastid DNA (at least per cell), others seem to decrease plastome copy numbers per cell and per organelle during chloroplast maturation. The amount of chloroplast DNA decreases during senescence.

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I. Introduction

Since the discovery of DNA in chloroplasts (Ris and Plaut 1962), its structure, abundance, and coding capacity have been extensively investigated (for reviews see Butterfass 1979; Herrmann and Possingham 1980; Sugiura 1995; Bendich 2004; Green 2011). The chloroplasts have evolved by endosymbiosis from once free-living cyanobacteria (Margulis 1970; Martin 2003). Compared to their free-living ancestors, the size of the genome (plastome) was greatly reduced during this process (to approximately 130–160 kbp; Sugiura 1995). For the most part this is due to the constant relocation of genes from the ancestral genome/plastome to the nuclear genome (Timmis et al. 2004; Kleine et al. 2009). However, basic genetic mechanisms of the nucleic acid metabolism and gene expression such as replication, transcription, and translation have been maintained (Mache and Lerbs-Mache 2001; Green 2011). The reported numbers of plastome copies per leaf cell vary from a few hundreds to more than 10,000 depending on the organism examined (Lamppa and Bendich 1979; Tymms et al. 1983; Leutwiler et al. 1984; Shaver et al. 2006; Zoschke et al. 2007). The number of plastome copies seems therefore to be regulated in a species-specific manner. The structure of the genome is not fully understood. A substantial part of the DNA can be isolated in form of circular DNA molecules having the expected size of the complete genome ('plastid chromosome'). In addition, there are linear and circular di- and oligomers, but also smaller than genome size and more complex molecules (Bendich 2007). Thus, if we mention in the following 'plastome copy numbers' or 'plastid/chloroplast genome copies', we refer to multiples of the DNA amount representing one genome, but not to multiples of the 'plastid chromosome'. Higher plants, with their many different tissues and cell types, have evolved mechanisms to adapt plastome copy numbers to the needs of the respective cell. This review addresses various aspects of the organization, structure, and amount of plastid DNA during leaf development.

Abbreviations: CND41 – 41 kDa chloroplast nucleoid DNA binding protein; DAPI – 4′,6-diamidino-2-phenylindole; NEP – Nuclear-encoded plastid RNA-polymerase; PEND – Plastid envelope DNA-binding protein; PEP – Plastid-encoded plastid RNA-polymerase; ppGpp – Guanosine 5′-diphosphate 3′-diphosphate; qPCR – Quantitative real-time PCR; RubBisCO – Ribulose-1,5-bisphosphate carboxylase oxygenase; SN-type – Scattered nucleoid type; TAC – Transcriptionally active chromosome

II. Plastid DNA Is Contained in Nucleoids

A. The Number of Nucleoids and Their DNA Content per Organelle

Erwin Baur, Carl Correns (both in 1909) and Otto Renner (1934) demonstrated by genetic experiments that chloroplasts are individual genetic elements. In 1962, by using electron microscopic and cytochemical methods, Ris and Plaut (1962) indeed showed the presence of DNA within one or more irregularly shaped bodies in the chloroplast of *Chlamydomonas moewusii*. Subsequently, it was shown that the plastid DNA is replicated in a semiconservative way and contains genes for ribosomal RNAs, transfer RNAs as well as soluble and membrane-bound proteins (for references see Sugiura 1992). The appearance of plastid DNA in sub-plastomic bodies further sparked scientific interest. Various methods were used to obtain information on the structure and organization of the plastid DNA within the chloroplast, which ranged from early analyses of plastids isolated from thymidine labeled tissue by microscopic autoradiography to the later use of a DNA-specific, fluorescent stain, DAPI (4′,6-diamidino-2-phenylindole) (Herrmann 1970; Herrmann and Kowallik 1970; Dann et al. 1971; Kowallik and Herrmann 1972; Coleman 1978, 1979; James and Jope 1978; Kuroiwa et al. 1981).

Similar to *E. coli* (Ogden et al. 1988; Landoulsi et al. 1990), plastid nucleoids are mostly situated at peripheral areas during

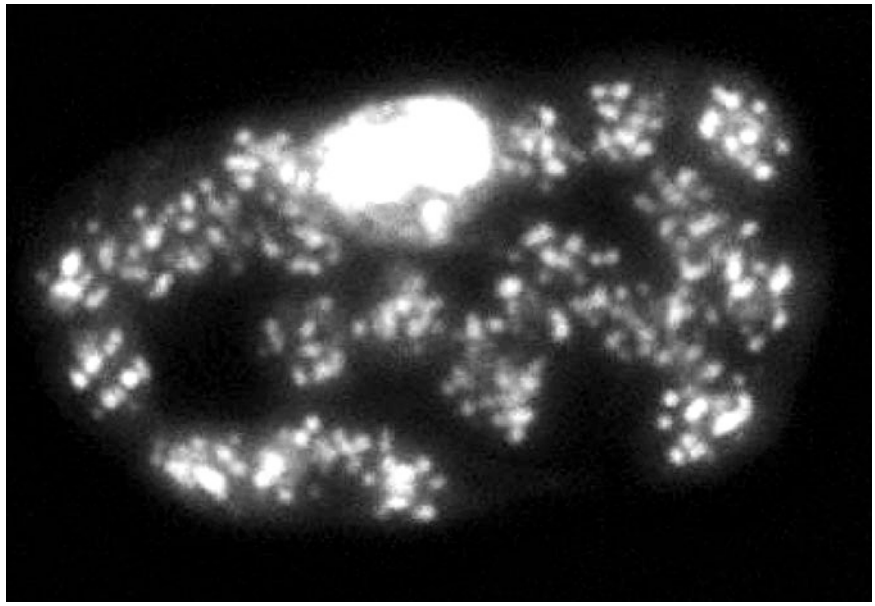

Fig. 11.1. DAPI-stained mesophyll cell of a young rosette leaf of *Arabidopsis thaliana*. All chloroplasts contain numerous nucleoids. The nucleus exhibits bright fluorescence due to its high DNA content. (Courtesy of R.G. Herrmann and H. Golczyk.)

active chloroplast division, since binding to the envelope membrane seems important for the replication of plastid DNA and equal distribution of the nucleoids (Kuroiwa et al. 1981; Miyamura et al. 1986). Later in development, the nucleoids were usually located within the organellar matrix associated with the thylakoids (Lindbeck et al. 1987). Membrane association is reported to enhance expression of several genes for photosynthetic components (Sato et al. 1993). The nucleoids of fully developed chloroplasts in leaves of angiosperms belong to the so-called SN-type (*s*cattered *n*ucleoids; Kuroiwa et al. 1981), which is distinguished by small nucleoids that are equally distributed throughout the plastid stroma (Fig. 11.1).

Interestingly, a correlation of the number of nucleoids, the amount of their DNA, and the size of the plastids has been reported (e.g., Herrmann 1970; Kowallik and Herrmann 1972; James and Jope 1978; Kuroiwa et al. 1981). Generally, polyploidization of the plastome within one organelle was shown by various isolation procedures, revealing multimeric copies and complex structures of the plastid DNA (Herrmann and Possingham 1980; Backert et al. 1995; Oldenburg and Bendich 2004; Scharff and Koop 2007). Kowallik and Herrmann (1972) furthermore suggested based on their in situ experiments, that nucleoids contain varying amounts of DNA even within one organelle. Similarly, individual polyploidization of plastid DNA of nucleoids, which can vary at least by a factor of ten, has been deduced from different fluorescent intensities after DAPI staining (Coleman 1978, 1979; James and Jope 1978; Miyamura et al. 1986).

Changes in DNA content, form, and number per organelle of nucleoids occur predominantly during chloroplast development. In proplastids and developing small chloroplasts only single or few mostly egg-shaped nucleoids have been observed. If kept in darkness, the nucleoids of the resulting etioplasts become cup-shaped and are often bound to the prolamellar body. Their size and DNA content increased about ten-times

with an average number of ten nucleoids per etioplast. However, during chloroplast differentiation the nucleoids divided rapidly. Therefore, the average number of nucleoids per chloroplast increased up to 30 in fully developed leaves containing similar DNA amounts as in etioplasts (Herrmann 1970; Herrmann and Kowallik 1970; Kowallik and Herrmann 1972; James and Jope 1978; Scott and Possingham 1980; Kuroiwa et al. 1981; Miyamura et al. 1986; Sato et al. 1993; Fujie et al. 1994; Kato et al. 2007; Rauwolf et al. 2010).

B. Protein Components of Plastid Nucleoids

Plastid nucleoids are compact structures in which the plastid DNA is associated and organized with proteins (Kuroiwa 1991). During chloroplast development and conversions between different plastid types, nucleoids undergo changes in form, size, and location within the organelle (see above; Hashimoto 1985; Kuroiwa 1991), presumably mediated by modifications in the composition and abundance of certain nucleoid proteins (Kuroiwa and Suzuki 1981; Briat et al. 1982; Hansmann et al. 1985; Reiss and Link 1985; Nemoto et al. 1988, 1990; 1991; Nakano et al. 1993). Since nucleoids lack distinct membrane or envelope structures, it is often unclear if identified proteins are indeed inherent nucleoid proteins, e.g., commonly nuclear-localized histones found in nucleoid preparations may have been nuclear contaminants (Phinney and Thelen 2005). However, it is conceivable that nucleoid proteins fall into certain categories depending on their function. Early biochemical analyses indeed showed several nucleoid-associated proteins involved in DNA-, membrane-, and protein-binding (Hansmann et al. 1985; Bülow et al. 1987; Lindbeck et al. 1987), involved in regulating gene expression and replication (Lam et al. 1988; Khanna et al. 1992), and presumably in DNA packaging (Nemoto et al. 1990; Baumgartner and Mullet 1991).

To date, only few plastid nucleoid-associated proteins have been characterized in more detail. Although histone-like proteins with biochemical properties similar to bacterial HU proteins have been reported in higher plants (Briat et al. 1984; Nakano et al. 1993; Yurina et al. 1995; Phinney and Thelen 2005) and algae (Crevel et al. 1989; Grasser et al. 1997; Kobayashi et al. 2002), the function of these proteins in higher plant nucleoid formation is not yet clear. Interestingly, proteomic studies of nucleoids in pea chloroplasts revealed that one of the most abundant proteins was DCP68 (Phinney and Thelen 2005; cf. Majeran et al. 2012). This protein is a sulfite reductase with plastid DNA-binding and condensing activities (Cannon et al. 1999) suggesting it to have a bifunctional role in reductive sulfur assimilation and being a major component of organellar nucleoid organization (Chi-Ham et al. 2002). Furthermore, this study identified approximately 35 other proteins possibly associated with nucleoids including a family of DNA gyrases, as well as proteins involved in DNA repair, and plastid transcription and translation (Phinney and Thelen 2005). A plastid envelope DNA-binding protein (PEND) is thought to attach the nucleoids to the envelope membrane early in chloroplast development in order to provide an anchor for plastome replication and segregation during organelle division (Sato et al. 1993, 1998; Terasawa and Sato 2005; Wycliffe et al. 2005). However, the PEND protein, was not detected in maize nucleoids which did also not contain the bisulfite reductase (Majeran et al. 2012). Other candidates for anchoring the DNA/nucleoid to the membrane are MFP1, CND4 and TCP34 (Nakano et al. 1993; Jeong et al. 2003; Weber et al. 2006). CND41 was identified in chloroplast nucleoids of cultured tobacco cells (Nakano et al. 1993) and was more abundant in actively dividing plastids than in mature organelles (Nakano et al. 1997). However, more recent studies point to CND41 being a DNA-binding protease involved in Rubisco degradation and the translocation of nitrogen during senescence (Murakami et al. 2000; Kato et al. 2004). Plastid nucleoids are thought to further contain Whirly ssDNA-

binding proteins (Krause et al. 2005), since they were co-purified with a chloroplast chromosome preparation (Pfalz et al. 2006) and shown to be associated with the thylakoid membrane (Prikryl et al. 2008). In Arabidopsis chloroplasts, Whirly proteins were shown to be important components of the plastid genome maintenance machinery, required for plastome stability (Maréchal et al. 2009; Cappadocia et al. 2010). Another nucleoid protein, YlmG, is proposed to be involved in chloroplast division and nucleoid partitioning and to have similar functions in cyanobacteria (Kabeya et al. 2010).

The eubacterial-type, plastid-encoded plastid RNA polymerase (PEP) can be isolated from plastids as both a soluble enzyme and an insoluble form, the '*t*ranscriptionally *a*ctive *c*hromosome' (TAC; e.g., Suck et al. 1996; Krause and Krupinska 2000; Pfalz et al. 2006). The TAC is a membrane bound protein/DNA complex (Gruissem and Tonkyn 1993) and should form a major part of the nucleoid. Proteomic data of both TAC and plastid nucleoids demonstrated that the PEP and other proteins are parts of both complexes (e.g., Krause and Krupinska 2000; Phinney and Thelen 2005; Pfalz et al. 2006; Majeran et al. 2012).

Although nuclear-encoded plastid RNA-polymerases (NEP) have not yet been found in TAC/nucleoid preparations (for review see Liere and Börner 2007; Steiner et al. 2011; Majeran et al. 2012), there are some indications from studies with transcription inhibitors that NEP is as well part of plastid nucleoids (Sakai et al. 1998; Sato et al. 2009).

A recent investigation into the proteome of nucleoids of proplastids and mature chloroplasts from maize leaves by mass spectrometry revealed a core proteome of more than 150 proteins (Majeran et al. 2012). In agreement with and extending previous studies the identified proteins have functions related to DNA (replication, organization, and repair) to RNA (synthesis, processing, splicing, and editing), and translation (ribosomal proteins, ribosome assembly factors). Comparison of the protein compositions of nucleoids from proplastids vs. mature chloroplasts indicated a shift from RNA metabolism to translation and homeostasis as predominant functions to occur in nucleoids during chloroplast development (Majeran et al. 2012; also for more discussion and references on nucleoid proteins).

III. Amount of Plastid DNA

A. Genome Copy Numbers per Plastid and Cell Vary Between Species and Tissues

Precise information about genome sizes has been obtained by sequencing whole plastid chromosomes. This data, together with the determination of the amounts of DNA per cell and organelle, revealed that plastids generally contain many copies of their genome. However, large differences exist among the species with respect to the number of plastome copies in the organelles and cells (Table 11.1). Part of the documented variability is due to different *nuclear* DNA levels. There is evidence for a positive correlation between nuclear ploidy levels and the number of plastome copies (see Butterfass 1979; Herrmann and Possingham 1980; Rauwolf et al. 2010 for discussion of older data). Therefore, the differences in the number of plastome copies between *Triticum monococcum* and *T. dicoccum* species (Dean and Leech 1982) and *Beta vulgaris* genotypes (Rauwolf et al. 2010) shown in Table 11.1 are discussed as a response to different nuclear genome ploidies. In a recent study, Rauwolf et al. (2010) presented thorough analyses of the DNA amounts per plastid and their changes during development of sugar beet leaves. On an average, the amounts determined for plastids with diameters from 1 to 8 μm ranged from 0.15 to 4.9×10^2 pg DNA. This corresponds to approximately 12–330 plastomes per organelle with about 4–7 copies per nucleoid. Interestingly, although the nuclear genome/plastome ratio changed during development, the plastome copy numbers seemed to depend on nuclear ploidy levels within the different developmental

Table 11.1. Plastome (plastid gene) copies per cell and organelle in mature green cells of different species

Species	Organ/tissue	Copies per cell	Copies per plastid	Method	Reference
Arabidopsis thaliana	Leaves 12-30d, hexaploid	1,200–1,700	20–35	qPCR	(Zoschke et al. 2007)
	Leaves 12–30d, dodecaploid	1,200–1,700	20–35		
	First and second leaves of 20-d-old plants		25	DAPI	(Rowan et al. 2009)
	First and second leaves of 20-d-old plants		24	qPCR	
	First and second leaves from 12- to 37-d-old plants	465–894			
Avena sativa	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 40 mm	16,500	101	DAPI	(Hashimoto and Possingham 1989a)
Beta vulgaris	Leaves		10–90	Autoradiography	(Herrmann 1970)
	Mature leaves 25–30 cm	1,888	29	DAPI	(Tymms et al. 1983)
	Trisomic leaf, very large plastids		216–324	Colorimetry	(Rauwolf et al. 2010)
	Mature leaves, 15–25 cm	3,297–3,688	121–132		
Hordeum vulgare	First leaf, 2d plus 6d light, plastids isolated 11–13 cm from base	3,300	55	Dot blot hybridization	(Baumgartner et al. 1989)
Medicago truncatula	Distal leaflet of mature leaves, isolated chloroplasts		47	DAPI	(Shaver et al. 2006)
	First leaf, 18d, greenhouse	362		qPCR	(Shaver et al. 2008)
Nicotiana excelsior	30 × 15 cm leaves		880	Colorimetry	Jope et al. (1978)
Nicotiana tabacum	Mature leaves, isolated chloroplasts		70	DAPI	(Shaver et al. 2006)
Oryza sativa	Second leaves, protoplasts, 156 h after imbibition, mature chloroplasts	800	45	DAPI	(Sodmergen et al. 1991)
Phaseolus vulgaris	First leaves, 21d, isolated chloroplasts		35	DAPI	(Kinoshita and Tsuji 1984)
Pisum sativum	Mature leaves, isolated chloroplasts		53	DAPI	(Shaver et al. 2006)
Solanum tuberosum	Leaves	3,000	22	Reassociation	(Scott et al. 1984)
Spinacia oleracea	Distal half of 10 cm leaf	4,900	57		Reassociation (Scott and Possingham 1980)
	Mature leaves, mesophyll cells in distal half of 10 cm long leaves	16,250	95	DAPI	(Lawrence and Possingham 1986)
Triticum monococcum	Mature cells of mature first leaves, isolated plastids	22,100	400	Colorimetry	(Dean and Leech 1982)
Triticum dicoccum	Mature cells of mature first leaves, isolated plastids	43,500	420		
Triticum aestivum	Mature cells of mature first leaves, isolated plastids	42,100	240		
	First leaves, 7d old seedlings, section 6–7 cm from leaf base, isolated plastids	50,000	310		Colorimetry (Boffey and Leech 1982)
Triticum aetivum	First leaves, 3d, tip, light, mesophyll		100	DAPI	(Miyamura et al. 1986)
Zea mays	Adult leaf, 58d, isolated plastids	1,456	13(27)[a]	DAPI	(Oldenburg and Bendich 2004)
	Second leaf, 17d, 16 h light/8h dark	800		qPCR	(Oldenburg et al. 2006)
Zea mays, Z. mexicana, Z. diploperennis,	Fully expanded first leaf blade, 8–12d after imbibition, isolated plastids		108	DAPI	(Zheng et al. 2011)
Zea mays	First leaf blade, 10d, 16 h light/8 h dark	1,600		qPCR	

[a]Calculation based on Vaccinia virus as DNA standard; value in brackets: calculation based on T4 phage as DNA standard

stages (Butterfass 1979; Herrmann and Possingham 1980; Rauwolf et al. 2010). Nuclear ploidy levels were shown to often correlate with changes in cell size and plastid numbers (Barow 2006) suggesting a basic control level of plastome copy numbers by the same or similar pathways (Rauwolf et al. 2010). Interestingly, the change in nuclear ploidy from the tetraploid *T. dicoccum* to the hexaploid *T. aestivum* (Dean and Leech 1982) and the massive endopolyploidization occurring during leaf development in Arabidopsis do not result in an increase of plastome copy numbers (Zoschke et al. 2007; Rowan et al. 2009) indicating that higher nuclear ploidy levels are not necessarily coupled with an increase in copy numbers of the chloroplast genome.

A larger part of the variability in plastid genome copies as shown in Table 11.1 is certainly due to technical problems as apparent from variations in the results obtained for the same species by different laboratories and methods (e.g., number of plastid genomes per cell in Arabidopsis: Zoschke et al. 2007; Rowan et al. 2009; *Beta vulgaris*: Tymms et al. 1983; Rauwolf et al. 2010; *Spinacia*: Scott and Possingham 1983; Lawrence and Possingham 1986; *Triticum aestivum*: Boffey and Leech 1982; Miyamura et al. 1990). DAPI stained plastid DNA needs for quantification a DNA standard. Depending on the standard, the calculated DNA amounts may differ by 100 % (Oldenburg and Bendich 2004). Also binding of protein to DNA and/or different degrees of compaction of DNA affects the fluorescence of DAPI (Sekine et al. 2002). PCR and other methods based on total leaf DNA have the disadvantage of being not suited to differentiate between the various cell types. Isolation of plastids/chloroplasts and protoplasts may lead to DNA degradation (for references and discussion of technical problems see below). Not only technical problems, also variable growth conditions (e.g., light, see below) or differences in the age and developmental stage (even though Table 11.1 contains only data from mature leaves) may lead to apparently conflicting results. Nevertheless, large interspecies differences with respect to plastome copy numbers exist (Table 11.1). There are species with relatively low amounts of plastid DNA, e.g., Arabidopsis with less than 2,000 chromosome copies per leaf cell and less than 50 copies per mature chloroplast (Leutwiler et al. 1984; Draper and Hays 2000; Zoschke et al. 2007; Rowan et al. 2009). Species like rye (*Avena sativa*) represent the other extreme with about 10,000 plastome copies per cell and more than 100 copies per chloroplast (Hashimoto and Possingham 1989a).

Green tissues of leaves, cotyledons and stems contain more plastome copies per organelle than non-green tissues like epidermis, root tissues, meristems or the vegetative and generative cells of pollen (Table 11.2). Since the number of plastids per cell is usually much lower in non-green tissues than in photosynthetically active tissues (e.g., Leutwiler et al. 1984; Pyke and Leech 1992, 1994; Pyke et al. 1994; Marrison et al. 1999; Pyke 1999; Rowan et al. 2004; Rauwolf et al. 2010), non-green cells contain much less plastid genomes than green cells (see references in Table 11.2; Lamppa and Bendich 1979; Cannon et al. 1986; Isono et al. 1997). Thus, for mesophyll cells of mature spinach leaves more than 16,000 plastome copies have been reported, while epidermis cells of the same leaves were found to possess only somewhat over 600 copies (Lawrence and Possingham 1986). Amyloplasts in potato tubers appear to be an exception. These non-green plastids were reported to contain with 195 plastome copies much more plastid DNA than leaf chloroplasts with only 22 copies (Scott et al. 1984). In a mixotrophic suspension culture of spinach cells, however, the copy number of plastomes in chloroplast-containing cells of the stationary phase was 5,940 contrasting with only 1,125 copies in amyloplast-containing cells of the log phase (Aguettaz et al. 1987). A similar ratio was calculated for plastome copies in leaf vs. root cells of Arabidopsis (Isono et al. 1997) and potato (Scott et al. 1984).

Table 11.2. Plastome (plastid gene) copies per cell and organelle in different tissues

Species	Organ/tissue	Copies per cell	Copies per plastid	Method	Reference
Arabidopsis thaliana	Seedlings, 3d, apical meristem, L1		28	DAPI	(Fujie et al. 1994)
	Seedlings, 3d, apical meristem, L2		19		
	Seedlings, 3d, apical meristem, L3		7		
	Seedlings, 3d, root		14		
	Seedlings, 3d, leaf primordia		38		
	Seedlings, 5d, first leaf		275		
	Pollen, vegetative and generative cell just after pollen mitosis I		14	DAPI	(Nagata et al. 1999)
	5th leaves of 14 d-old plants	1,000		qPCR	(Draper and Hays 2000)
	Cotyledons 2–27 d	1,100–1,500		qPCR	(Zoschke et al. 2007)
	Leaves 4–37d	1,000–1,700	20–35	qPCR	(Zoschke et al. 2007)
Medicago truncatula	Cotyledons, 10d, greenhouse	342		DAPI	(Shaver et al. 2008)
	First leaf, 10d, greenhouse	831			
Solanum tuberosum	Leaves	3,000	22	Reassociation	(Scott et al. 1984)
	Tubers	7,800	195		
	Roots	650			
Spinacia oleracea	Mature leaves, mesophyll cells in distal half of 10 cm long leaves	16,250	95	DAPI	(Lawrence and Possingham 1986)
	Mature leaves, epidermis cells in distal half of 10 cm long leaves	625	50		
Triticum aestivum	First leaves, 3d, shoot apex, light		45	DAPI	(Miyamura et al. 1986)
	First leaves, 3d, tip, light, mesophyll		100		
	First leaves, 3d, tip, light, epidermis		3		
Zea mays	Middle of stem, 13d, isolated plastids	5,280	96	DAPI	(Oldenburg and Bendich 2004)
	Juvenile leaf, 13d, isolated plastids	1,400	28		

B. Variation in Amount and Structure of Plastid DNA During Leaf Development

1. The Fate of Plastid DNA Differs Between Species

There is convincing evidence for the increase in the number and size of plastids and in the amount of DNA per plastid, and consequently per cell, during the earliest stages of leaf development when green, photosynthetically active chloroplasts develop from the smaller, undifferentiated proplastids in the presence of light (Table 11.3). This holds true for both dicotyledenous and monocotyledenous plants. Among the latter, only leaves of graminaceous species have been investigated so far. They are particularly suitable for studies on leaf development since new cells are produced only from the basal meristem; i.e., the leaf cells are in a linear array with the youngest near the base and the oldest at the tip of the blade (Boffey et al. 1979). However, the position of a cell within leaf indicates its age also in dicots. Therefore, several studies on plastome copies in leaf cells included not only leaves of different ages but also material from different leaf sections (Table 11.3). An increase in plastome copies is also observed during the development of etioplasts from proplastids in young cells; i.e., when leaves develop in complete darkness (Bennett and Radcliffe 1975; Baumgartner et al. 1989; Hashimoto and Possingham 1989b; Miyamura et al. 1990; Oldenburg et al. 2006; Shaver et al. 2008; Zheng et al. 2011). A rise in the amount of DNA per developing chloroplast/etioplast is only possible if the rate of plastid DNA replication exceeds the rate of plastid division, a condition that is normally not fulfilled in non-green tissues (Table 11.2).

Also light seems to affect the DNA content in a species-specific manner (Table 11.3). The reduction of plastid DNA copies during chloroplast maturation per chloroplast and cell as detected by DAPI staining depends on light in maize and *M. truncatula* (Oldenburg et al. 2006; Shaver et al. 2008; Zheng et al. 2011), a phenomenon that was also observed in barley leaves by dot blot hybridization (Baumgartner et al. 1989). The light-induced decrease of DNA per cell was neither observed in leaves of rye (*Avena sativa*), soybean, and wheat (Cannon et al. 1986; Hashimoto and Possingham 1989a; Miyamura et al. 1990), nor in cultured leaf discs of spinach. In the latter material, light stimulated the synthesis of chloroplast DNA (Hashimoto and Possingham 1989b).

After having reached a high level per chloroplast and cell early in leaf development, the chloroplast DNA may adopt different, species-specific courses during further leaf development: (1) it may keep the high level until senescence; (2) copy numbers per plastid may decline to a lower level caused by ongoing plastid division without replication of the DNA while the copy number per cell remains relatively stable, subsequently the DNA may keep the lower level in all chloroplasts until senescence; (3) copy numbers per plastid *and per cell* may be reduced to a lower level by ongoing plastid division without replication of the DNA and in addition due to active degradation of part of the DNA, subsequently the DNA may keep the lower level per cell and in all chloroplasts until senescence; (4) the DNA content per chloroplast may rapidly decline due to active degradation and plastid division resulting in the complete loss of DNA in most chloroplasts long before senescence. Those four courses are proposed based on published data (Table 11.3), however, they remain speculation. It is obvious that there are differences between the species with respect to the fate of plastid DNA during leaf maturation. Yet, in most cases (Table 11.3) there are not enough data available to unequivocally group the species into one out of those four routes, since, unfortunately, comprehensive data sets are missing concerning the earliest and/or latest stages of leaf development; i.e., just those stages which are expected to show drastic alterations in plastid DNA amounts.

An example for course 1 (see above) may be the fate of plastid DNA in sugar beet (*Beta vulgaris*) leaves. Rauwolf et al. (2010) report a steady increase in the DNA content

Table 11.3. Plastome (plastid gene) copies per cell and organelle – developmental changes

Species	Organ/tissue	Copies per cell	Copies per plastid	Method	Reference
Arabidopsis thaliana	Seedlings, 3d, leaf primordia		38	DAPI	(Fujie et al. 1994)
	Seedlings, 5d, first leaf		275		
	Seedlings, 10d, first leaf		590		
Arabidopsis thaliana	Cotyledons 2–27d, leaves 4–37d	1,000–1,700	20–35	qPCR	(Zoschke et al. 2007)
Arabidopsis thaliana	Entire shoots from 13-d-old seedlings, isolated chloroplasts		57	DAPI	(Rowan et al. 2009)
	First and second leaves of 20-d-old plants		25	DAPI	
	Entire shoots from 13-d-old seedlings, isolated chloroplasts		77	qPCR	
	First and second leaves of 20-d-old plants, isolated chloroplasts		24	qPCR	
	Juvenile leaves < 3 mm	255–301		qPCR	
	First and second leaves from 12–37-d-old plants	465–894		qPCR	
Avena sativa	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 2 mm	3,280	73	DAPI	(Hashimoto and Possingham 1989a)
	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 5 mm	6,110	97		
	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 10 mm	11,400	132		
	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 20 mm	16,000	116		
	Protoplasts from first leaves of 4-d-old seedling, continuous light; distance from base 40 mm	16,500	101		
	Protoplasts from first leaves of 4-d-old seedling, continuous darkness; distance from base 40 mm	24,900	163		
	Protoplasts from first leaves of seedlings grown for 4 d in darkness followed by 24 h light	25,500	156		
Beta vulgaris ssp. vulgaris	Young leaves, isolated chloroplasts		13	Density gradient centrifugation, colorimetry	(Pascoe and Ingle 1978)

Beta vulgaris	Leaves		10–90	Autoradiography	(Herrmann 1970)
Beta vulgaris	Young leaves 2–3 cm	1,118	104	Reassociation	(Tymms et al. 1983)
	Mature leaves 25–30 cm	1,888	29		
Beta vulgaris	Leaves, small plastids		10–30	Colorimetry	(Rauwolf et al. 2010)
	Leaves, large plastids		110–130		
	Trisomic leaf, very large plastids		216–324		
	Young leaves, 0.2–0.5 cm	66–100	9–13		
	Young leaves, 2.5–3.5 cm	750–1,556	13–18		
	Mature leaves, 15–25 cm	3,297–3,688	121–132		
Hordeum vulgare	First leaf, 2d plus 2d light, plastids isolated from basal 5 mm	1,700	140	Dot blot hybridization	(Baumgartner et al. 1989)
	First leaf, 2d plus 2d light, plastids isolated 10–20 mm from base	7,000	220		
	First leaf, 2d plus 6d light, plastids isolated 11–13 cm from base	3,300	55		
	First leaf, 4d darkness, plastids isolated from basal 5 mm	1,550	130		
	First leaf, 4d darkness, plastids isolated 10–20 mm from base	5,900	210		
	First leaf, 8d darkness, plastids isolated 11–13 cm from base	6,200	220		
Medicago truncatula	Distal leaflet of young leaves, isolated chloroplasts		122	DAPI	(Shaver et al. 2006)
	Distal leaflet of mature leaves, isolated chloroplasts		47		
Medicago truncatula	Cotyledons, 5 d, darkness, isolated plastids		21	DAPI	(Shaver et al. 2008)
	Cotyledons, 3 d darkness, 3 h light		364		
	Cotyledons, 3d darkness, 9 h light		180		
	Cotyledons, 3d darkness, 24 h light		51		
	Cotyledons, 5d, 16 h light/8h dark		8		
	Cotyledons, 3d, darkness	1,047		qPCR	
	Cotyledons, 3d, darkness, 2 h light	2,285			
	Cotyledons, 3d, darkness, 4 h light	2,109			
	Cotyledons, 3d, darkness, 16 h light	1,957			
	Cotyledons, 4d, greenhouse	899			
	Cotyledons, 10d, greenhouse	342			
	Cotyledons, 18d, greenhouse	204			
	First leaf, 8d, greenhouse	577			
	First leaf, 10d, greenhouse	831			

(continued)

Table 11.3. (continued)

Species	Organ/tissue	Copies per cell	Copies per plastid	Method	Reference
	First leaf, 14d, greenhouse	293			
	First leaf, 18d, greenhouse	362			
	30 × 15 cm leaves		880		
Nicotiana tabacum	Young leaves, isolated chloroplasts		130	DAPI	(Shaver et al. 2006)
	Intermediate leaves		190		
	Mature leaves, isolated chloroplasts		70		
Oryza sativa	Second leaves, protoplasts, 60 h after imbibition, young developing chloroplasts	320	20	DAPI	(Sodmergen et al. 1991)
	Second leaves, protoplasts, 96 h after imbibition, mature chloroplasts	1,800	100		
	Second leaves, protoplasts, 156 h after imbibition, mature chloroplasts	800	45		
	Second leaves, protoplasts, 168 h after imbibition, mature chloroplasts	550	30		
Phaseolus vulgaris	First leaves, 7d, isolated chloroplasts		52	DAPI	(Kinoshita and Tsuji 1984)
	First leaves, 13d, isolated chloroplasts		45		
	First leaves, 21d, isolated chloroplasts		35		
Pisum sativum	Seedlings 7d, isolated chloroplasts, light -grown		224	Mass of DNA bands after gel electrophoresis	(Bennett and Radcliffe 1975) (corrected values; see Boffey and Leech 1982)
	Seedlings 10 d, isolated chloroplasts, light grown		224		
	Seedlings 7d, isolated etioplasts, dark- grown		400		
	Seedlings 10 d, isolated etioplasts, dark-grown		864		
Pisum sativum	Young leaves	6,106	244	Reassociation	(Lamppa and Bendich 1979)
Pisum sativum	Young leaves, isolated chloroplasts		135	DAPI	(Shaver et al. 2006)
	Mature leaves, isolated chloroplasts		53		
Spinacia oleracea	Basal half of 2 cm leaf	3,300	353	Reassociation	(Scott and Possingham 1980)
	Distal half of 2 cm leaf	5,700	240		
	Distal half of 5 cm leaf	5,700	112		

	Distal half of 8 cm leaf	4,400	63		
	Distal half of 10 cm leaf	4,900	57		
	Chloroplasts isolated from tip of 2 cm long leaves		205	DAPI	(Lawrence and Possingham 1986)
	Chloroplasts isolated from tip of 5 cm long leaves		135		
	Chloroplasts isolated from tip of 10 cm long leaves		80		
Triticum aestivum	First leaves, 7d old seedlings, section 1–2 cm from leaf base, isolated plastids	<40,500	810	Colorimetry	(Boffey and Leech 1982)
	First leaves, 7d old seedlings, section 2–3 cm from leaf base, isolated plastids	>51,000	1,020		
	First leaves, 7d old seedlings, section 4–5 cm from leaf base, isolated plastids	50,000	320		
	First leaves, 7d old seedlings, section 6–7 cm from leaf base, isolated -treated plastids	50,000	310		
Triticum aestivum	First leaves, 3d, shoot apex, light		45	DAPI	(Miyamura et al. 1986)
	First leaves, 3d, shoot apex, darkness		32		
	First leaves, 3d, leaf base, light, mesophyll		78		
	First leaves, 3d, leaf base, darkness, mesophyll		72		
	First leaves, 3d, 10 mm from leaf base, light, mesophyll		97		
	First leaves, 3d, 10 mm from leaf base, darkness, mesophyll		125		
	First leaves, 3d, tip, light, mesophyll		100		
	First leaves, 3d, tip, darkness, mesophyll		110		
	First leaves, 3d, leaf base, light, epidermis		45		
	First leaves, 3d, leaf base, darkness, epidermis		32		
	First leaves, 3d, 10 mm from leaf base, light, epidermis		3		
	First leaves, 3d, 10 mm from leaf base, darkness, epidermis		3		
	First leaves, 3d, tip, light, epidermis		3		
	First leaves, 3d, tip, darkness, epidermis		3		
Zea mays	Base of stem, 8d, isolated plastids	3,300	165 (348)[a]	DAPI	(Oldenburg and Bendich 2004)

(continued)

Table 11.3. (continued)

Species	Organ/tissue	Copies per cell	Copies per plastid	Method	Reference
	Middle of stem, 8d, isolated plastids	7,095	225 (473)[a]		
	Juvenile leaf, 8d, isolated plastids	1,590	106 (224)[a]		
	Base of stem, 13d, isolated plastids	2,376	36 (75)[a]		
	Middle of stem, 13d, isolated plastids	5,280	96 (203)[a]		
	Juvenile leaf, 13d, isolated plastids	1,400	28 (60)[a]		
	Adult leaf, 58d, isolated plastids	1,456	13 (27)[a]		
Zea mays	Base of stalk, 17d, 16 h light/8 h dark	1,500		qPCR	(Oldenburg et al. 2006)
	Base of stalk, 17d, darkness	380			
	Top of stalk, 17d, 16 h light/8 h dark	1,300			
	Top of stalk, 17d, darkness	1,820			
	First leaf, 17d, 16 h light/8h dark	220			
	First leaf, 17d, darkness	1,820			
	Second leaf, 17d, 16 h light/8h dark	800			
	Second leaf, 17d, darkness	2,180			
Zea mays	Stalk, small isolated plastids		113	DAPI	(Zheng et al. 2011)
	Stalk, large isolated plastids		221		
Zea mexicana	Stalk, small isolated plastids		71		
	Stalk, large isolated plastids		262		
Zea diploperennis	Stalk, small isolated plastids		60		
	Stalk, large isolated plastids		183		
Zea mays	First leaf blade, 10d, 16 h light/8 h dark, isolated plastids		78		
	First leaf blade, 10d, darkness, isolated etioplasts		665		
	First leaf blade, 10 d darkness, then 4 h light, isolated plastids		832		
	First leaf blade, 10 d darkness, then 4 h light, isolated plastids		490		
	First leaf blade, 10 d darkness, then 8h light, isolated plastids		246		
	First leaf blade, 10 d darkness, then 24 h light, isolated plastids		80		
	First leaf blade, 10d, 16 h light/8 h dark		1,600	qPCR	
	First leaf blade, 10d, darkness		1,200		
	First leaf blade, 10 d darkness, then 24 h light		1,600		

[a]Calculation based on Vaccinia virus as DNA standard; value in brackets: calculation based on T4 phage as DNA standard

of plastids and cells during leaf development reaching highest levels in mature leaves confirming and extending older data (Herrmann 1970; Herrmann and Kowallik 1970; Kowallik and Herrmann 1972; cf. Tymms et al. 1983). Also rye plastid DNA may follow this way. Hashimoto and Possingham (1989a) measured the fluorescence of DAPI-stained plastid DNA and calculated the number of plastomes in individual chloroplasts and cells along the developmental gradient in the blades of primary leaves. They observed a rise of plastome copies both per chloroplast and per cell over the first basal 10 mm without further alteration in the level per cell during maturation of leaves and chloroplasts up to the tip. There was a slight decline in the DNA content per plastid between 10 and 30 mm, but no further change up to the leaf tip. The fate of chloroplast DNA in tobacco (*Nicotiana tabacum)* leaves may meet course 1 (Li et al. 2006) or course 2 (Rowan and Bendich 2009). Courses 2–4 are similar to each other: after a rise follow a descent in plastid DNA levels before senescence starts, but rate and extent of the decline in DNA content differ. Most species are reported to exhibit a decline in the DNA content per plastid during leaf maturation (Table 11.3). The fate of plastid DNA may follow course 2 in pea, wheat and spinach. The drop in plastome copy numbers per organelle during leaf maturation is suggested to result from the division of plastids without corresponding replication of DNA. There is no indication for significant reductions of plastid DNA per cell and therefore not for active degradation of DNA in these species (Lamppa et al. 1980; Boffey and Leech 1982; Lawrence and Possingham 1986; Miyamura et al. 1990). Barley may adopt course 3 (Baumgartner et al. 1989), also *Medicago truncatula* (although the observed decline in plastid DNA per leaf cell is only about 50–60 %; Shaver et al. 2008), and rice (*Oryza sativa*; Sodmergen et al. 1989, 1991). During the first 96 h after imbibition, number and size of chloroplasts and the amount of plastid DNA increased in the second rice leaf reaching a level of about 100 copies per plastid and 1,800 per cell. While chlorophyll content, chloroplast number and size remained stable, the number of plastome copies per chloroplast and cell dropped down to approximately 20 and 550, respectively, during the following 72 h. At this point of time, 168 h after imbibition, DAPI did not stain the chloroplast DNA anymore. Aging of the second leaves starts later at 240 h after imbibition. Therefore, degradation of plastid DNA was suggested to trigger senescence (Sodmergen et al. 1991).

We have categorized barley and rice as course 3 because the lowest reported plastome numbers per chloroplast are 50 and 20, respectively (Baumgartner et al. 1989; Sodmergen et al. 1991). All mature chloroplasts in cells from the middle section of primary barley leaves showed intensive fluorescence of the DNA/nucleoids after DAPI staining (Scott et al. 1982). It is not clear from the presented data how the number of 20 plastome copies per organelle fits to the observation that almost all mature chloroplasts could not be stained by DAPI in rice (Sodmergen et al. 1991). Like maize (*Zea mays*, Oldenburg and Bendich 2004; Oldenburg et al. 2006; Zheng et al. 2011), and perhaps Arabidopsis (Rowan et al. 2004, 2009), rice could also be an example for course 4, or vice versa, chloroplast DNA in all those species might actually meet fate 2 or 3. The proposed courses 3 and 4 differ from each other in the amount of DNA per chloroplast after its decline but well before the onset of senescence: a low level in most chloroplasts compared to young, dividing plastids in case of course 3 and none in most chloroplasts in case of course 4. If the lack of DAPI fluorescence would indeed indicate the absence of DNA from chloroplasts, then rice, maize and Arabidopsis would adopt course 4, since for all three species the decline in plastid DNA content is reported to result in chloroplasts without DAPI-stainable DNA (the vast majority of chloroplasts was found to contain no DNA as far as it is detectable by DAPI staining; Sodmergen et al. 1991; Oldenburg and Bendich 2004; Rowan et al. 2004; Evans et al. 2010). DAPI staining

combined with a highly sensitive detection technique was demonstrated to detect DNA of the size of a single plastid genome (Miyamura et al. 1986). This does not necessarily mean that such sensitivity can be achieved under all conditions. In other species (which we would classify as species that adopt course 1 or 2), DNA/nucleoids can easily be observed after DAPI staining in virtually all chloroplasts of mature leaves (e.g., Scott and Possingham 1980; Scott et al. 1984; Cho et al. 2004; Rauwolf et al. 2010; Fulgosi et al. 2012). Thus, a dramatic reduction or even lack of DNA in all or most mature chloroplast (courses 3 and 4) is not a *general* feature of chloroplast and leaf development in contrast to a recent proposal (Rowan and Bendich 2009).

2. Do Mature Chloroplasts Lack DNA?

Data of several studies challenge the idea that there is no or nearly no DNA in mature chloroplasts of potential course four-species like maize and Arabidopsis. DNA blot hybridization and quantitative real-time PCR (qPCR) contradict the conclusions drawn from DAPI data by indicating no or only minor changes in the DNA content of chloroplasts during development (Draper and Hays 2000; Li et al. 2006; Zoschke et al. 2007; Rowan et al. 2009; Evans et al. 2010; Zheng et al. 2011). Rowan et al. (2009) proposed that the disagreement between data obtained by different methods might result from the fact that only green tissue was usually analyzed by DAPI staining whereas qPCR and hybridization were applied to DNA originating from all leaf tissues. It is easily imaginable that in one cell type (e.g., in green mesophyll cells) the DNA per chloroplast decreases during leaf maturation and the nuclear DNA remains stable, while in other cells (e.g., all non-green cells) the nuclear DNA increases due to endopolyploidization, but the number of plastome copies per plastid does not change. Rowan et al. (2009) suggested that under such conditions quantitative PCR with total DNA as template would not detect the decline of chloroplast DNA. However, a four-fold decline of chloroplast DNA in 50 % of the cells (about 50 % of cells in wheat leaves are mesophyll cells, Dean and Leech 1982; Arabidopsis leaves consist of about 50 % green and 50 % non-green cells, Rowan et al. 2009), and a stable plastid DNA content in the remaining 50 % of cells (non-green tissues contain much less DNA per plastid and per cell, Table 11.2) would be easily detectable by PCR.

More recently, a second explanation was proposed for the deviating results of DAPI staining on one hand and blot hybridization and PCR on the other hand – the potential existence of copies of chloroplast sequences within the nuclear (and mitochondrial) genome (Zheng et al. 2011). However, copies of chloroplast DNA sequences in nuclear and mitochondrial genomes were taken into account in the qPCR and hybridization studies by Li et al. (2006) and Zoschke et al. (2007); yet, no changes in chloroplast DNA content were observed during leaf development.

Thirdly, a possible explanation for the contradicting results may be that under certain conditions DAPI staining does not provide correct results. Rowan and Bendich (2009) could rule out that DNase acting during isolation on chloroplasts would lead to the observed loss of DNA, since the demise of chloroplast DNA was also observed by DAPI staining in situ. However, Evans et al. (2010) suggested that DAPI might have no access to chloroplasts at later stages of development and therefore does not stain their DNA. In addition, changes in structure and organization of chloroplast DNA during leaf development might lead to altered interactions with DAPI (Sekine et al. 2002; Oldenburg and Bendich 2004; Rowan et al. 2004; Shaver et al. 2006).

Data obtained in another series of experiments are also not in agreement with the lack of DNA in most chloroplasts of mature leaves suggested by the missing DAPI staining. The lack of DNA in chloroplasts would preclude transcription. Yet, active chloroplast transcription is commonly observed in mature

chloroplasts. The transcription rates of several Arabidopsis chloroplast genes (including house-keeping and photosynthesis genes) were measured by run-on assays with chloroplasts from cotyledons and rosette leaves of different ages. The activities of all genes were highest in young and mature leaves exhibiting full photosynthetic activity (6–18-day-old) and still reached 25–50 % of their maximal values in old leaves at the beginning of senescence (Zoschke et al. 2007). Similarly, the transcriptional activity of chloroplast genes was determined in chloroplasts isolated from different sections of maize leaves representing the developmental gradient from very young to mature cells. Transcription of all investigated genes was highest in the cells at the tip of leaves, i.e. in the oldest cells with mature chloroplast (Cahoon et al. 2004). Furthermore, analyses of the transcriptional activity of chloroplast genes in barley during plant development and artificially induced senescence (prolonged dark treatment) showed active transcription of most tested genes in old or senescent leaves (Krupinska and Falk 1994; Krause et al. 1998). According to the data obtained by DAPI staining, most mature chloroplasts do not contain DNA, while a few chloroplast showed still a high fluorescence indicating the presence of many genome copies (Oldenburg and Bendich 2004; Zheng et al. 2011). These few chloroplasts might theoretically be responsible for the observed transcription activities in chloroplast isolated from mature or senescent leaves (Krupinska and Falk 1994; Krause et al. 1998; Cahoon et al. 2004; Zoschke et al. 2007). Why should transcription be needed in a few chloroplasts and not in the vast majority of chloroplasts? Chloroplasts without DNA seem imaginable since most mitochondria of higher plants do not possess the complete genome and might function (for a short time?) even without DNA (Preuten et al. 2010; Wang et al. 2010). Yet, the situation of mitochondria is not comparable to the situation of chloroplasts since mitochondria can theoretically exchange components like genes and gene products by frequent fission and fusion, i.e. the individual mitochondria seem to exist only for short periods of time (Arimura et al. 2004; Sheahan et al. 2005). Fusion of chloroplasts has not been observed, and stromules interconnecting chloroplasts (Gray, Chap. 9) might not be formed frequently enough to be a substitute for organellar fusion as a means of exchange (see Chap. 7; Hanson and Sattarzadeh 2011). At least in mature leaves of barley *all* chloroplasts were found to be transcriptionally active. By a combination of autoradiography, light microcopy and electron microscopy, Siemenroth et al. (1981) showed that all chloroplasts in 8–10-day-old primary leaves incorporated on a similar scale labeled uridine into RNA.

Taken together, the data suggest that all chloroplasts in mature leaves do contain substantial amounts of DNA, and the apparent loss of DNA in chloroplasts during maturation and long before senescence (‘course four’) may be an artefact due to technical problems with staining the DNA by DAPI. Obviously, the situation calls for more carefully designed experiments.

3. Chloroplast DNA Levels Decline During Senescence

Regardless of the reported differences in the fate of chloroplast DNA during leaf development, the data show consistently that leaf cells maintain hundreds or thousands copies of the plastome (Table 11.3) and that chloroplast genes are active at least until the start of pigment degradation, i.e. the visible onset of senescence (e.g., Jiang et al. 1993; Zoschke et al. 2007; Evans et al. 2010). There are only a few studies, which followed the further fate of chloroplast DNA during senescence. In soybean *(Glycine max)*, the chloroplast DNA content per organelle and per cell starts to decline when Rubisco large subunit (*rbcL)* mRNA levels, Rubisco protein levels and Rubisco activity decrease (Jiang et al. 1993). In Arabidopsis, the level of *rbcL* mRNA starts to drop down and *rbcL* transcription declines at about the stage when the degradation of chlorophyll is initiated, while

there is still no significant decline of chloroplast DNA. At this stage, the accumulation of *psbA* transcripts remained high, though *psbA* transcription was damped (Zoschke et al. 2007). During later stages of senescence, in yellow Arabidopsis leaves having lost more than 80 % of their chlorophyll, the levels of chloroplast DNA and *rbcL* mRNA further decrease, whereas the number of chloroplasts and the *psbA* mRNA level remain stable (Evans et al. 2010). Similarly, a steady decrease of DAPI staining of plastid nucleoids and of the *rbcL* gene as detected by DNA-blot hybridization was observed during natural senescence of maple (*Acer pseudoplatanus*) leaves (Fulgosi et al. 2012). Taken together, the data suggest that senescing leaf cells keep plastid DNA as long as they keep their chloroplasts.

IV. Conclusions

Plastids normally contain many copies of their genome. To date the reason of maintaining high copy numbers of the plastome and their changes resulting from developmental, cellular (e.g., nuclear ploidy levels), and environmental cues remains elusive. It has been suggested that the adjustment of plastome copy numbers is a regulatory response to varying demands of plastid gene expression (Bennett and Radcliffe 1975; Scott and Possingham 1983), in particular of the genes for ribosomal RNA (Bendich 1987). However, the transcriptional activity of plastid genes seems not generally to be correlated with plastome copy numbers (Eberhard et al. 2002; Zoschke et al. 2007). A massive increase in plastome copy numbers per organelle and cell occurs very early in leaf development accompanied by a rise in the number of nucleoids per plastid. This increase is likely owing to the increased demand for plastid gene products during chloroplast biogenesis as mentioned above, but also to provide enough plastome copies for distribution to the daughter organelles during the phase of very active plastid division (Kuroiwa 1991). The available data suggests a species-specific fate of chloroplast DNA later during leaf development. Some species may further increase plastome numbers (at least per cell) during leaf maturation, while others may decrease the amount of plastid DNA per organelle and per cell before senescence. During senescence, the amount of chloroplast DNA per cell and organelle decreases. There is no proof for the assumption that degradation of DNA triggers senescence in leaves (Sodmergen et al. 1989), since rather large copy numbers of the plastome and chloroplast gene expression can still be observed at later stages of senescence. We have now a rather detailed understanding of the process of plastid division (Miyagishima 2011), but near to nothing is known about replication of plastid DNA (Parent et al. 2011) or the enzymes that degrade plastid DNA (Sodmergen et al. 1989). An attractive and challenging field of future research will be to find out how cells can control plastid numbers and plastid DNA levels in response to endogenous and environmental cues.

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Chapter 12

The Ins and Outs of Chloroplast Protein Transport

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Summary

Much of the chloroplast proteome is encoded in the nuclear genome and needs to be imported post-translationally. Information for the organellar targeting of these imported proteins lies in an N-terminal leader sequence, the transit peptide, which is specifically bound by receptor components at the chloroplast surface. These receptor components are part of the TOC (*t*ranslocon at the *o*uter envelope membrane of *c*hloroplasts) complex, which, together with the TIC (*t*ranslocon at the *i*nner envelope membrane of *c*hloroplasts) machinery, mediates the translocation of precursor proteins into chloroplasts. Apart from the receptors, these complexes incorporate channel, motor and regulatory functions. Many components of this TOC/TIC apparatus have been identified. Multiple isoforms of the TOC receptors (and possibly of some other components) enable the operation of different import pathways with different substrate preferences, perhaps so that non-abundant proteins can be imported without serious competition from highly-abundant proteins of the photosynthetic apparatus. The different import pathways might also play a role in the differentiation of different plastid types. While much research has focused on these canonical TOC/TIC-mediated import routes, a number of studies have revealed alternative protein transport pathways to chloroplasts that employ different mechanisms; one of these passes through the endoplasmic reticulum and the Golgi apparatus. Other recent studies have revealed several protein targeting pathways leading to the envelope itself.

I. Introduction

A. Chloroplasts and Protein Import

Chloroplasts are the most prominent and intensively-studied members of a diverse group of organelles, the plastids, found ubiquitously in plants and a variety of algae (Whatley 1978; Keeling 2010). They contain chlorophyll and are responsible for the light and carbon reactions of photosynthesis, as well as many important biosynthetic functions (Nelson and Ben-Shem 2004; López-Juez and Pyke 2005). Other plastid types include the amyloplasts, which amass large quantities of starch and play important roles in energy storage and gravitropism, and the chromoplasts, which accumulate carotenoid

Abbreviations: aaRS – Aminoacyl-tRNA synthetase; AKR2A – Ankyrin repeat-containing protein 2A; APG1 – Albino or pale green mutant 1; BamA – β-barrel assembly machinery A; CAH1 – Carbonic anhydrase 1; ceQORH – Chloroplast envelope quinone oxidoreductase homologue; CIA2 (-5) – Chloroplast import apparatus 2 (-5); ClpC – Caseinolytic protease, subunit C; Com44/Cim44 – Chloroplast outer/inner membrane proteins, 44 kD; Cpn60 – Chaperonin, 60 kD; DEPC – Diethylpyrocarbonate; Fd – Ferredoxin; FNR – Ferredoxin-$NADP^+$ reductase; GAP – GTPase activating protein; GEF – Guanine nucleotide exchange factor; Hip – Hsp70-interacting protein; Hop – Hsp70/Hsp90-organizing protein; Hsp70 (-93, -100) – Heat-shock protein, 70 kD (93 kD, 100 kD); IDP – Intrinsically disordered protein; LHCII – Light-harvesting complex protein of photosystem II; MGD1 – Monogalactosyldiacylglycerol synthase 1; OEP–Outerenvelopeprotein,kD;PAGE–Polyacrylamide gel electrophoresis; PIC1 – Permease in chloroplasts 1; POTRA – Polypeptide transport associated; *ppi1* (*-2*, *-3*) – *Plastid protein import 1* (*-2*, *-3*); PreP – Presequence protease; SAM (Sam) – Sorting and assembly machinery; SP1 (*sp1*) – Suppressor of *ppi1* locus 1; SRP – Signal recognition particle; SSU (pSSU) – Rubisco small subunit (precursor of); Sti1 – Stress-inducible 1; Tat – Twin-arginine translocase; TIC (Tic) – Translocon at the inner envelope membrane of chloroplasts; TIM (Tim) – Translocase of the inner mitochondrial membrane; TOC (Toc) – Translocon at the outer envelope membrane of chloroplasts; TOM (Tom) – Translocase of the outer mitochondrial membrane; TPP – Thylakoidal processing peptidase; TPR – Tetratricopeptide repeat; TROL – Thylakoid rhodanese-like protein; VIPP1 – Vesicle-inducing protein in plastids 1

pigments and act as attractants in flowers and fruits (Neuhaus and Emes 2000; López-Juez and Pyke 2005).

Like mitochondria, chloroplasts evolved through endosymbiosis. They are believed to be descendent from an ancient photosynthetic prokaryote related to extant cyanobacteria (Larkum et al. 2007; Reyes-Prieto et al. 2007). While the modern organelle retains a fully-functional, endogenous genetic system, the organellar genome is greatly reduced and typically encodes just ~100 different proteins (Martin et al. 2002; Timmis et al. 2004). As a result, most chloroplast proteins must be imported from the cytosol. Because all plastids within a particular organism contain the same small set of organellar genes, it are the imported proteins that control the functions and developmental fate of each organelle (López-Juez 2007).

Approximately 3,000 different proteins are needed to develop a fully-functional chloroplast, and most (>90%) of these are encoded in the nucleus and synthesized on free cytosolic ribosomes (Keegstra and Cline 1999; Leister 2003). Typically, chloroplast proteins are made in precursor form, each one having an amino-terminal targeting signal called a transit peptide. These precursors, or preproteins, are then transported into the organelle in an energy-consuming, post-translational targeting process termed chloroplast protein import. Import is mediated by hetero-oligomeric protein complexes in the outer and inner envelope membranes called TOC and TIC (*t*ranslocon at the *o*uter/*i*nner envelope membrane of *c*hloroplasts), respectively (Fig. 12.1) (Soll and Schleiff 2004; Bédard and Jarvis 2005; Kessler and Schnell 2006; Inaba and Schnell 2008; Jarvis 2008; Li and Chiu 2010). The various components of the TOC and TIC complexes are discussed in detail in the following sections; for a list of the components, the reader is referred to Table 2 of Jarvis (2008). Chloroplast import is somewhat similar to mitochondrial protein import, which is mediated by the functionally analogous TOM and TIM (*t*ranslocase of the *o*uter/*i*nner *m*itochondrial membrane) complexes (Neupert and Herrmann 2007; Schmidt et al. 2010). In both cases, preproteins are threaded through the membranes in unfolded conformation. However, the main components of the chloroplast and mitochondrial protein import machineries are not closely related. Once a chloroplast preprotein reaches the organellar interior (the stroma), the transit peptide is removed, allowing the remaining part of the protein to fold into its functional conformation or engage one of several internal sorting pathways (see Sect. IV) (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Cline and Dabney-Smith 2008; Jarvis 2008; Li and Chiu 2010).

B. Transit Peptides

Most nucleus-encoded proteins of the chloroplast interior require an amino-terminal transit peptide to gain access to the organelle (Bruce 2000, 2001; Armbruster et al. 2009). Transit peptides are functionally analogous to the cleavable, amino-terminal presequences that mediate protein import into mitochondria (Neupert and Herrmann 2007; Schmidt et al. 2010). While the mature part of a chloroplast preprotein can influence import efficiency (Dabney-Smith et al. 1999; Rial et al. 2002), it is the transit peptide that specifically interacts with the import machinery (Sveshnikova et al. 2000; Hinnah et al. 2002; Inaba et al. 2003; Smith et al. 2004). In fact, transit peptides are very effective at mediating the import of heterologous passenger proteins into chloroplasts (Schreier et al. 1985; Van den Broeck et al. 1985; Lee et al. 2006, 2009a).

In spite of the apparent specificity of the chloroplast import process, transit peptides are remarkably heterogeneous in relation to amino acid sequence and total length (Bruce 2000, 2001). They possess no readily discernable blocks of sequence conservation, and lengths vary from 20 to >100 residues. Superficially, their only common characteristics appear to be an abundance of hydroxylated residues (serine in particular) and a deficiency of acidic residues, giving them a net positive charge. In this respect, transit

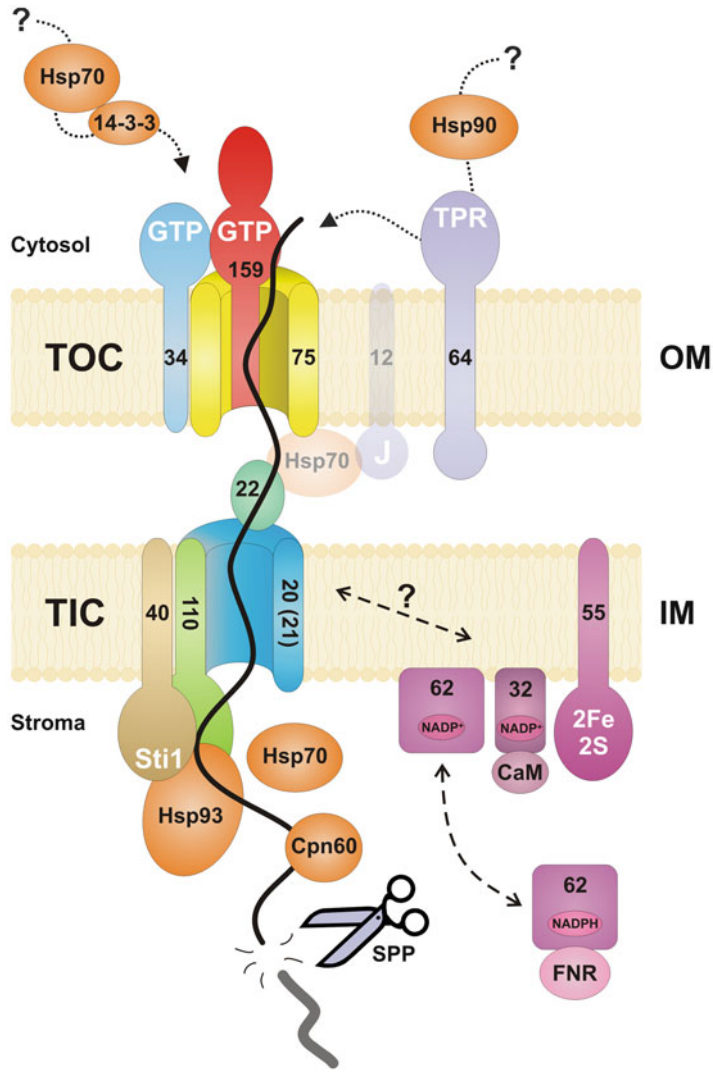

Fig. 12.1. The TOC and TIC complexes of the chloroplast protein import apparatus. An illustration showing the TOC and TIC translocons in the outer and inner envelope membranes (OM and IM, respectively). Individual components are identified by their molecular weights (*black text*), while some key functional domains are indicated (*white text*). Toc159, Toc34 and Toc75 together form the core TOC complex in the outer membrane. While Toc159 and Toc34 are responsible for preprotein recognition, Toc75 forms the outer envelope channel. The 14-3-3, Hsp70 and Hsp90 chaperones are proposed to interact with unfolded preproteins (forming so-called "guidance complexes") to maintain their import competence and direct them to the Toc34 or Toc64/OEP64 receptors. Tic22 is thought to provide a link between the TOC and TIC complexes, facilitating preprotein passage through the intermembrane space; however, the existence of Hsp70 and Toc12 in the intermembrane space is in doubt, as recent evidence indicates that both proteins are stromal. Inner envelope channels might be formed by Tic110 and/or Tic20/Tic21, or by cooperation between these components (as indicated). Tic110 also works together with Tic40 in the recruitment and regulation of stromal chaperones, such as Hsp93 and Hsp70; together, these components form a motor complex for protein import propulsion. SPP cleaves the transit peptide on the stromal side, while other chaperones (e.g., Cpn60 and Hsp70) facilitate protein folding or aid intraorganellar routing. A redox-regulator, comprising Tic32, Tic62 and Tic55, might be involved in fine-tuning the import process, working in conjunction with Ferredoxin-$NADP^+$ reductase (FNR) and calmodulin (CaM). (This figure has been adapted from Jarvis (2008).)

peptides are remarkably similar to mitochondrial presequences, and so it is not entirely clear how organellar specificity is achieved in plants (Macasev et al. 2000; Chew and Whelan 2004; Bhushan et al. 2006). In fact, a substantial number of preproteins are purposely "dual-targeted" to both chloroplasts and mitochondria (see Sect. V) (Silva-Filho 2003; Duchêne et al. 2005; Carrie et al. 2009), which clearly illustrates the functional similarities between the two types of targeting sequence.

Although mitochondrial presequences do not share a conserved consensus sequence, they do possess a characteristic secondary structure: they have the capacity to form amphipathic helices, and this is important for their interaction with receptors of the TOM complex (Brix et al. 1997; Abe et al. 2000). However, chloroplast transit peptides do not appear to possess secondary structure in aqueous solution (Krimm et al. 1999; Wienk et al. 2000). Instead, it has been suggested that they evolved specifically to have properties of a "perfect random coil", perhaps to aid interaction with cytosolic factors and/or the import machinery (von Heijne and Nishikawa 1991). It was recently reported that a minimal length of 60 N-terminal residues in unfolded conformation is required for efficient translocation, and, in cases where the transit peptide is shorter than this, the N-terminal part of the mature protein must also be unfolded (Bionda et al. 2010). An alternative possibility is that transit peptides adopt a characteristic structure only upon interaction with the outer envelope membrane, which is the only membrane containing galactolipids exposed to the cytosol (Krimm et al. 1999; Wienk et al. 2000; Bruce 2001). Indeed, transit peptides seem to interact strongly with artificial membranes containing chloroplast lipids in vitro (Bruce 1998), while an *Arabidopsis thaliana* mutant deficient in a chloroplast-specific galactolipid exhibited inefficient chloroplast protein import (Chen and Li 1998). Nonetheless, the role of envelope lipids in the import mechanism, if any, remains to be established.

Transit peptides have been dissected in a variety of mutational and deletion studies, leading to hypotheses that they possess a number of functional domains or motifs (Reiss et al. 1989; Pilon et al. 1995; Rensink et al. 2000; Lee et al. 2006, 2008, 2009a). However, no consensus of opinion has emerged from this work, and so the functional, defining features of a chloroplast transit peptide remain elusive. In spite of this, numerous computer programs are available that can be used to identify transit peptides with reasonable accuracy (Emanuelsson et al. 2007; Nakai and Horton 2007). Analysis of the Arabidopsis genome sequence using such tools yielded chloroplast proteome estimates ranging from ~2,000 to >4,000 proteins (Leister 2003; Richly and Leister 2004; Haas et al. 2005).

II. Events at the Outer Envelope Membrane

A. TOC Complex Composition

1. Identification of TOC Components

Attempts to find the envelope components involved in chloroplast protein import started in earnest about 20 years ago, and since then extensive biochemical studies have been performed with isolated *Pisum sativum* (pea) chloroplasts. In the early studies, thermolysin was found to be useful as it digests only those protein domains normally exposed to the cytosol at the chloroplast outer membrane. Significantly, this treatment can inhibit the ability of chloroplasts to bind precursors, which implied the existence of functional receptor proteins at the outer envelope surface involved in the import process (Cline et al. 1984, 1985; Friedman and Keegstra 1989). Quantitative analysis of precursor binding gave further evidence that chloroplast protein import is mediated by membrane-localized receptors, since the binding is saturable (Friedman and Keegstra 1989). There then followed attempts to isolate components of the protein import apparatus.

In the early 1990s, the core components were first detected in pea chloroplasts (Waegemann and Soll 1991; Perry and Keegstra 1994). It was not long before researchers from several laboratories actually identified the main proteins of the TOC and TIC complexes (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994; Schnell et al. 1994; Wu et al. 1994; Seedorf et al. 1995; Lübeck et al. 1996). Among the earliest components identified, three are of the outer membrane and one is of the inner membrane. They are named according to their molecular weights, thus: Toc34, Toc75, Toc159 and Tic110 (Schnell et al. 1997). It should be noted that the Toc159 protein was initially identified as an 86 kD proteolytic fragment, termed Toc86. It was not until the *Arabidopsis thaliana* genome was sequenced that it was recognized that Toc86 is only a part of a much larger full-length protein (Bölter et al. 1998; Chen et al. 2000).

Among these first-identified components, Toc34 and Toc159 are both GTPases, while Toc75 possesses properties of a channel; these three components are all integral proteins of the outer envelope membrane. Precursor binding and outer envelope translocation are driven by a TOC complex comprising these three proteins, as was shown in an in vitro assay using a reconstituted translocation system in lipid vesicles (Schleiff et al. 2003a). Further analyses like density gradient centrifugation, gel filtration, and blue native PAGE confirmed that the TOC core complex consists of Toc34, Toc75 and Toc159, and showed that it was between 500 kD and 1 MD in size, in pea and Arabidopsis (Schleiff et al. 2003b; Kikuchi et al. 2006; Chen and Li 2007). Moreover, the stoichiometry of the TOC complex components was reported to be 4–5:4:1 (Schleiff et al. 2003b) or 3:3:1 (Kikuchi et al. 2006), between Toc34, Toc75 and Toc159, respectively. Differences between these stoichiometric estimates may be due to the dynamic nature of complex composition (Becker et al. 2004a), the formation of a TOC complex superdimer of 800–1,000 kD, the presence of additional, unidentified components (Kikuchi et al. 2006), the degradation of Toc159 (in the ~500 kD complex, Toc159 was present as the 86 kD fragment) (Schleiff et al. 2003b), or the application of different techniques. To date, there is still no consensus on the true constitution of the TOC complex.

The structure of the core TOC complex was elucidated by electron microscopic analysis, which revealed a toroid shape with a thick ring surrounding a central cavity, and a finger domain in the center which separates the central cavity into four pore-like structures (Schleiff et al. 2003b). It was speculated that each pore-like structure is made up of one Toc34 molecule and one Toc75 molecule, and that the central finger is formed by one Toc159 molecule; this corresponded to the proposed stoichiometry of 4–5:4:1. The complex particle was estimated to have a diameter of 13 nm and a height of 10–12 nm (Schleiff et al. 2003b).

As the identity of the main components of the TOC complex became clear (Fig. 12.1), researchers concentrated more on investigating the specific functions of individual Toc proteins. Since the completion of the genome sequencing project (Arabidopsis Genome Initiative 2000), Arabidopsis has increasingly been adopted as a model system for plant development and cell biology research. In the import field, this allowed in vivo studies to be performed (Jarvis et al. 1998; Bauer et al. 2000; Gutensohn et al. 2000), which led to a better understanding of the mechanisms of chloroplast protein import.

2. The Receptor Proteins, Toc34 and Toc159

The Toc159 and Toc34 proteins are integral membrane proteins of the outer envelope membrane, and both are substantially exposed to the cytosol. Each protein has a C-terminal membrane anchor and a homologous GTP-binding domain. They are responsible for preprotein recognition; both proteins can bind preproteins directly and thus they are considered to be receptors (Perry and Keegstra 1994; Sveshnikova et al. 2000; Smith et al. 2004).

a. Toc34

Toc34, together with Toc159, was first identified by its association with precursors bound to isolated pea chloroplasts (Kessler et al. 1994; Schnell et al. 1994). Toc34 has two domains: a GTPase domain which is exposed in the cytosol, and a very short, C-terminal hydrophobic membrane span for localization in the outer membrane. Crystal structure analyses have been performed on *P. sativum* Toc34 and on atToc33, a Toc34 homologue in *A. thaliana* (note that the "at" prefix denotes species of origin). An internal cavity in atToc33 was identified, which might act as an interaction site for precursor binding (Sun et al. 2002; Koenig et al. 2008). Importantly, these studies showed that Toc34 can homodimerize through its GTPase domain, a fact also suggested by gel filtration and pull-down assays (Sun et al. 2002; Weibel et al. 2003; Yeh et al. 2007). An interesting possibility is that Toc34 also heterodimerizes with Toc159, through interaction of the homologous GTPase domains; indeed, such Toc34-Toc159 interactions were detected in biochemical analyses (Bauer et al. 2002; Smith et al. 2002; Wallas et al. 2003) and in yeast (Aronsson et al. 2010), and were verified in planta (Rahim et al. 2009). In other GTPase systems, GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) are usually involved in the transitions between the active and inactive forms of the protein, by stimulating GTP hydrolysis and replacement of GDP with GTP (Bourne et al. 1991); however, so far no such factors have been identified in the chloroplast import system.

The dimerization of Toc34 revealed by the crystal structure suggested that each single Toc34 molecule within a dimer might act as a GAP on the interacting monomer (Sun et al. 2002). This is similar to the regulation that occurs between the signal recognition particle (SRP) and its receptor of the ER translocation system (Keenan et al. 2001). However, the consequences of TOC receptor dimerization have been tested, using mutants, with variable results (Weibel et al. 2003; Yeh et al. 2007). Weibel et al. found that atToc33-R130A abolishes dimer formation without changing the efficiency of GTP hydrolysis, while Yeh et al. observed that the same mutation leads to a significant decrease in GTPase activity. The latter study found that the dimerization property is influenced by protein sample aging in vitro, a phenomenon that was also observed by Koenig et al. (2008), and this might explain the differing results from the two groups. However, Koenig et al. observed only minor GTPase activation upon dimerization, which led them to propose that the homodimer requires an additional factor as a co-GAP. However, a more recent report suggested that Toc34 homodimerization limits the nucleotide exchange rate, instead of stimulating hydrolysis, and that preprotein binding disrupts the dimer in order to promote GDP-GTP exchange (Aronsson and Jarvis 2011; Oreb et al. 2011). This contrasted somewhat with earlier studies showing that preprotein binding strongly stimulates GTP hydrolysis (Jelic et al. 2002, 2003), which has led to the suggestion that transit peptides perform a GAP function (Reddick et al. 2007). Regardless of these inconsistencies, studies on dimerization-defective atToc33 point mutants in organello, using chloroplasts from transgenic Arabidopsis plants, showed that the dimerization is important for the initiation of the preprotein translocation process (Lee et al. 2009b). However, such mutations do not obviously affect chloroplast development in planta or plant growth (Aronsson et al. 2010). Thus, the exact function of Toc34 dimerization remains to be determined.

Functional analysis of Toc34 has also been performed with Arabidopsis mutants, showing its important role in plastid import in vivo. There are two Toc34 homologues in Arabidopsis, termed atToc33 and atToc34. The *plastid protein import 1* (*ppi1*) mutant, lacking atToc33, was the first protein import apparatus mutant to be found, and its analysis was significant as it illustrated that a translocon component identified by biochemical approaches is actually functional in vivo (Jarvis et al. 1998). Such *ppi1* mutants

show a striking chlorotic phenotype, altered chloroplast ultrastructure, and compromised protein import in vitro (Jarvis et al. 1998; Gutensohn et al. 2004). On the other hand, the atToc34 mutant, *ppi3*, shows no obvious phenotype in aerial parts, but significant growth defects have been observed in the roots (Constan et al. 2004a). Collectively, these data demonstrate that the Toc34 receptor plays a central role in plastid protein import. The functions of atToc33 and atToc34 are partially redundant, as indicated by the fact that the double mutation, *ppi1 ppi3*, is embryo lethal (Constan et al. 2004a; Hust and Gutensohn 2006), and by the demonstration that the *ppi1* phenotype can be recovered by the overexpression of atToc34 (Jarvis et al. 1998). However, it is also suggested that atToc33 acts more specifically in the import of precursors of the photosynthetic apparatus (so-called photosynthetic preproteins), whereas atToc34 is involved more in non-photosynthetic preprotein import (Kubis et al. 2003) (Fig. 12.2; discussed in detail in Sect. II.D).

b. Toc159

Like Toc34, Toc159 is also regarded as a receptor, but it has a more complex structure: in addition to the central GTPase (G) domain, which shares about 30% identity with that of Toc34, it possesses a large acidic (A) domain at the N-terminus, and a large C-terminal membrane (M) domain. The A-domain is extremely unstable and thus Toc159 was identified initially as an 86 kD fragment lacking the entire A-domain (Hirsch et al. 1994; Bölter et al. 1998). The function of the A-domain is unclear; Toc159 protein lacking the A-domain can efficiently complement the atToc159 knockout mutant (*ppi2*) phenotype in Arabidopsis (Lee et al. 2003; Agne et al. 2009, 2010), indicating that the A-domain is not essential in vivo. However, isolated chloroplasts with intact Toc159 did perform more efficient preprotein import than those in which Toc159 A-domain had been proteolysed (Bölter et al. 1998), indicating that this non-essential domain does play a role in the import process. A recent report suggested that the A-domain can exist in a free, highly-phosphorylated form in the cytosol, separate from the other Toc159 domains, although the biological significance of this is presently unclear (Agne et al. 2010).

The A- and G-domains of Toc159 are exposed to the cytosol. Toc159 was degraded to a 52 kD M-domain fragment after protease treatment of isolated chloroplasts, a treatment which removes the exposed parts of outer membrane proteins (Hirsch et al. 1994; Kessler et al. 1994; Chen et al. 2000). This also indicated that the M-domain is membrane-embedded. Interestingly, unlike other membrane-spanning protein domains, the Toc159 M-domain lacks a clear hydrophobic stretch and is rather hydrophilic. In vitro import experiments showed that preproteins can be efficiently imported into isolated chloroplasts even when Toc159 has been degraded to just the 52 kD M-domain (Chen et al. 2000). Moreover, using a protoplast transient expression system as well as transgenic Arabidopsis plants expressing a series of deletion mutants, the M-domain was found able to complement the import defect associated with the loss of Toc159, and to partially recover the mutant phenotype of *ppi2* plants (Lee et al. 2003). This suggests that the M-domain is the minimal domain required for Toc159 function.

Toc159 has been reported to exist in both soluble and membrane-bound forms (Hiltbrunner et al. 2001; Ivanova et al. 2004), implying that the receptor might bind preproteins in the cytosol and target them to the chloroplast membrane. However, whether this soluble form exists or is relevant is in doubt, as it has been reported that it is no longer found after higher-speed centrifugation is used to isolate Toc159-containing membranes (Becker et al. 2004a), and that it may in fact simply correspond to the free A-domain, as discussed earlier (Agne et al. 2009, 2010).

In Arabidopsis, the Toc159 receptor is encoded by a gene family with four members, and the corresponding proteins are termed atToc159, atToc132, atToc120 and atToc90

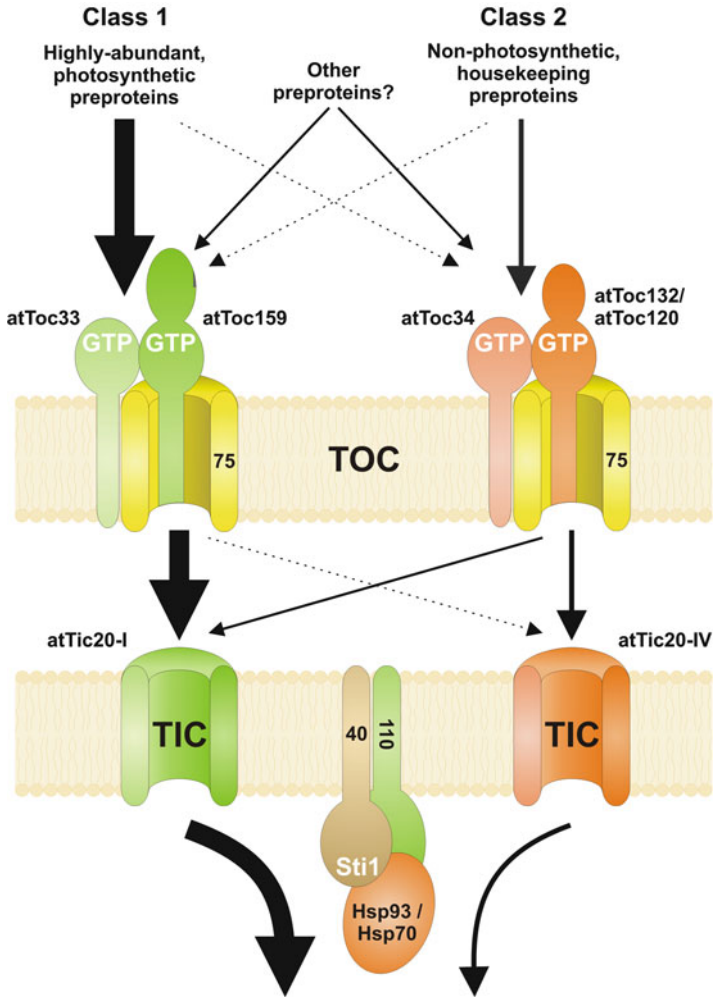

Fig. 12.2. Substrate-specific pathways for protein import into chloroplasts. Multiple isoforms of the TOC receptors exist in Arabidopsis and other species. These are believed to associate differentially to form distinct translocon complexes with different substrate (preprotein) specificities. The most abundant isoforms of the Toc159 and Toc34 receptors in Arabidopsis (atToc159 and atToc33, respectively) associate to form a TOC complex with specificity for highly-abundant, photosynthetic preproteins (*Class 1*). By contrast, the less abundant isoforms, atToc132/120 and atToc34, associate to form a different TOC complex with specificity for lower abundance, non-photosynthetic or housekeeping preproteins (*Class 2*). The *dotted crossing arrows* indicate that the aforementioned substrate specificities are not absolute, while there may even be a third class of preproteins that do not show a particular preference for either pathway. Recent evidence suggests that these different, substrate-specific import pathways may extend down to the level of the inner membrane. It is hypothesized that the two principal Arabidopsis isoforms of the putative channel protein, Tic20, form distinct TIC complexes with different properties: the atTic20-I complex having specificity for photosynthetic preproteins, and the atTic20-IV complex having specificity for non-photosynthetic proteins. A common motor complex, comprising Tic110, Tic40 and Hsp93/Hsp70, provides the driving force for translocation through both pathways. (This figure has been adapted from Jarvis (2008) and Hirabayashi et al. (2011).)

(Bauer et al. 2000; Ivanova et al. 2004; Kubis et al. 2004). The different isoforms share significant similarity in the G- and M-domains, but are rather divergent in the A-domain. Among them, atToc90 has only a truncated A-domain, while atToc132 and atToc120 are more closely related to each other than to atToc159 (Ivanova et al. 2004).

Genetic analyses showed that they do perform important roles in chloroplast protein import in vivo. The Arabidopsis atToc159 mutant, *ppi2*, possesses the strongest phenotype, and is albino due to severely disrupted chloroplast development (Bauer et al. 2000; Kubis et al. 2004). While the atToc132 and atToc120 single mutants have only weak or no mutant phenotypes, respectively, the double mutant exhibits an albino phenotype almost as severe as that of *ppi2*, indicating these two proteins together play an important role in chloroplast biogenesis (Ivanova et al. 2004; Kubis et al. 2004). Moreover, it has been proposed that atToc159 functions more specifically in photosynthetic protein import (like atToc33), and that atToc132/120 is involved more in non-photosynthetic protein import (like atToc34) (Fig. 12.2; discussed in detail in Sect. II.D). The function of atToc90 has not been fully clarified as yet, and its knockout mutant does not show any obvious mutant phenotypes (Hiltbrunner et al. 2004; Kubis et al. 2004). However, it seems that atToc90 is partially functionally redundant with atToc159; it was reported that the phenotype of *ppi2* can be partially recovered by the overexpression of atToc90, and that *toc90* knockout mutations can slightly enhance the phenotype of *ppi2* (Hiltbrunner et al. 2004; Infanger et al. 2010). It will be interesting to elucidate whether atToc90 is functionally related to atToc132/120 as well.

3. The Channel Protein, Toc75

Toc75 is generally regarded as the translocation channel at the outer envelope membrane, where it is the most abundant protein (Eckart et al. 2002; Vojta et al. 2004). It is deeply embedded within the outer membrane and possesses a typical β-barrel structure (Schnell et al. 1994; Tranel et al. 1995; Hinnah et al. 1997), as well as the ability to bind transit peptides directly (Hinnah et al. 2002). At the beginning, it was thought that the full length of Toc75 contributes to the β-barrel structure, but later it became clear that it has a more complex structure. Toc75 belongs to the conserved BamA (β-*b*arrel *a*ssembly *m*achinery A) family of proteins (Ertel et al. 2005; Gentle et al. 2005; Hsu and Inoue 2009) The BamA family can trace its origin to gram-negative bacteria, and it also exists in the mitochondria of eukaryotes. Like other family members, Toc75 can be divided into two parts: a C-terminal β-barrel domain as predicted previously, and an N-terminal domain with three POTRA (*po*lypeptide *tra*nsport associated) repeats (Sánchez-Pulido et al. 2003; Ertel et al. 2005; Gentle et al. 2005). The β-barrel domain contains 16–18 transmembrane strands for channel formation (Hinnah et al. 2002; Ertel et al. 2005), while the POTRA domain may be involved in preprotein recognition and/or complex assembly (Ertel et al. 2005). Using artificial lipid membranes, Toc75 was found to form a voltage-sensitive channel with a pore diameter of about 14–26 Å (Hinnah et al. 2002). Nonetheless, the exact topology of Toc75 remains unclear. Interestingly, a fraction of the Toc75 protein pool has been reported to exist in free form outside of TOC complexes (Kouranov et al. 1998), indicating that Toc75 may possess some functions beyond standard preprotein import (Tu et al. 2004). However, another report argued that such free Toc75 actually does not exist, or at least not in photosynthetic plastids (Kikuchi et al. 2006).

In the Arabidopsis genome, there are at least three Toc75-homologous sequences, *atTOC75-III*, *atTOC75-IV*, and *atTOC75-I*, which are named according to their chromosomal locations (Jackson-Constan and Keegstra 2001). In addition, there is a less related homologue, termed OEP80 (*o*uter *e*nvelope *p*rotein, 80 kD), formerly known as atToc75-V (Eckart et al. 2002; Inoue and Potter 2004). Based on sequence similarity and expression levels, atToc75-III is believed to be the major orthologue of pea Toc75, serving as the main channel of the Arabidopsis TOC complex. Its importance in Arabidopsis has also been verified in that *toc75-III* knockout mutations lead to embryo lethality (Baldwin et al. 2005; Hust and Gutensohn 2006). In fact, embryo development in *toc75-*

III null mutants is arrested at an extremely early stage, demonstrating the necessity of plastid protein import in early embryo development. Knockdown of *atTOC75-III* expression by RNA interference (RNAi) and a hypomorphic *toc75-III* mutant allele both produce plants with reduced chlorophyll content (Stanga et al. 2009; Huang et al. 2011), indicating an important role for atToc75-III in chloroplast biogenesis beyond embryogenesis. By contrast, atToc75-IV is expressed at very low levels and its knockout mutant does not show any obvious abnormal phenotypes under normal growth conditions. However, altered etioplast ultrastructure and a reduced de-etiolation efficiency indicated that atToc75-IV has some roles during dark growth of plants (Baldwin et al. 2005). The third homologue, *atTOC75-I*, proved to be a pseudogene with no expression, due to a gypsy/Ty3 transposon insertion (Baldwin et al. 2005).

Phylogenetic analyses suggested that OEP80 and Toc75 belong to distinct families, and it was for this reason (combined with a lack of information on its function) that the former was renamed from atToc75-V to OEP80 (Inoue and Potter 2004). OEP80 is not considered to be a TOC component, although it may be responsible for the insertion of β-barrel proteins (e.g., Toc75) into the outer membrane, with a similar function to its mitochondrial homologue, Sam50/Tob55 (Gentle et al. 2004; Inoue and Potter 2004; Huang et al. 2011). Paralleling the situation for atToc75-III, Arabidopsis *oep80* knockout mutations are embryo-lethal, indicating an important role for OEP80 in embryo development, while RNAi-mediated knockdown of *AtOEP80* expression causes chlorosis in plants (Patel et al. 2008; Huang et al. 2011). Differences in the stage of embryo arrest between the *toc75-III* and *oep80* knockout mutants (the latter abort considerably later in development), and in the severity of chlorosis in the knockdown lines (atToc75-III RNAi plants are much paler than AtOEP80 RNAi plants), may indicate a more specialized role for OEP80 (Patel et al. 2008; Huang et al. 2011).

4. Other TOC Components

More recently, Toc12 and Toc64 were identified as putative new components of the TOC complex. However, their exact roles in the import apparatus have yet to be determined.

Through proteomic studies of the outer membrane of pea chloroplasts and co-immunoprecipitation assays, Toc12 was identified as a new protein associated with the TOC apparatus (Becker et al. 2004b). It was described as an integral outer membrane protein with a large soluble part facing the intermembrane space. It is a DnaJ-like protein with a conserved J-domain that can interact with Hsp70 and enhance its ATP hydrolysis activity. The protein was shown to be associated with Toc64 and Tic22, and thus was proposed to form part of an intermembrane space complex acting as a "bridge" between the outer and inner membrane translocons. It was hypothesized that Toc12 facilitates translocation across the intermembrane space by stimulating an intermembrane space-localized Hsp70 (imsHsp70) (Qbadou et al. 2007). However, more recently, this hypothesis has been challenged by a study which demonstrated that the originally-found Toc12 in pea is actually a truncated form of a pea DnaJ-J8 protein (Chiu et al. 2011). In Arabidopsis, DnaJ-J8 seems to be a stromal protein with a transit peptide. Moreover, Arabidopsis *DnaJ-J8* T-DNA insertion mutants do not show any obvious defects in chloroplast protein import (Chiu et al. 2011). Further doubt is cast on the aforementioned TOC-TIC bridging model by the fact that a gene coding for the imsHsp70 has never been found in Arabidopsis (Ratnayake et al. 2008; Su and Li 2008). Thus, the mechanism of translocation through the intermembrane space remains to be established.

Toc64 was first identified in the isolated pea TOC complex after cross-linking (Sohrt and Soll 2000). It is suggested to dynamically associate with the complex, contrasting with the other stably-present core TOC components (Schleiff et al. 2003b). Topology studies showed that Toc64 is anchored in the outer membrane by three transmembrane

spans, thereby presenting a C-terminal TPR (*t*etratrico*p*eptide *r*epeat) domain to the cytosol and a central domain (with amidase homology) to the intermembrane space (Qbadou et al. 2007). In vitro biochemical studies indicated that these two domains may enable Toc64 to perform bipartite functions: the TPR domain might serve as a receptor for preproteins carried by the cytosolic factor, Hsp90 (see Sect. II.B) (Qbadou et al. 2006, 2007); the intermembrane space domain might assist the translocation of preproteins across the intermembrane space, together with other components (see previous paragraph on Toc12) (Qbadou et al. 2007). However, in vivo studies do not support the importance of Toc64 in protein import. There are three Toc64 isoforms in Arabidopsis: atToc64-III, atToc64-I and atToc64-V. The first of these was shown to localize in chloroplasts, and it shares the highest sequence identity with the original Toc64 isolate from pea (Chew et al. 2004); on the other hand, atToc64-I (AMI1) is a cytosolic protein acting as a typical amidase (Pollmann et al. 2003, 2006), while atToc64-V (mtOM64) is localized in the mitochondrial outer membrane, perhaps replacing a receptor of the TOM translocon, Tom70, that is found in yeast and mammals but not in plants (Chew et al. 2004; Lister et al. 2007). Surprisingly, even the triple mutant of Arabidopsis Toc64 homologues does not display any abnormal phenotypes, as judged by a variety of criteria (Aronsson et al. 2007), clearly showing that Toc64 is not essential for protein import in Arabidopsis. Moreover, knockouts lacking Toc64 in moss also do not present any obvious defects, with the possible exception of a slight deformity in chloroplast shape (Hofmann and Theg 2005b). However, because its presumed mitochondrial counterpart, Tom70 (the role of which is well established), is also not essential for cell survival in yeast (Hines et al. 1990), further work will be necessary before a final conclusion can be reached on Toc64 participation in import. It has been proposed that Toc64 should be renamed as OEP64 (*o*uter *e*nvelope *p*rotein, 64 kD) until its function has been clearly determined (Hofmann and Theg 2005b; Aronsson et al. 2007).

B. Cytosolic Factors

Protein translocation pathways into organelles can be grouped according to the use of two fundamentally different mechanisms: co-translational transport, which happens when translocation is closely linked to translation, as in SRP-dependent transport into the ER; and, post-translational transport, in which cytosolic factors may be required to assist precursor targeting to the organellar membrane, as in mitochondrial or peroxisomal import (Wickner and Schekman 2005). Since the import of chloroplast proteins is generally considered to be a post-translocation process, it is suggested that various cytosolic factors are involved. Several clues support this notion: the fact that precursors (especially of hydrophobic membrane proteins) produced in the cytosol are not in their final conformation and thus tend to aggregate or be degraded (Wickner et al. 1999; Lee et al. 2009c); the differing requirements for functionality of transit peptides between in vivo and in vitro conditions (Rensink et al. 1998; Lee et al. 2002); the differences in import behaviour seen in vitro when using precursors translated in rabbit reticulocyte or wheat germ lysates (May and Soll 2000; Schleiff et al. 2002). Indeed, in vitro evidence suggests that several chaperone proteins form "guidance complexes" that facilitate preprotein targeting to the chloroplast. However, the conditions under which such complexes exhibit significance in vivo remain to be established. Quite recently, the light-harvesting complex protein, LHCII, was reported to be translated near the border of chloroplasts in the green alga, *Chlamydomonas reinhardtii*, suggesting mRNA targeting combined with cotranslational transport as an alternative mechanism for protein import into chloroplasts (Uniacke and Zerges 2009).

1. Hsp70

Cytosolic Hsp70 (*h*eat-*s*hock *p*rotein, 70 kD) is one of the chaperones proposed to facilitate chloroplast protein transport. Interestingly, over 75% of chloroplast transit peptides are

predicted to contain at least one Hsp70 binding site (Rial et al. 2000). Direct interactions between Hsp70s and transit peptides have also been demonstrated both in vitro (Ivey et al. 2000; Rial et al. 2000; Zhang and Glaser 2002) and in vivo (Lee et al. 2009c). This supports the notion that the cytosolic chaperone is involved in keeping preproteins in an unfolded, competent form, which is important for protein import (Walker et al. 1996). However, the consequences of Hsp70 binding for protein import are still unclear. It has been shown that the unfolding process is not strictly linked with Hsp70 (Ruprecht et al. 2010). Moreover, recent evidence shows that a cytosolic Hsp70 in Arabidopsis can associate with accumulated cytosolic precursors that are targeted for degradation through the 26S ubiquitin proteasome system (Lee et al. 2009c); this indicates that the binding of Hsp70 might not simply escort the preproteins to the chloroplast membrane. Nonetheless, Hsp70 does seem to play a role in protein translocation in cooperation with other cytosolic factors, such as 14-3-3.

2. 14-3-3

The 14-3-3 family of proteins are regulatory molecules and chaperones that specifically bind to phosphorylated proteins to mediate a variety of signal transduction processes, as well as protein translocation (Gokirmak et al. 2010). Many transit peptides contain a phosphopeptide binding motif for 14-3-3 proteins. It was reported that 14-3-3 can form a "guidance complex" together with Hsp70 and preproteins, which can significantly increase in vitro import efficiency for certain phosphorylatable precursors (May and Soll 2000). The "guidance complex" containing 14-3-3 was also suggested to be important for determining the specificity of import to chloroplasts versus mitochondria in plants, since 14-3-3 cannot interact with plant mitochondrial preproteins (May and Soll 2000). However, mutation of the putative 14-3-3-binding phosphorylation site in transit peptides does not affect import efficiency and fidelity in vivo (Nakrieko et al. 2004; Lee et al. 2006), indicating that this "guidance complex" system is not essential.

Reflecting the unique problem faced by plant cells in differentiating between two different endosymbiotically-derived organelles, the protein import receptor components of mitochondria in plants are significantly different from those in other organisms (i.e., yeast or animals), as well as from those in chloroplasts (Macasev et al. 2000; Schleiff and Becker 2011). In spite of these receptor differences, some chloroplast preproteins can be efficiently imported into pea mitochondria in vitro, but not in vivo (Cleary et al. 2002). This indicates that special mechanisms must be utilized to achieve the specificity of import in vivo, and that components of such mechanisms are absent or inactive in vitro. Apart from the aforementioned 14-3-3 guidance hypothesis, another strategy that may be employed to achieve targeting specificity is transport of mRNA towards the destination organelle, such that preproteins are produced at the periphery of the correct organelles (Marc et al. 2002; Chew and Whelan 2004; Uniacke and Zerges 2009). However, there is no evidence yet concerning whether this is a general phenomenon for chloroplast protein import in plants.

3. Hsp90

In animals, Hsp90 and/or Hsp70 chaperones carry some preproteins to mitochondria via the Tom70 receptor (Young et al. 2003). Likewise, Hsp90 is proposed to deliver preproteins to chloroplasts in plants as part of another "guidance complex" (Qbadou et al. 2006, 2007). There are two major differences between the two plastidic "guidance complexes": one is that Hsp90 binds to preproteins that are not necessarily phosphorylated; the other is that, unlike 14-3-3, which carries preproteins directly to Toc34, Hsp90 makes use of Toc64 as the initial docking site and then afterwards passes the preproteins on to Toc34 (Qbadou et al. 2006). However, the precursor of the 33 kD subunit of the oxygen evolving complex, OE33, a protein which was shown to be transported to Toc64 by Hsp90 in vitro (Qbadou et al. 2006), is imported with normal efficiency into chloroplasts of *toc64* knockout mutants (Aronsson et al. 2007);

this indicates that the putative Hsp90-Toc64-based targeting mechanism is also not essential. Therefore, alternative systems differing from those described above might also be present, employing different components such as the newly-identified TPR-containing chaperone receptor, OEP61 (*o*uter *e*nvelope *p*rotein, 61 kD) (Kriechbaumer et al. 2011).

4. Actin

Recently, actin has been found to interact directly with Toc159 on the cytosolic side of the outer envelope membrane. In fact, many TOC/TIC components and VIPP1 (*v*esicle-*i*nducing *p*rotein in *p*lastids 1) were found associated with actin in a co-immunoprecipitation assay (Jouhet and Gray 2009a). It was proposed that this actin-TOC-TIC-VIPP1 complex may facilitate the trafficking of cytosolic preproteins to the thylakoid membrane (Jouhet and Gray 2009b). This is interesting, as the use of actin as a transport "highway" might well resolve the problems of targeting specificity and efficiency discussed above. However, myosin, the essential "motor" protein for actin-based motility (Ross et al. 2008), has not been found to interact with the chloroplast envelope (Jouhet and Gray 2009a), and so the significance of these observations remains to be established.

C. Models for Protein Translocation Through the TOC Complex

There are two main models for the mode of action of the TOC complex: the "targeting model" and the "motor model". The main difference between these two models is the role played by Toc159 in the translocation process.

The "targeting model" was originally proposed based on the finding that Toc159 exists in a free, cytosolic form as well as at the outer membrane. Based on this observation, cytosolic Toc159 was proposed to serve as a soluble receptor for preproteins, targeting them to the chloroplast surface (Hiltbrunner et al. 2001; Bauer et al. 2002; Smith et al. 2002, 2004). In the model, docking of Toc159 at the outer membrane is mediated by its interaction with Toc34, through their G-domains, and is dependent on GTP binding and hydrolysis activity (Bauer et al. 2002; Smith et al. 2002). Once docked, the GTPase activity of Toc159 and/or Toc34 was suggested to promote insertion of the preprotein into the channel protein, Toc75. Finally, Toc159 would become dissociated from the TOC complex to enable another targeting cycle. In this model, Toc159 plays two roles, as a preprotein carrier and in the triggering of translocation, while Toc34 acts as the docking site for the Toc159-preprotein complex. Evidence supporting the model includes: first, Toc159 was found to be the main component (along with Toc75) associated with preproteins during the early stages of import in cross-linking experiments (Perry and Keegstra 1994; Ma et al. 1996; Akita et al. 1997); second, the insertion of Toc159 into the membrane depends on the formation of a heterodimer with Toc34 (Bauer et al. 2002; Smith et al. 2002). However, as mentioned above (see Sect. II.A.2), the existence of the cytosolic form of Toc159 has been challenged (Becker et al. 2004a; Agne et al. 2009, 2010). Nonetheless, Toc159 may still act as a membrane-bound receptor in the model, as suggested by the fact that Toc159 can bind precursors at the envelope of isolated chloroplasts in import assays (Smith et al. 2004).

In the "motor model", membrane-bound Toc34 acts as the first receptor for the preprotein, and it dynamically associates with Toc159 in a manner regulated by its GTPase activity. By contrast, Toc159 plays a major role as a GTP-dependent motor in the translocation process (Schleiff et al. 2003a; Becker et al. 2004a). This model was suggested by experiments using the TOC translocon reconstituted into artificial membranes, which implied that Toc34 recognizes preproteins initially, while the minimal functional unit comprises just Toc159 and Toc75 (i.e., the motor and the channel) (Schleiff et al. 2003a; Becker et al. 2004a). This model is consistent with the stoichiometric and structural

analyses of the TOC complex described earlier (see Sect. II.A.1), which suggested that Toc159 is located in the center of the complex as a monomer, where it may serve as a catalytic motor and a docking site for four surrounding Toc34 molecules (Schleiff et al. 2003b). However, observations that Toc159 lacking the G-domain can still promote preprotein import (Chen et al. 2000; Lee et al. 2003) conflict with the proposed central role of Toc159 GTPase activity in this model. Moreover, recent studies on transgenic plants expressing Toc159 G-domain point-mutants imply that Toc159 acts more as a molecular switch than as a motor (Wang et al. 2008; Agne et al. 2009).

The two models differ considerably in detail. Nonetheless, it seems possible that what actually occurs in vivo shares elements with both models. For example, both receptors might be involved in the recognition and translocation processes of protein import, perhaps acting in a partially redundant way (Inaba and Schnell 2008; Jarvis 2008; Aronsson and Jarvis 2011). One feature that both models possess is a key role for GTP cycling at the receptors, which functions to control the interactions of the receptors and the preprotein, and to initiate or drive preprotein insertion into the channel. The importance of receptor GTP cycling has been shown by several G-domain mutation studies in vitro (Chen and Schnell 1997; Bauer et al. 2002; Wallas et al. 2003), and also suggested by the observation that the non-hydrolysable GTP analogues can inhibit the import process (Schnell et al. 1994; Young et al. 1999). However, the mechanism of import through the TOC apparatus may be more complicated, as indicated by recent studies which investigated the role of GTPase function in vivo (Wang et al. 2008; Agne et al. 2009; Lee et al. 2009b; Aronsson et al. 2010). Surprisingly, the transgenic expression of atToc159 mutants with defects in GTP binding and/or hydrolysis can efficiently complement the *ppi2* mutant phenotype (Wang et al. 2008; Agne et al. 2009); in addition, non-hydrolysable GTP analogues still strongly inhibit protein import in these transgenic plants, indicating that GTPases other than Toc159 are involved in the import process. Similarly, GTPase- and dimerization-defective mutants of atToc33 are effective at complementing the *ppi1* mutant phenotype (Lee et al. 2009b; Aronsson et al. 2010). The fact that G-domain-defective forms of both atToc159 and atToc33 retain in vivo functionality raised the possibility that the two GTPases might be partially redundant; however, complementation analysis shows that is not the case (Aronsson et al. 2010). An alternative explanation is that the presence of both receptor types is required to maintain the structural integrity of the complex, but that only one of the two receptors needs to have normal GTPase functionality in order for import to proceed (Aronsson et al. 2010). To better elucidate the functions of the receptors, additional experimentation will need to be performed, such as the mutation of the G-domains of both main receptors simultaneously.

D. Regulation of Import Through Substrate-Specific Pathways at the Outer Membrane

As mentioned above (in Sect. II.A.2), one of the notable characteristics of the TOC receptors in Arabidopsis is that they are encoded by small gene families, which is in contrast with the Toc75 channel protein (as it is generally regarded that only atToc75-III plays a significant role in canonical import pathways), and with most TIC components. In this regard, it is relevant that not only highly-abundant, photosynthetic preproteins need to be imported, but also lower abundance (but nevertheless essential) non-photosynthetic or housekeeping preproteins. Considering these facts, it is easy to imagine that import is organized by using separate TOC complexes with different substrate-specific receptors (Fig. 12.2), in order to ensure the efficient import of all proteins and avoid potentially damaging competition effects between precursors of widely differing abundances. Further significance of such specificity might be in controlling the differentiation of different plastid types; consistent with this notion, it

has been shown that while non-photosynthetic proteins can be imported into both chloroplasts and root plastids, their photosynthetic counterparts are generally more readily imported into chloroplasts (Wan et al. 1996; Yan et al. 2006). As detailed below, an accumulating amount of evidence has shown that such regulatory mechanisms do operate, and that different groups of receptors are responsible for importing specific preprotein substrates.

Firstly, the phenotypic analysis of Arabidopsis TOC receptor mutants indicated that the receptors may form two groups: one comprising atToc33 and atToc159, involved in the import of photosynthetic preproteins; and another comprising atToc34 and atToc132/120, involved in the import of non-photosynthetic, housekeeping preproteins (Jarvis et al. 1998; Bauer et al. 2000; Kubis et al. 2003; Constan et al. 2004a; Ivanova et al. 2004; Kubis et al. 2004). As mentioned earlier, the *ppi1* (or *toc33*) mutant has a striking chlorotic leaf phenotype, whereas *ppi3* (*toc34*) has only a specific defect in root development (Jarvis et al. 1998; Kubis et al. 2003; Constan et al. 2004a). Accordingly, *ppi2* (*toc159*) exhibits strong defects in chloroplasts and only mild defects in root plastids, while *toc132 toc120* exhibits the strongest phenotypes in root plastids (Yu and Li 2001; Kubis et al. 2004). Furthermore, it has been shown that atToc132 is involved in root gravitropism (Stanga et al. 2009).

Secondly, while atToc159 and atToc33 are highly expressed in leaves, the expression levels of atToc132, atToc120 and atToc34 are higher in roots. Moreover, in general, atToc159 and atToc33 are the dominant isoforms in their families (Kubis et al. 2003; Vojta et al. 2004). These tissue-specific expression patterns fit well with the proposed substrate specificities of the receptors, and also help to meet the huge demand for import capacity during the light-dependent biogenesis of highly-abundant photosynthetic proteins in chloroplasts.

Thirdly, transcriptomic and proteomic studies performed on the aforementioned TOC receptor mutants were consistent with the model. For example, the *ppi1* (*toc33*) and *ppi2* (*toc159*) mutants displayed down-regulated expression of photosynthetic genes/proteins, but relatively normal expression of non-photosynthetic genes/proteins (Bauer et al. 2000; Kubis et al. 2003, 2004; Kakizaki et al. 2009). These observations suggested that photosynthetic proteins could not be efficiently imported into plastids in these mutants, and that plastid-to-nucleus signaling pathways were consequently activated to prevent the futile expression of further organellar components that would be unable to reach their final destination.

Fourthly, a range of different experiments provided more direct evidence of specificity for different types of preprotein. For example, in vitro import assays using isolated *ppi1* chloroplasts showed that atToc33 is preferentially involved in the import of photosynthetic preproteins (Kubis et al. 2003). Moreover, in vivo targeting studies revealed similar functional preferences for atToc159 (Smith et al. 2004). In vitro pull-down experiments and functional assays supported the notion that this specificity is due to the preferential interaction of both receptors with photosynthetic precursors (Jelic et al. 2003; Smith et al. 2004).

Finally, co-immunoprecipitation data indicated that atToc159 and atToc132/120 exist in two distinct complexes. Furthermore, atToc159 exhibited preferential association with atToc33, while atToc132/120 was more prone to interact with atToc34 (Ivanova et al. 2004).

Although it seems that the specificity is related to the interaction between the transit peptide and the receptors (Smith et al. 2004; Yan et al. 2006), the mechanistic detail has yet to be clarified. No obvious defining features could be recognized upon examining the two transit peptide groups (Kubis et al. 2004; Vojta et al. 2004). However, some specific motifs in the transit peptide of the Rubisco small subunit precursor (pSSU) have recently been shown to be linked to the atToc159-dependent import pathway (Lee et al. 2009a). In addition, a recent report indicated that the Toc159 A-domain plays a significant role in defining

substrate-specific import pathways in vivo and in vitro (Inoue et al. 2010). Remarkably, transgenic overexpression of atToc132 lacking the A-domain (which may consequently lack selectivity), but not the full-length atToc132, can partially recover the phenotype of *ppi2* (*toc159*) Arabidopsis plants, indicating that the A-domain is responsible for selectivity. Interestingly, spectroscopic studies showed that the Toc159 A-domains display characteristics of intrinsically disordered proteins (IDPs), which usually function in highly dynamic protein-protein interactions (Richardson et al. 2009). However, the physiological significance of the Toc159 A-domain remains unclear, as it is not essential for plant development (Lee et al. 2003; Agne et al. 2009, 2010).

A recent work by Ling et al. (2012) has shed new light on how the protein import machinery is dynamically regulated to control the differentiation of different plastid types. A forward genetic screen in Arabidopsis identified a ubiquitin E3 ligase embedded in the plastid outer envelope membrane. This protein, termed SP1 (for suppressor of *ppi1* locus 1), was shown to mediate the ubiquitination of TOC proteins (particularly the receptors), thereby promoting their turnover by the ubiquitin-proteasome system (UPS). Mutant *sp1* plants were found to complete developmental transitions that involve plastid type interconversions (e.g., de-etiolation, when etioplasts transform into chloroplasts) inefficiently, implying an important role for SP1, the UPS, and the TOC machinery in governing organellar proteome changes. It was proposed that SP1 allows for the rapid replacement of TOC receptors of one type with different receptor isoforms, in order to accommodate and orchestrate changing protein import requirements (Ling et al. 2012).

Other regulatory mechanisms act to control the TOC components, in order to meet changing demands for protein import during different developmental stages and under different growth conditions. For example, the expression levels of atToc33 and pea Toc75 are much higher during early developmental stages (Tranel et al. 1995; Jarvis et al. 1998), presumably to fulfil the massive requirement for protein import at such times when chloroplast biogenesis activity is intense (Dahlin and Cline 1991). On the other hand, under temperature-stress conditions, import rates are down-regulated, which correlates with declining expression of TOC/TIC components (Dutta et al. 2009); this may serve to decelerate photosynthetic activity in order to avoid production of reactive oxygen species which might otherwise cause damage to the plant (Apel and Hirt 2004). However, the mechanisms underlying such regulatory expression changes are still largely unknown. To date, only one transcription factor with a role in chloroplast protein import has been identified: CIA2 (*c*hloroplast *i*mport *a*pparatus 2) was found to promote the expression of chloroplast translation components, and to regulate import by controlling the expression levels of certain TOC components, thereby helping to fulfil the vast demand for proteins in developing chloroplasts (Sun et al. 2001, 2009). Clearly, it will be of considerable interest to identify more regulators involved in chloroplast protein import in the future.

III. Events at the Inner Envelope Membrane

A. Overview of the Inner Membrane Translocation Machinery

While translocation through the outer envelope membrane is proposed to happen at multiple different TOC complexes, depending on preprotein specificity, it has generally been assumed that these different import pathways converge at the inner envelope membrane with a single TIC translocon (Kovacheva et al. 2005; Jarvis 2008). This translocon is composed of a channel, formed by Tic110 and/or Tic20 and Tic21, a motor complex comprising Tic110, Hsp93 and the Tic40 co-chaperone, and possibly also a redox-regulator with three components, Tic55, Tic32 and Tic62 (Fig. 12.1) (Jarvis 2008; Kovacs-Bogdan et al. 2010). Tic22 is

localized in the intermembrane space and is thought to link the TOC complex to the TIC complex, perhaps aiding formation of a TOC/TIC supercomplex. Such supercomplexes might form at contact sites between the outer and inner envelope membranes, where the distance from the chloroplast surface to the stroma is minimized and the import path is shortest (Schnell and Blobel 1993; Perry and Keegstra 1994); preproteins would then be able to pass through the outer and inner membrane channel components at the same time.

As soon as the transit peptide of a preprotein emerges from the TIC channel, it is bound and cleaved by the stromal processing peptidase (SPP), a zinc-binding metalloendopeptidase of the M16/pitrilysin family (Schnell and Blobel 1993; Richter and Lamppa 1998; Richter et al. 2005). Thereafter, the transit peptide is degraded and the mature protein is released and allowed to adopt its three-dimensional structure with the help of molecular chaperones (Jackson-Constan and Keegstra 2001), or engage downstream, intraorganellar targeting pathways (see Sect. IV) (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Jarvis 2008). Evidence suggests that Cpn60 (*c*ha*p*eroni*n*, 60 kD) and Hsp70 are amongst the chaperones that facilitate protein folding or intraorganellar routing following import (Yalovsky et al. 1992; Madueño et al. 1993; Kessler and Blobel 1996).

Chloroplast inner envelope membranes are very similar to thylakoid membranes in terms of lipid composition. While outer envelope membranes are rich in phosphatidylcholine, like all extrachloroplastic membranes, inner envelope membranes as well as thylakoids contain much reduced amounts of phosphatidylcholine but increased amounts of monogalactosyldiacylglycerol (Block et al. 1983). Inner envelope membranes and thylakoids are developmentally related, and derive evolutionarily from the cyanobacterial chloroplast progenitor. Similarly, many components of the TIC machinery (namely, Tic20, Tic21, Tic22, Tic32, Tic55 and Tic62) have a cyanobacterial origin, and so are likely to have acquired new functions as they were recruited to the translocon (Reumann and Keegstra 1999; Reumann et al. 2005; Kalanon and McFadden 2008; Gross and Bhattacharya 2009). It has even been suggested that these components have retained their original functions in addition to their newer roles in protein import (Gross and Bhattacharya 2009); thus, Tic55 might be involved in chlorophyll *a* degradation, Tic32 and Tic62 might have metabolic dehydrogenase activities with specific substrates, Tic20 might be an ion channel, while Tic21 may function as an iron transporter (Gross and Bhattacharya 2009). In contrast, Tic110 has no cyanobacterial homologues and is therefore thought to have a eukaryotic origin. Its ubiquity amongst plastid-containing species suggests that it evolved very early on, and that it formed part of an ancient host-specific translocon together with Toc34 and Toc75. Tic40 is proposed to be a more recent acquisition, as it is less widely distributed, has eukaryote-derived co-chaperone activity, and seems to play an auxiliary role by accelerating the process of import (Kalanon and McFadden 2008; Gross and Bhattacharya 2009).

B. Energy Requirements and Different Stages of Translocation

Based on energetic requirements determined in vitro using isolated chloroplasts, protein import can be divided into several discrete steps. With an ATP requirement of 100 μM in the intermembrane space (along with a requirement for GTP, as used by the TOC receptors) (Olsen et al. 1989; Olsen and Keegstra 1992; Young et al. 1999), so-called "early import intermediates" are formed. Under such conditions, preproteins are inserted through the TOC complex and establish contacts with the intermembrane space exposed TIC components (Kouranov et al. 1998). In vitro, preproteins can be arrested at this stage by limiting ATP supply or by employing fully-energized chloroplasts at low temperatures (Leheny and Theg 1994); such manipulation enabled the further

subdivision of this early import intermediate step into three discrete sub-stages (Inoue and Akita 2008). After their emergence from the Toc75 channel, Tic22 might be one of the first points of contact for preproteins with the TIC machinery. Tic22 is a soluble protein with a largely unknown function, but it may be involved in guiding preproteins to the inner membrane channel (Kouranov et al. 1998). A putative intermembrane space HSP70 (imsHsp70) component was suggested to deliver the energy for progression to the early import intermediate step, stimulated by the J-domain co-chaperone component, Toc12 (Marshall et al. 1990; Becker et al. 2004b). However, as was discussed in Sect. II, the relevance of this hypothesis is in considerable doubt, as a gene for an imsHsp70 has never been identified (Ratnayake et al. 2008; Su and Li 2008), and Toc12 seems to be a truncated form of the stromal protein, DnaJ-J8 (Chiu et al. 2011). Thus, the question of how ATP is actually used in the intermembrane space remains unanswered.

Preproteins are subsequently threaded though the inner envelope membrane channel, composed of either Tic110 or Tic20/Tic21, or perhaps even a combination of these (Kouranov et al. 1998; Heins et al. 2002; Teng et al. 2006; Balsera et al. 2009). For complete translocation through the inner envelope channel, about 1 mM ATP is needed in the stroma (Pain and Blobel 1987; Olsen et al. 1989; Theg et al. 1989). This is thought to be used by stromal chaperones (heat shock proteins), such as Hsp93 and Hsp70, that cooperate with Tic110 and the Tic40 co-chaperone (Kessler and Blobel 1996; Akita et al. 1997; Nielsen et al. 1997b; Shi and Theg 2010). The mechanism of translocation has been suggested to be of a thermal ratchet type, with chaperones binding to freshly exposed parts of the translocating preprotein, thus preventing backward movement (Glover and Tkach 2001; Neupert and Brunner 2002; Kovacheva et al. 2005; Chou et al. 2006). In contrast with mitochondrial protein import, chloroplast import does not employ a transmembrane protonmotive force (Pain and Blobel 1987; Theg et al. 1989).

C. Translocation Through the Inner Membrane Channel

Tic110 is one of the most abundant components of the TIC apparatus (Vojta et al. 2004), and, based on electrophysiological studies, a role for the protein as a translocation channel in the inner membrane has been suggested (Heins et al. 2002; Balsera et al. 2009). This notion is supported by the fact that the single-copy gene in Arabidopsis is essential (as is the gene for the main TOC channel, atToc75-III), with the knockout mutants aborting at the globular stage with a raspberry-like embryo phenotype (Inaba et al. 2005; Kovacheva et al. 2005). Tic110 has been proposed to form a cation-selective, β-barrel channel with a pore diameter of 1.7 nm in liposomes in vitro (Heins et al. 2002). However, there has been considerable disagreement concerning the structural nature of this protein, as another investigation showed that it is composed primarily of α-helices, and that it is anchored in the inner membrane by two N-terminal hydrophobic transmembrane domains (Inaba et al. 2003). In the latter topology, a large hydrophilic domain is oriented towards the stroma and is thought to recruit Tic40 and stromal chaperones for the propulsion of preprotein import (Kessler and Blobel 1996; Inaba et al. 2003; Chou et al. 2006). A later study attempted to resolve these inconsistencies, and concluded that the large hydrophilic part contains four amphipathic helices that contribute to the channel (Balsera et al. 2009). Loops protruding into the intermembrane space might interact with Tic22 and TOC components to aid formation of TOC/TIC supercomplexes at contact sites.

Another component that has been suggested to form the TIC channel is Tic20 (Kouranov et al. 1998). It was originally proposed to share weak homology with bacterial amino acid transporters and mitochondrial Tim proteins; however, a careful analysis of consensus sequences recently concluded that Tic20 is not evolutionarily related to these other proteins (Kasmati et al. 2011). Tic20 possesses four α-helical transmembrane domains, similar to the

mitochondrial inner membrane channel components, Tim22 and Tim23 (Rassow et al. 1999; Kalanon and McFadden 2008; Kasmati et al. 2011). Tic20 has been found to interact with preproteins at a slightly later stage than Tic22 in cross-linking studies, and to be part of TOC/TIC supercomplexes (Ma et al. 1996; Kouranov and Schnell 1997; Kouranov et al. 1998). Antisense down-regulation of the main Tic20 isoform in Arabidopsis generated plants with defects in chloroplast biogenesis and protein import (Chen et al. 2002), while complete knockout mutants are albino and severely impaired in the import of photosynthetic preproteins (Teng et al. 2006; Kikuchi et al. 2009; Hirabayashi et al. 2011; Kasmati et al. 2011). In Arabidopsis, Tic20 is encoded by four genes that fall into two distinct groups based on sequence similarity and phylogenetic considerations (Kasmati et al. 2011). The atTic20-I and atTic20-IV proteins, together with the original Tic20 isolated from pea, are part of the main group with demonstrable importance in chloroplast biogenesis. It is proposed that atTic20-I is primarily responsible for the import of photosynthetic preproteins in shoots, and that atTic20-IV imports mainly non-photosynthetic preproteins in roots (Fig. 12.2) (Kikuchi et al. 2009; Hirabayashi et al. 2011). Double *tic20-I tic20-IV* knockout mutations are gametophytic- and embryo-lethal, indicating that atTic20-IV can partially compensate for the loss of atTic20-I in the *tic20-I* mutant (Hirabayashi et al. 2011; Kasmati et al. 2011); this demonstrates the partially redundant and essential functions of atTic20-I and atTic20-IV. In the second group, neither atTic20-II nor atTic20-V seems to be vital for chloroplast biogenesis, since *tic20-II tic20-V* double mutants are indistinguishable from wild type (Kasmati et al. 2011).

Interestingly, even though Tic20 is essential for protein import and plant viability, it appears to be much less abundant than the other putative or actual channel components in Arabidopsis, namely atToc75-III and atTic110 (Vojta et al. 2004). This suggests that Tic20 associates with only a subset of translocon complexes, and that its role might also be fulfilled by other components, such as Tic21, which has been proposed to perform a similar channel function during later development (Teng et al. 2006; Gross and Bhattacharya 2009). However, blue native PAGE analyses indicated that the Tic20 protein is present in a large, 1 MD complex together with Tic21 and preprotein (Kikuchi et al. 2009); Tic21 was only loosely associated with the complex, whereas Tic20 seemed to be a core component. Interestingly, Tic110 was not present in the 1 MD complex, and was instead present in a distinct, smaller complex of 200–300 kD. Thus, it was suggested that Tic20 together with Tic21 functions in a large channel complex (perhaps including other, unidentified components), whereas Tic110 instead acts later on in the import process, functioning in association with Tic40 and chaperones in a distinct motor complex (Fig. 12.2) (Kikuchi et al. 2009). Arabidopsis knockout mutants lacking either the main Tic20 isoform (atTic20-I) or Tic21 display similar defects in the import of photosynthetic preproteins, supporting the notion that the two proteins function together (Kikuchi et al. 2009).

Tic21 was identified as CIA5 (*c*hloroplast *i*mport *a*pparatus 5) in a screen for Arabidopsis plants defective in the import of a selectable marker into chloroplasts (Teng et al. 2006). The *tic21* knockout displays inefficient chloroplast protein import, with precursors seen to accumulate in the cytosol. The mutant has an albino phenotype, and so is viable only on an external carbon source. Interestingly, the *tic21 tic20-I* double mutant showed no additive phenotypic effects, relative to the single mutants, supporting the aforementioned hypothesis that Tic21 works in conjunction with Tic20 (Teng et al. 2006). It was suggested that Tic20 might play a channel role in early plant development, with Tic21 taking over the same function in later development (Teng et al. 2006); however, as already discussed, this idea is inconsistent

with the fact that the two proteins have been found together in the same complex (Kikuchi et al. 2009).

A protein called PIC1 (*p*ermease *i*n *c*hloroplasts 1) was described as a possible iron channel in the inner envelope membrane, and was found to be identical to Tic21 (Duy et al. 2007). Arabidopsis *pic1* mutants accumulated ferritin in the chloroplasts, which is a protein that binds iron in order to prevent iron loss or oxidative stress caused by free iron ions. Similarly, ferritin expression was up-regulated in the mutants, and other proteins related to iron stress and metabolism were differentially regulated. A yeast iron uptake defective mutant could be complemented with PIC1, further supporting a role of PIC1/Tic21 in iron transport across the inner envelope membrane (Duy et al. 2007). Consistently, plants overexpressing PIC1 accumulated free iron ions in the stroma, leading to oxidative stress, iron accumulation in flower tissue, and differential expression of genes associated with metal transport (Duy et al. 2011). It is known that protein components containing iron are important for chloroplast protein import, since diethylpyrocarbonate (DEPC), a chemical that inactivates iron-sulfur proteins, is reported to have a negative effect on protein import (Caliebe et al. 1997; Row and Gray 2001; Boij et al. 2009). Therefore, it is feasible that a block in iron import, and therefore in the biogenesis of iron-sulfur clusters, would affect protein import indirectly, similar to DEPC. However, it was found that genes related to iron homeostasis, encoding ferritin and copper superoxide dismutases, are not only up-regulated in PIC1/Tic21 mutants, but also in other pale mutants with defects in chloroplast biogenesis, such as *tic20-I* and *alb3* (Kikuchi et al. 2009). Thus, further investigation is required to determine whether disturbed iron homeostasis leads to diminished protein import, or diminished protein import leads to disturbed iron homeostasis. An alternative possibility is that the PIC1/Tic21 protein has a dual role, and that it acts in both of these processes (Gross and Bhattacharya 2009).

D. Import Propulsion at the Inner Envelope Membrane

A large portion of the C-terminal domain of Tic110 is oriented towards the stroma and has been reported to bind transit peptides as they emerge from the pore (Jackson et al. 1998; Inaba et al. 2003). Molecular chaperones are recruited to this Tic110 stromal domain, and these are believed to consume the energy, in the form of ATP, that is used to drive protein import at the stromal side, and to participate in the folding of newly-imported proteins (Kessler and Blobel 1996; Akita et al. 1997; Nielsen et al. 1997a; Chou et al. 2006). In mitochondria, it is well established that a matrix Hsp70 ATPase (mtHsp70) delivers the energy for preprotein translocation at the site of the inner membrane translocon (Neupert and Brunner 2002). However, even though chloroplast-localized Hsp70 does exist, it has generally been suggested that Hsp93 is the principal component of the TIC motor complex, since it associates with Tic110 (Akita et al. 1997; Nielsen et al. 1997a). The role of chloroplast Hsp70 (cpHsp70) has long been unclear, until recent studies clarified its function (Shi and Theg 2010; Su and Li 2010).

In Arabidopsis, knockouts of two cpHsp70 isoforms, *hsp70-1* and *hsp70-2*, were both shown to be defective in protein import (Su and Li 2008, 2010). Moreover, the phenotypes of *hsp70-1 hsp93-V* and *hsp70-1 tic40* double mutants were found to be more severe than those of the corresponding *hsp93-V* and *tic40* single mutants, respectively (Su and Li 2010) (note that the *hsp93-V* mutant lacks the main Hsp93 isoform in Arabidopsis; see below); this suggests that cpHsp70-1 has an important role in protein import propulsion, partially overlapping with the parallel system of Tic40/Hsp93. Since the *hsp70-1 tic40* genotype is lethal, whereas *hsp93-V tic40* causes only a pale phenotype, it seems that, in the *tic40* knockout background, cpHsp70-1

but not Hsp93-V becomes an essential and limiting factor for protein import propulsion. The cpHsp70-2 protein might be a minor isoform in Arabidopsis, partially redundant with cpHsp70-1 because the double knockouts are lethal (Su and Li 2010). Similarly, in moss, cpHsp70-deficient mutants displayed inefficient chloroplast protein import, as did another mutant with a deficiency in chloroplast-localized isoforms of the GrpE co-chaperone (Shi and Theg 2010). Related GrpE proteins promote nucleotide exchange at Hsp70 in prokaryotic systems, and play a well-established role in mitochondrial protein import in conjunction with mtHsp70 (Neupert and Brunner 2002). Moreover, immunoprecipitation studies showed that moss cpHsp70 associates with preproteins in a complex with Hsp93 and Tic40 (Shi and Theg 2010).

Hsp93, also named ClpC, is part of the Hsp100 family of molecular chaperones. Besides its function in protein import, it is also part of the Clp protease complex in chloroplasts, recognizing and unfolding substrate proteins that are destined for degradation (Shanklin et al. 1995). It forms hexameric rings in the presence of ATP, through which clients (either preproteins engaged in import, or other proteins targeted for degradation) may be threaded, thereby moving them towards their stromal destination or the Clp proteolytic core (Schirmer et al. 1996; Jackson-Constan et al. 2001). In Arabidopsis, two isoforms of Hsp93 exist, called atHsp93-V (ClpC1) and atHsp93-III (ClpC2). The former has a much higher expression level than atHsp93-III, and *hsp93-V* knockout mutants are pale and have a reduced protein import capacity, while *hsp93-III* knockout mutants are indistinguishable from wild type (Constan et al. 2004b; Sjögren et al. 2004; Kovacheva et al. 2005, 2007). Since *hsp93-III hsp93-V* double mutants are embryo lethal, and because the two proteins are very similar at the amino acid level (91% identical) (Kovacheva et al. 2007), these Arabidopsis homologues are believed to have largely redundant, overlapping functions, with atHsp93-V being the major isoform and atHsp93-III partially compensating for its loss in the *hsp93-V* mutant.

Tic40 is a homologue of the Com44/Cim44 protein originally identified in *Brassica napus*, and can be covalently linked to Tic110 via a disulfide bridge under oxidizing conditions (Stahl et al. 1999). It is anchored in the chloroplast inner envelope membrane by its N-terminal transmembrane domain, and projects a large hydrophilic C-terminal domain into the stroma, similar to Tic110 (Stahl et al. 1999; Chou et al. 2003). This stromal region contains a TPR domain through which it can interact with Tic110, as well as an Sti1-like co-chaperone domain of the type found in eukaryotic Hip/Hop co-chaperones (Chou et al. 2003, 2006; Bédard et al. 2007). It has been shown that Tic40 is in a complex not only with Tic110 but also with Hsp93, and that these three proteins all function at similar times in the import process, thus establishing a link between Tic110 and the energy-delivering chaperones (Chou et al. 2003). The current model suggests that Tic40 binds favourably to Tic110 when a transit peptide is bound to the stromal domain of Tic110 (Inaba et al. 2003; Chou et al. 2006). The transit peptide is then released from Tic110, upon binding of Tic40, and passed to hexameric Hsp93 which pulls the preprotein through the central pore. The Sti1 domain of Tic40 can stimulate the ATPase activity of Hsp93 and, thus, also the process of threading. Interestingly, the Sti1 domain of Tic40 can be functionally replaced in planta with the Sti1 domain of mammalian Hip (*H*sp70-*i*nteracting *p*rotein), for which an ATPase-stimulating function has never before been reported (Bédard et al. 2007).

Recently, it has been found that a variety of preproteins destined for the chloroplast inner envelope membrane (including Tic110, Tic21 and also Tic40 itself) accumulate as soluble, stromal intermediates in *tic40* mutant chloroplasts following in vitro import (Chiu and Li 2008). It has therefore been suggested that Tic40 has an additional function in the post-import re-insertion of certain

proteins that are destined to the inner envelope membrane (see Sect. IV) (Li and Schnell 2006; Tripp et al. 2007; Vojta et al. 2007b; Viana et al. 2010).

E. Redox Regulation of the TIC Machinery

A considerable body of literature indicates that the TIC translocon is regulated by the redox status of the chloroplast (Fig. 12.1) (Balsera et al. 2010). Light induces photosynthetic electron transfer which leads to an overall reduced state of the stroma during the day as NADPH accumulates. Consumption of NADPH at night leads to an oxidized stroma with a higher concentration of $NADP^+$. The import machinery might be directly regulated by the metabolic state of the stroma via the $NADP^+$/NADPH ratio (Stengel et al. 2009; Balsera et al. 2010). In maize chloroplasts, precursors of different isoforms of ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR) were imported differentially under light or dark conditions: photosynthetic isoforms, pFdI and pFNRI, were equally imported in light- and dark-exposed chloroplasts, whereas non-photosynthetic isoforms, pFdIII and pFNRII, were mis-sorted to the intermembrane space under light conditions (Hirohashi et al. 2001). This suggests that the non-photosynthetic isoforms might interfere with photosynthesis and, therefore, that systems have evolved to prevent their import under light conditions.

Two proteins that associate with the TIC machinery and have the ability to bind NADPH, namely Tic32 and Tic62, have been described (Küchler et al. 2002; Hörmann et al. 2004). These components are thought to modulate protein import in response to changing $NADP^+$/NADPH ratios in the stroma. Tic32, a member of the family of short chain dehydrogenases, associates with Tic110 and with Tic40 at the stromal side of the inner envelope membrane, and has binding sites for both NADPH and calmodulin (Hörmann et al. 2004). Association of Tic32 with the TIC apparatus is dependent on the $NADP^+$/NADPH ratio, with Tic32 binding under oxidized conditions (high $NADP^+$/NADPH ratio) and dissociating from the TIC translocon under reduced conditions (low $NADP^+$/NADPH ratio) (Chigri et al. 2006; Kovacs-Bogdan et al. 2010). A reduction of protein import in the presence of ophiobolin A and ionomycin, which both disrupt calcium signaling, has been attributed to Tic32 (Chigri et al. 2005, 2006). Interestingly, calmodulin and NADPH binding to Tic32 are mutually exclusive, suggesting that a calcium signal is relayed via calmodulin only under oxidizing conditions when Tic32 is associated with the TIC apparatus (Chigri et al. 2006). The reported influence of calcium on Tic110 channel activity in vitro allows speculation that Tic32 delivers calcium via calmodulin directly to Tic110 under certain redox conditions in order to modulate protein import activity (Balsera et al. 2009).

Tic62 has several properties that are very similar to those of Tic32. It binds Tic110 and preproteins at the stromal side of the envelope, and has an N-terminal binding site for NADPH (Küchler et al. 2002). Its association with the TIC machinery is dependent on the $NADP^+$/NADPH ratio, with Tic62 being bound under oxidized conditions and dissociating under reduced conditions, just like Tic32 (Stengel et al. 2008). Like TROL (*t*hylakoid *r*ho*d*anese-*l*ike protein), which tethers FNR to thylakoids for the reduction of $NADP^+$ in the terminal step of photosynthetic electron transport, Tic62 possesses a C-terminal FNR-binding site (Küchler et al. 2002; Juric et al. 2009; Alte et al. 2010). Under reducing conditions in the stroma, Tic62 dissociates from the TIC apparatus, thereby increasing its affinity for FNR and leading to its preferential association with the thylakoids (Stengel et al. 2008). Interestingly, the Tic62-bound FNR appears not to be involved in photosynthetic electron transport even when bound to the thylakoids (Benz et al. 2009). Whether Tic62 has two distinct functions, one at the thylakoids and another at the TIC complex, or the capacity to relay thylakoid signals to the TIC translocon, is currently unknown (Benz et al. 2009).

Thioredoxins are small proteins that mediate the formation of disulfide bridges upon redox changes. Thioredoxin isoforms have been found associated with the inner envelope membrane, leading to the suggestion that the TIC machinery might be subject to thiol-based redox regulation (Ferro et al. 2003). N-ethylmaleimide, a compound that modifies cysteines and therefore interferes with thiol-based redox regulation, has been found to influence chloroplast protein import (Row and Gray 2001). Similarly, a variety of reducing agents such as glutathione and dithiothreitol can influence protein import, suggesting that regulatory disulfide bridges may exist within or between translocon components, including Toc159, Toc34 and Toc75 (Pilon et al. 1992; Seedorf and Soll 1995; Stengel et al. 2009). Hsp93, both from cyanobacteria and from higher plants, contains conserved cysteines with the potential to form intra- or intermolecular disulfide bridges (Mata-Cabana et al. 2007; Balsera et al. 2009); these may occur in the nucleotide binding domains of Hsp93, possibly leading to modulation of ATPase activity upon disulfide bridge formation (Balsera et al. 2009). Tic110 is able to form intramolecular disulfide bridges under oxidizing conditions, probably mediated by a stromal thioredoxin (Balsera et al. 2009). Disulfide bridges between Tic110 and Tic40 have also been found under oxidizing conditions (Stahl et al. 1999); Tic40 has only one cysteine at its C-terminus that might stabilize its binding to Tic110 and/or Hsp93 (Bédard et al. 2007; Balsera et al. 2010). Since these three components interact to form the motor complex, it can be speculated that disulfide bridges play a role in stabilizing or destabilizing complex formation under certain redox states in the chloroplast.

Tic55 was discovered in a complex with Tic110, and was found to associate with translocating preproteins along with other translocon components (Caliebe et al. 1997). It can bind to thioredoxins and contains some conserved cysteines that have the potential to form disulfide bridges (Bartsch et al. 2008). Additionally, Tic55 contains a Rieske-type iron-sulfur center. Rieske centers are known to be inhibited by DEPC, and it has been reported that DEPC treatment leads to a reduced import efficiency of pSSU; thus, Tic55 might be involved in the regulation of protein import via an electron transfer process, or act as a sensor of oxidative stress (Caliebe et al. 1997). However, knockout mutants of Arabidopsis atTic55-II, which is the orthologue of the originally-described pea Tic55, displayed neither visible abnormalities nor defects in chloroplast protein import (Boij et al. 2009). Moreover, the aforementioned negative effect of DEPC on protein import could also be observed in the *tic55-II* mutant, and so it can be concluded that, at least in Arabidopsis, DEPC has a chloroplast target different from Tic55 (Boij et al. 2009). Additional doubts over the participation of Tic55 in protein translocation were raised when two independent laboratories failed to detect the protein in import complexes (Kouranov et al. 1998; Reumann and Keegstra 1999).

F. Processing of Preproteins in the Stroma

Soon after the N-terminal part of the preprotein has emerged from the TIC machinery, the transit peptide is removed by the stromal processing peptidase (SPP), a metalloendopeptidase of the M16 family (other family members include subunit β of the mitochondrial processing peptidase, MPP, and *Escherichia coli* pitrilysin) (Vandervere et al. 1995; Richter and Lamppa 1998; Richter et al. 2005). SPP recognizes a stretch of basic residues with weak sequence homology or conservation of physicochemical properties near the C-terminus of the transit peptide (Emanuelsson et al. 1999; Richter and Lamppa 2002; Rudhe et al. 2004). It cleaves the transit peptide from the mature sequence using the catalytic activity of its zinc-binding domain, and then an additional proteolytic step releases transit peptide fragments from SPP; these are then degraded by a presequence protease termed PreP (Richter and Lamppa 2002, 2003; Moberg et al. 2003).

The SPP protein is evolutionarily well conserved, as related sequences are found in algae and malaria parasites (Richter et al. 2005). An ancestral activity was probably inherited with the original endosymbiont, as SPP-related sequences even exist in cyanobacteria. Interestingly, SPP is encoded by a single-copy gene in Arabidopsis, and so it must be able to bind to a wide range of transit peptides with highly variable sequences (Richter and Lamppa 1998; Bruce 2001; Jarvis 2008).

Antisense-mediated down-regulation of *SPP* gene expression in Arabidopsis or tobacco plants led to a variety of abnormal phenotypes, ranging from albinism to seedling lethality (Wan et al. 1998; Zhong et al. 2003). Chloroplasts of the antisense lines displayed abnormal ultrastructure and were less abundant than those in wild-type cells (Wan et al. 1998; Zhong et al. 2003). Similarly, in rice, a point mutation affecting a conserved glutamate residue of SPP caused visible chlorosis associated with small, abnormal chloroplasts (Yue et al. 2010). Interestingly, chloroplast protein import efficiency was compromised in the SPP antisense lines (Wan et al. 1998; Zhong et al. 2003); this might reflect the fact that most TIC components and Toc75 possess a transit peptide, and so rely on SPP for their correct maturation, or indicate that transit peptide cleavage is an integral component of the chloroplast import mechanism. Complete loss of SPP in Arabidopsis knockout mutants led to embryo abortion at the 16-cell stage, further emphasizing the importance of transit peptide cleavage for organelle development and plant growth (Trösch and Jarvis 2011).

IV. Intraorganellar Protein Transport Pathways

A. Internal Sorting of Plastid Proteins

Chloroplasts are complex organelles comprising several distinct suborganellar compartments; as a consequence, the internal routing of chloroplast proteins is necessarily a complex process. While envelope proteins may utilize variations of the TOC/TIC import pathway to reach their final destination (see Sects. IV.B and IV.C below), proteins destined for the thylakoid membrane or lumen additionally employ one of four distinct targeting pathways. In spite their undoubted importance, these thylakoidal pathways are beyond the scope of this chapter, and so the following paragraph provides a brief overview only, for the sake of completeness. Readers are referred to the following reviews for more detailed information (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Cline and Dabney-Smith 2008).

Proteins destined for the thylakoid lumen engage the Sec ("Secretory") and Tat (*t*win-*a*rginine *t*ranslocase) pathways, and possess an additional cleavable targeting signal, just downstream of the transit peptide. Unlike transit peptides, such lumenal targeting peptides are similar to the signal peptides that mediate inner membrane transport in bacteria; they are removed by a type I signal peptidase termed thylakoidal processing peptidase (TPP). The Sec pathway is powered by ATP hydrolysis at the SecA motor protein, and accepts only unfolded proteins. By contrast, the Tat pathway uses the thylakoidal proton gradient as its energy source, and is able to deliver folded proteins; the importance of this pathway may relate to the transport of proteins that acquire their final conformation (through co-factor binding or oligomerization) in the stroma. On the other hand, thylakoid membrane proteins utilize either the SRP-dependent pathway or the so-called "spontaneous" insertion pathway. The former consumes GTP as a result of a critical interaction between the SRP and its membrane receptor (both are GTPases), and is mainly concerned with the insertion of polytopic light-harvesting complex proteins. The "spontaneous" pathway, however, seems to proceed without energy consumption or the involvement of a proteinaceous transport machinery.

In contrast with the TOC/TIC pathway, at least three of these thylakoidal pathways are closely related to protein transport systems in bacteria, which gives rise to the concept of conservative sorting: i.e., the transport of nucleus-encoded, thylakoid proteins occurs in two sequential, independent translocation steps, at the envelope and at the thylakoids, with the latter having been retained (or conserved) from the prokaryotic ancestor of the organelle. Recent evidence suggests that similar conservative sorting may operate at the level of the inner envelope membrane (see Sect. IV.C).

B. Sorting to the Outer Envelope Membrane

Most outer envelope membrane proteins do not possess a transit peptide; rather, they are directed to the membrane by intrinsic targeting information. There are several different mechanisms for targeting to the outer membrane (Hofmann and Theg 2005c), and the most prominent of these is probably that used by proteins such as OEP7/14 (*o*uter *e*nvelope *p*rotein, 7/14 kDa) and Toc64/OEP64. Targeting information in such proteins lies within an amino-terminal transmembrane domain, which bears superficial resemblance to signal peptides for ER translocation (Lee et al. 2001; Hofmann and Theg 2005c). Adjacent to the transmembrane domain there is a charged region that seems to play a crucial role in differentiating these proteins from those destined for the ER. Despite initial suggestions that such proteins insert "spontaneously" into the membrane, it now seems clear that they utilize a proteinaceous import machinery and consume nucleoside triphosphates during insertion (Tu and Li 2000; Hofmann and Theg 2005a). In fact, competition studies (with preproteins possessing transit peptides), and cross-linking results, revealed that Toc75 is employed during outer membrane insertion (Tu et al. 2004); the relevant Toc75 may correspond to a fraction that is disassociated from other TOC components (Kouranov et al. 1998). Toc75 involvement in outer membrane insertion parallels the situation in mitochondria, where the TOM channel, Tom40, is similarly employed (Rapaport 2005). More recently, the cytosolic protein, AKR2A (*ank*yrin *r*epeat-containing protein 2A), was identified as a cytosolic sorting factor in this targeting pathway (Bae et al. 2008; Bédard and Jarvis 2008). The AKR2A protein is proposed to act as a chaperone, preventing aggregation of client proteins and guiding them to the envelope membrane. Interestingly, AKR2A was also reported to be involved in the insertion of a peroxisomal membrane protein (Shen et al. 2010), suggesting that it may be important for the targeting of a broad class of membrane proteins (Zhang et al. 2010).

Targeting of Toc34 is also mediated by an intrinsic signal, but in this case the relevant transmembrane domain is situated at the C-terminus (i.e., it is a tail-anchored protein). As with OEP7/14 and similar proteins, insertion appears to require both envelope proteins and an energy source (Tsai et al. 1999). Moreover, competition studies suggest that Toc34, OEP7/14 and Toc64/OEP64 may all employ the same insertion mechanism (Tu and Li 2000; Hofmann and Theg 2005a, c). Toc34 insertion has been reported to depend on previously-inserted Toc34, as well as on membrane lipids, and to follow a different pathway from that used by another tail-anchored protein, OEP9 (a 9 kD outer envelope protein of unknown function) (Qbadou et al. 2003; Dhanoa et al. 2010). Quite a different targeting mechanism is employed by the Toc159 protein, a fact which may be related to its large, atypical M-domain. Membrane insertion of Toc159 is thought to involve a homotypic G-domain interaction with Toc34 already integrated into the membrane, as well as the channel protein, Toc75 (Bauer et al. 2002; Smith et al. 2002; Wallas et al. 2003). The G-domain interactions and protein insertion via this pathway are controlled by GTPase cycling at the receptors. That the M-domain alone could associate with the

outer membrane in vivo suggests that some targeting information must reside in this domain (Lee et al. 2003). As there are no typical transmembrane spans within the M-domain, its association with the membrane is most likely dependent on the TOC complex, although a short hydrophobic segment near the C-terminus could interface with the core of the lipid bilayer (Inaba and Schnell 2008).

Toc75 employs an even more unusual mechanism for membrane insertion. This protein possesses an unusually large, bipartite targeting signal: the N-terminal domain is a standard transit peptide, while the C-terminal domain mediates intraorganellar targeting (Tranel et al. 1995; Tranel and Keegstra 1996). The second domain contains a poly-glycine stretch that arrests translocation so that the preprotein can disengage from the translocon and undergo membrane integration (Inoue and Keegstra 2003). The transit peptide domain is cleaved by SPP as normal, whereas the second domain is removed by a TPP-related type I signal peptidase that resides in the envelope as well as in the thylakoids (where it additionally plays a role in the maturation of thylakoidal proteins) (Inoue et al. 2005; Shipman and Inoue 2009; Shipman-Roston et al. 2010). How Toc75 becomes integrated into the outer membrane following maturation is unclear. Insertion and topogenesis of similar β-barrels in the mitochondrial outer membrane is mediated by the sorting and assembly machinery (SAM) (Neupert and Herrmann 2007; Schmidt et al. 2010). At the core of the SAM complex is the BamA homologue, Sam50, and it is hypothesized that a related protein may have an analogous role in chloroplasts, with one candidate being OEP80 (Eckart et al. 2002; Inoue and Potter 2004; Gentle et al. 2005; Patel et al. 2008).

C. Sorting to the Intermembrane Space and Inner Envelope Membrane

Most proteins destined for the intermembrane space or inner envelope membrane possess a cleavable, amino-terminal targeting sequence. Thus, targeting to these destinations is quite different from outer membrane insertion.

Targeting to the intermembrane space has been studied for two components, Tic22 and MGD1 (*m*onogalactosyl*d*iacylglycerol synthase 1), which employ different targeting pathways to reach their destinations (Kouranov et al. 1999; Vojta et al. 2007a). While both proteins have a targeting sequence, only that of MGD1 seems to be cleaved by SPP; together with the energetic requirements for its import, this indicates that MGD1 partially enters the stroma. By contrast, Tic22 is cleaved by an as yet unknown protease in the intermembrane space, implying that it does not pass through the TIC channel; in fact, there is even uncertainty over the involvement of the TOC machinery during Tic22 transport.

For targeting to the inner envelope membrane, two pathways exist. In the stop-transfer pathway, proteins do not completely enter the stroma; instead, a hydrophobic transmembrane domain arrests translocation in the channel, leading to lateral release of the protein into the membrane (Brink et al. 1995; Knight and Gray 1995; Tripp et al. 2007). This pathway might be especially important for polytopic proteins that are prone to aggregation, such as the triose phosphate/phosphate translocator. By contrast, in the post-import pathway, proteins insert into the inner envelope membrane only after complete translocation into the stroma, where they form a soluble intermediate (Lübeck et al. 1997; Li and Schnell 2006; Tripp et al. 2007). Similar mechanisms operate in mitochondria, where targeting to the inner membrane employs stop-transfer and conservative sorting pathways (Neupert and Herrmann 2007); the latter pathway is analogous to the post-import pathway, and its name is a reference to the fact that it employs machinery that is at least partly of prokaryotic origin.

For both pathways, stop-transfer and post-import, a cleavable transit peptide is required, implying initial involvement of the TOC/TIC

apparatus (Knight and Gray 1995; Lübeck et al. 1997; Stahl et al. 1999). Recent studies on the inner envelope protein, APG1 (*a*lbino or *p*ale *g*reen mutant 1), which employs the stop-transfer pathway, revealed that membrane targeting information lies exclusively in the transmembrane domain, and that this domain alone is sufficient to direct stop-transfer insertion (as opposed to the use of the post-import route), even in the context of heterologous passenger proteins (Viana et al. 2010).

Tic40 and Tic110 have been used to study the post-import pathway (Li and Schnell 2006; Tripp et al. 2007). Both proteins are anchored in the inner membrane by N-terminal transmembrane spans, projecting large C-terminal domains towards the stroma. During transport, they form post-SPP intermediates lacking the transit peptide in the stroma (Inaba et al. 2005; Bédard et al. 2007). Actually, Tic40 possesses a bipartite targeting signal, although the role of the second domain is uncertain, as a serine/proline-rich region of the mature protein and the adjacent transmembrane domain seem to control membrane insertion (Li and Schnell 2006; Tripp et al. 2007). The former may interact with the latter to form a membrane insertion loop, while in Tic110 a similar structure may be formed by the two, closely-juxtaposed transmembrane helices. Efficacy of the targeting signals in Tic40 is dependent upon context within the protein sequence, suggesting that post-import pathway signals are complex, which is perhaps necessary to avoid stop-transfer insertion (Viana et al. 2010). Stromal events in the post-import pathway may be assisted by the Hsp93 chaperone (Vojta et al. 2007b), while reinsertion is dependent on proteinaceous membrane components of unknown identity (Li and Schnell 2006). Bearing in mind the previously-described conservative sorting pathways at the thylakoids and in mitochondria, it is intriguing that a second Sec translocase (in addition to the well-characterized thylakoidal system) was recently identified in chloroplast envelopes (Skalitzky et al. 2011). Evidence has also been presented that resident Tic40 (and possibly also Tic110) plays a role in inner membrane protein insertion (Inaba et al. 2005; Chiu and Li 2008).

V. Dual-Targeting and Non-canonical Protein Transport to Chloroplasts

A. Dual-Targeting of Proteins to Chloroplasts and Other Organelles

While the majority of chloroplast proteins are targeted quite specifically to plastids, in recent years it has become increasingly apparent that a significant number of proteins make their way to more than one destination (Peeters and Small 2001; Silva-Filho 2003; Mackenzie 2005; Carrie et al. 2009). Transport to both chloroplasts and mitochondria is the most common form of dual-targeting (with ~50 proteins having been reported to do this), but there are also proteins that reside in the nucleus, ER or peroxisomes as well as in chloroplasts (Levitan et al. 2005; Sapir-Mir et al. 2008; Krause and Krupinska 2009). The prevalence of such multi-destination transport suggests that protein targeting is not as inflexible as was once thought, and has been taken as evidence in support of a hypothesis that accounts for the evolutionary relocation of organellar genes to the nucleus, a model which depends on the "minor mistargeting" of large numbers of proteins to multiple destinations (Martin 2010).

Although there are exceptions (Ueda et al. 2008), dual-targeting to chloroplasts and mitochondria typically involves one of two different mechanisms (Peeters and Small 2001). In the first of these, alternative transcript splicing and/or differential transcriptional or translational initiation is employed to produce proteins that possess different N-terminal leader sequences with distinct targeting properties. Alternatively, a single mRNA may be produced encoding a single protein which possesses an ambiguous leader

sequence, that is competent for import into both chloroplasts and mitochondria. The functions of dual-targeted proteins include DNA and RNA synthesis and processing, protein synthesis, and cellular stress response (Mackenzie 2005; Carrie et al. 2009). The most striking example of dual-targeting occurs amongst the aminoacyl-tRNA synthetases (aaRSs), where 17 of the 24 organellar proteins in Arabidopsis are targeted to both chloroplasts and mitochondria (Duchêne et al. 2005); only two are uniquely chloroplastic. Plant cells originally inherited three aaRS genes, one from each of the three ancestral genomes (nuclear, mitochondrial and plastidic). Remarkably, there are no examples in Arabidopsis where all three still coexist, indicating that extensive exchange and loss of aaRS genes has occurred during evolution, such that the proteins are now shared between two, or even all three, of the compartments that possess translational machinery.

The ambiguous transit peptides of preproteins that are dually targeted to both endosymbiotic organelles have been examined in some detail (Peeters and Small 2001; Pujol et al. 2007; Berglund et al. 2009a, b). In general, they seem to have properties that are very similar to, but intermediate between, those of proteins targeted exclusively to either chloroplasts or mitochondria. In the N-terminal region, serine content is more similar to that in chloroplast transit peptides, while arginine content is more similar to that in mitochondrial presequences. Dual targeting peptides also show enrichment of phenylalanine and leucine residues, but they seem to lack a shared or common functional-domain architecture (Berglund et al. 2009a, b). Evidence also suggests that the extent to which a given protein is dual-targeted is influenced by the mature domain of the preprotein, as well as by developmental factors (Mackenzie 2005; Carrie et al. 2009). Software has been developed for the in silico prediction of ambiguous targeting peptides, and its use suggests that as many as ~400–500 proteins may be dual-targeted to chloroplasts and mitochondria in Arabidopsis and other plants (Mitschke et al. 2009). Competition assays suggest that dual-targeted proteins employ the same organellar import machineries as organelle-specific proteins (Berglund et al. 2009b).

B. Non-canonical Protein Transport to Chloroplasts

Until quite recently, transit peptide-dependent import was considered to be the sole protein transport route leading to the chloroplast interior. However, it is now clear that several alternative targeting signals and pathways exist (Radhamony and Theg 2006; Jarvis 2008). In fact, a recent study estimated that over 10% of the chloroplast proteome comprises proteins that lack a typical transit peptide (Armbruster et al. 2009). Proteomic studies played an important role in the identification of these non-canonical pathways (Kleffmann et al. 2004). For example, a protein named ceQORH (*c*hloroplast *e*nvelope *q*uinone *o*xido*r*eductase *h*omologue) was identified in the Arabidopsis envelope proteome, and was found to associate with the inner envelope membrane in spite of the fact that it lacks a transit peptide. In fact, its extreme amino-terminus is not required for import; instead, an internal sequence of ~40 residues controls localization (Miras et al. 2002). The protein does require proteinaceous components and ATP for its targeting, but the canonical TOC/TIC apparatus is not involved (Miras et al. 2007). Another inner membrane protein, Tic32/IEP32 (*i*nner *e*nvelope *p*rotein, 32 kD), was similarly found to lack a transit peptide (Nada and Soll 2004), and it too could localize properly without assistance of the TOC machinery. Competition analysis suggested that ceQORH and Tic32 follow different import pathways (Miras et al. 2007).

Proteomic analysis also led to the identification of a large number of chloroplast proteins with predicted signal peptides for ER translocation (Kleffmann et al. 2004). Chloroplast protein traffic through the endomembrane system is well documented in

organisms that have complex plastids, such as algae and apicomplexan parasites (Nassoury and Morse 2005). Such organelles are derived from secondary endosymbioses, and consequently are surrounded by multiple membranes including remnants of the secondary endosymbiont's cell membrane and the host ER. It is therefore inevitable that chloroplast protein traffic passes through the endomembrane system in these species, and it does so under the guidance of bipartite leader sequences comprising a signal peptide for ER transport followed by a transit peptide for chloroplast import. However, plant chloroplasts are not surrounded by such extra membranes, and so until recently similar trafficking was not thought to occur in plants. However, physical and functional associations between the ER and the outer envelope membrane are well documented in plants (Crotty and Ledbetter 1973; Whatley et al. 1991; Benning et al. 2006; Andersson et al. 2007). Moreover, indirect evidence for chloroplast protein transport through the ER and Golgi in plants has existed for some time, with glycoproteins and proteins with apparent signal peptides having been found in plastids (Gaikwad et al. 1999; Chen et al. 2004; Asatsuma et al. 2005).

Firm evidence for such targeting was provided recently by thorough analyses of proteins such as Arabidopsis CAH1 (*c*arbonic *an*hydrase 1) (Villarejo et al. 2005; Nanjo et al. 2006). This stromal protein was strongly predicted to have a signal peptide; accordingly, it could not be imported directly by isolated chloroplasts, but instead was taken up co-translationally by ER microsomes and processed to its mature size (Villarejo et al. 2005). In addition, glycosylated CAH1, as well as several other glycoproteins, was identified in the chloroplast stroma, implying that some proteins are transported through the Golgi en route to the chloroplast. Indeed, application of brefeldin A (a chemical that interferes with Golgi-mediated vesicle traffic) obstructed CAH1 transport within the endomembrane system. It is not clear how proteins following this pathway enter the chloroplast, as they do not seem to possess bipartite targeting signals. Some data suggest that the signal peptide itself provides the necessary targeting information (Chen et al. 2004), while others have argued that surface characteristics of the mature protein play a role (Kitajima et al. 2009). The proteins may be released into the intermembrane space, following vesicle fusion with the outer membrane, thereafter entering an unknown translocon, the TIC machinery (Scott and Theg 1996), or vesicles that pinch off from the inner envelope membrane (Benning et al. 2006; Benning 2009). Such vesicle fusion would inevitably deliver lipids to the chloroplast as well, although the significance of this in the context of other mechanisms of lipid transport remains to be seen.

VI. Concluding Remarks

Research on chloroplast protein import has provided a wealth of data in recent years, leading to a greatly enhanced understanding of the molecular steps underlying the process, but also to a certain amount of confusion over conflicting results. A major challenge for future research will be to construct consensus models that rationalize the contradictory results and explain as much of the available information as possible. While our knowledge concerning G-domain function in the TOC receptors has increased considerably thanks to recent work, the precise mode of action is still debated. The ATP requirement in the intermembrane space, a long-standing fact, will require renewed attention now that doubt has been cast on the existence of ATP-processing components previously assumed to reside in the intermembrane space. We have learnt much about the function, regulation and topology of several putative inner envelope channel proteins, but there is still no agreement concerning their capabilities for preprotein transport, or indeed their functional interactions with each other. Moreover, while a consensus view is emerging concerning the existence of substrate-specific import pathways, the molecular basis for TOC receptor (and possibly also TIC channel) selectivity will

require further work. It is commonly agreed that a complex and important process like chloroplast protein import must be tightly regulated, and a lot of work has been done to unveil putative redox-regulatory processes at the TIC apparatus. However, the mechanistic details of such regulatory networks are currently lacking. We hope and expect that future research focusing on these fascinating challenges will bring the consensus of opinion we are waiting for, increasing our knowledge about chloroplast protein import which is such a crucial process in plant life.

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Part IV

Leaf Senescence and Chloroplast Dismantling

Chapter 13

Defining Senescence and Death in Photosynthetic Tissues

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Summary

Having clear concepts of senescence and aging, as well as the diverse related terminology, will foster these and related fields by facilitating communication within and between the academic constituencies that study them. Moreover, it will enable those working on photosynthetic tissues to contribute more to and draw more help from other fields ranging from gerontology to evolution. Senescence is a very important developmental process in the life cycle of organisms, especially photosynthetic organisms where it is often very dramatic. Senescence is commonly viewed as an increase in the probability that an organism will die as it ages chronologically (demographic view); however, plant physiologists/molecular biologists go a step further to define senescence as internal processes that actively cause death (physiological view). During senescence, a very wide range of changes (senescence syndrome) occur at the cell, organ and organismic levels; however, many are not causal and therefore not senescence per se. Although chloroplast degradation during senescence may not cause death, the decline in photosynthesis is very important and may limit agricultural/biomass productivity. Senescence is actively driven by the nucleus (necrogenic processes), but it also involves shutdown of life-supporting (biostatic) processes. Death, the endpoint of senescence, can be defined generally as the collapse of homeostasis. At the cell level, death appears to be the loss of the plasma membranes' ability to retain/exclude molecules. Exactly how senescence brings the cells that comprise organs/organisms to death is not clear; however, programmed cell death processes participate in the end stage.

I. Introduction

A. The Conceptual Framework of Senescence

In order for a field to flourish and have a high impact, it needs a clear, well-founded terminology to serve as conceptual framework and for communication. The key terms that need definition at the outset are senescence, senescence syndrome and aging, which are related. However, it is necessary to look beyond these terms to a new, complementary vocabulary (necrogenesis, biostasis, Sect. II.B below) that will better designate the causal processes that are being uncovered. In addition, it is important to explain and integrate the plethora of related terminology, particularly the numerous cell death processes (Table 13.1). Lastly, if death is the endpoint of senescence, we need a clear concept of what that is.

Senescence has been a very useful conceptual "handle" adopted by biologists to designate some very important degenerative/end-of-life developmental processes; however, the term senescence has widespread, variable usage both outside the scientific literature and in several disparate scientific communities. This lack of interchange/coordination has caused some communication problems (Sect. II.B below). I will favor a physiological definition (Sect. II.B below), because it applies quite visibly to photosynthetic organisms, it is mechanistic and it is already in widespread usage among the primary readership of this

Abbreviations: PCD – Programmed cell death

Table 13.1. Terminology – senescence and related terms (processes)

Term	Abbreviated description/definition	Reference
General concepts		
Senescence (dictionary)	The organic (animate) process of growing older	Dictionary
Senescence (physiological)	Active, endogenously-driven degeneration/loss of functions that lead to death	Leopold (1961); Noodén and Leopold (1978); Sects. I.B and II.B
Senescence (demographic)	Defined statistically as an increased probability of dying with increased age	Sect. II.B
Senescence (evolutionary)	Same as demographic senescence	Roach (2004)
Senescence syndrome	All of the many changes that accompany physiological senescence, whether primary or not	Noodén (1988a); Sects. I.B, IV
Negative senescence	Decline in mortality with age after reproductive maturity	Vaupel et al. (2004); Sect. III.D
Cellular senescence	Permanent arrest of the cell cycle/mitosis	Vicencio et al. (2008); Sect. III.D
Mitotic senescence	Similar to cellular senescence above	Gan (2007); Sect. III.D
Negligible senescence	Lack of age-related increases in mortality risks/decreases in vitality	Finch (1990); Sect. III.D
Aging (dictionary)	The process of growing older or to become older (animate or inanimate)	Dictionary
Aging (biological)	Passive accumulation over time of damage that may affect function and survival	Noodén and Leopold (1978); Murphy and Partridge (2008); Sects. I.C and II.B
Cellular processes		
Programmed cell death (PCD)	Endogenously-driven (programmed) self destruction in cells	
Developmental cell death	Refers to PCD as it functions in development	
PCD type1	Same as apoptosis below	Kroemer et al. (2009); Noodén (2004a)
PCD type 2	Same as autophagy below	Kroemer et al. (2009); Noodén (2004a)
PCD type 3	Same as necrosis/oncosis below	Kroemer et al. (2009); Noodén (2004a)
Apoptosis	Cell shrinkage; nuclear and chromatin condensation and fragmentation; plasma membrane blebbing	Kroemer et al. (2009); Noodén (2004a); Sect. III.C
Necrosis	Swelling of cytoplasm and organelles; rupture of plasma membrane Same as oncosis below	Kroemer et al. (2009); Noodén (2004a); Sect. III.C
Oncosis	Same as necrosis above	Kroemer et al. (2009); Noodén (2004a); Sect. III.C
Autophagy	"Self eating"; massive vacuolation of the cytoplasm (double-membrane autophagic vacuoles; endocytosis)	Kroemer et al. (2009); Noodén (2004a); Sect. III.C
Hypersensitivity	Rapid death of groups of cells associated with resistance to pathogen infection	Heath (2000)
Senescence patterns	Descriptive adjectives can be combined with senescence to designate particular manifestations of senescence, e.g., monocarpic, polycarpic, leaf, flower, fruit, autumnal, progressive, nonsequential, and top senescence	Leopold (1961); Sect. III.B

series. I will explain the other ideas, provide a rationale for the physiological definition of senescence and in the process argue for this definition as the standard.

In addition, much can be gained from transferring information from the huge literatures relating to animals, microorganisms, human aging, medical pathology, ecosystems and evolution into the research thinking on photosynthetic organisms; however, these tend to be somewhat isolated communities, each with a similar yet different terminology. Reciprocally, those studying photosynthetic organisms can extend/enhance their impact by understanding the views of other constituencies and addressing them when practical. In other words, if the plant senescence and programmed cell death researchers wish to be seen as relevant in these other communities and have broader access to research support, they will have to understand and work with these other views of these terms/concepts.

Increasingly, molecular findings will need to be explained in broader contexts, i.e., the organism and ecosystems (natural and agricultural), to be relevant, and this conceptual integration will help to pave the way for the future (Noodén 1984, 2004a). Indeed, this integration can be useful in advancing research on crop yields and biomass accumulation, and it will become more so as systems biology emerges to the forefront.

B. Senescence and the Senescence Syndrome

For many decades, plant physiologists and biochemists have viewed senescence primarily as: endogenously controlled degenerative changes that lead to the death of an organ or organism (Leopold 1961; Noodén and Leopold 1978). This narrows the focus of the term senescence to causal (primary) processes.

From an early stage, it was recognized that ultimately this conceptual definition had to be translated into the biochemical specifics of what causes death; however, that research is still underway. Along the way, it was also recognized that many of the changes occurring during senescence did not cause death but accommodated or simply accompanied the senescence process, i.e., were secondary or peripheral. The term senescence syndrome describes this broad collection of accompanying changes with no attempt to designate which are causal (Noodén 1988a). The phenomena that we recognize as senescence can also be defined in terms of counteracting causal processes, first, activation of those processes (necrogenesis) that actively cause death, and second, shutdown of those processes (biostasis) that prevent death/maintain life (Sect. II.B below).

Other life science researchers (particularly those in fields of ecology, evolution and human gerontology) see senescence differently, most often in statistical terms (demography). Indeed, there is a sharp divide between plant physiologists/biochemists and the others, because we have focused on plant systems (e.g., leaf senescence and monocarpic plants [one reproductive phase, Sect. II.B below]) where senescence is characterized by dramatic visible changes (Fig. 13.1) and orderly, internally controlled processes often manifested as a decrease in photosynthesis (Sect. IV.C below).

C. Aging

Aging, like senescence, has been widely used outside the scientific community (Sect. II.B below), but in the plant physiology/molecular biology community, it usually refers simply to the passive accrual of changes (some, but not all, are detrimental) over time (see Noodén 2004a). These changes, sometimes termed ‘wear and tear’ are mostly (but not entirely) exogenous. Aging leading to decreased functionality may increase the vulnerability to death, but does not cause death directly, at least not quickly. There should be no confusion about the role of chronological age (aging) in senescence; extensive data indicate that generally internal factors, not chronological age, cause death during senescence (Sect. II.C below; Fig. 13.1; Molisch 1938; Noodén et al. 2004).

Fig. 13.1. A senescent intact, attached leaf from the shrub *Philadelphus grandiflorus* and a disk that was excised and cultured while the leaf was still green. The failure of the excised disk to senesce demonstrates the influence of surrounding tissues (context) on the senescence of component parts of the leaf. (From Stahl 1909)

II. Senescence Versus Aging: Evolution of the Concepts

A. Overview

Senescence and aging are by far the most confusing and problematic concepts that we have to deal with. This can easily become the root of miscommunication, generate unnecessary controversy and eventually inhibit research. Senescence is intertwined with aging conceptually, so they need to be considered together. Both carry with them a lot of connotative meaning from their widespread use outside the biological community, and this can be viewed as "baggage" that limits development of precise scientific meanings. In order to understand these terms and the literature that employs them in the fields of ecology, evolution and gerontology, it seems important to look at their developmental history.

B. Senescence, Aging and Necrogenesis/Biostasis

An Overview of Evolution of the Concepts of Senescence and Aging – Three pervasive influences have governed the development of the concepts of aging and senescence: *First*, the public (nontechnical) usage of aging vastly outweighs all of the scientific uses, and it seems best not to fight this. Even with this constraint, the concept of aging can still be useful in biological science (more about that below in Sect. II.C). *Second*, these concepts have developed mostly independently (with little cross talk) in separate life science research communities. *Third*, as expected, these ideas have been shaped by the phenomena that these research communities had to deal with. For example, gerontologists, ecologists and evolutionists dealt mainly with organisms that had multiple reproductive episodes accompanied by a slow decline to death (polycarpy), whereas plant physiologists dealt mainly with organs (especially leaves) and monocarpic organisms (a single reproductive episode followed by death) that undergo rapid and often very visible degeneration to death.

The terms monocarpy and polycarpy predate the related terms semelapary and iteropary by >100 years (Noodén 1980), so they would normally take precedence over the later duplicating terms. Moreover, they also convey their intrinsic meaning in a more readily recognizable way, so hereafter, I will use monocarpy and polycarpy preferentially.

The Gerontologists' Views of Senescence and Aging – Our forerunners have given a lot of careful thought to what senescence and aging are. For example, Medawar (1957, p. 46) states in his classic book that 'Ageing' hereafter stands for mere ageing, and has no other innuendo. I shall use the word 'senescence' to mean ageing accompanied by the decline

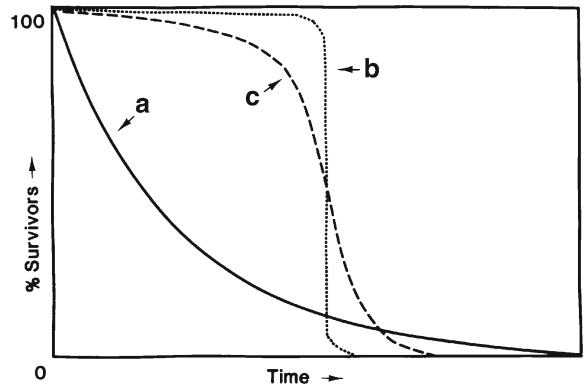

Fig. 13.2. Survival curves for organisms with different longevity patterns. (**a**) Survival curve at a constant rate of mortality (50% per unit of time). (**b**) Survival curve for populations with abrupt termination of all members by senescence (probable physiological senescence). (**c**) Survival curve of a population that exhibits increased susceptibility (demographic senescence) to death with age. (Redrawn from Pearl and Miner 1935.)

in bodily facilities and sensibilities and energies which ageing colloquially entails. "Senescence, may then be defined as that change of bodily facilities and sensibilities and energies which accompanies ageing, and which renders the individual progressively more likely to die from accidental causes of random incidence" (Medawar 1957, p. 55). Comfort (1964, p. 6) comes to similar conclusions: "Senescence is probably best regarded as a general title for the group of effects which, in various phyla, lead to a decreasing expectation of life with increasing age." and he draws heavily on graphic demographic representations. This leads us to the demographic concept of senescence which holds that senescence is simply an increased probability of dying with increased age.

Senescence is often intermingled with other manifestations of aging such as decreased fecundity (Comfort 1964; Finch 1990; Roach 2004). Fecundity is certainly important in shaping the evolution of senescence, but decreased fecundity does not per se cause senescence. On the contrary, developing reproductive structures induce senescence in many plants (see internal/correlative controls below in this section). In other words, it is important to distinguish the factors that actually cause senescence from those that influence the evolution of senescence.

Returning briefly to aging- in his wide-ranging magnum opus, Finch (1990) noted that "The term aging, like many words in ordinary usage, is haunted by assumptions that can bias scientific thinking." "Because many age-related changes have no adverse effect on vitality or lifespan, I avoid the word **aging** in this book. You won't miss it!". "Senescence is used mainly to describe age-related changes in an organism that adversely affect its vitality and functions, but most importantly, increase the mortality rate as a function of time."

These ideas formed the foundation of the subsequent thinking about senescence; however, those threads have sometimes been lost between generations. Nonetheless, I think all can agree that senescence is a progressive deterioration in functionality (changes in vitality with age), and this can render the organism more vulnerable to death or even cause death directly.

Survivorship Curves (Fig. 13.2) Illustrate Senescence. Here, curve a represents organisms where life is terminated mainly by external forces, accidents such as predation at a constant rate (Leopold 1961; Comfort 1964; Finch 1990; Roach 2004). Curve b (almost a right-angle survivorship curve) represents monocarpic organisms that die off rapidly,

almost simultaneously in large populations, after reproduction. Curve c (humans, trees and many polycarpic organisms) is somewhat intermediate. Pattern a is exogenously driven (e.g., by predation), whereas pattern b is endogenously controlled. Organisms displaying pattern b include soybeans, canola, maize, squids, marsupial shrews and some fish species; this degeneration leading to death is clearly active and endogenous (Molisch 1938; Comfort 1964; Noodén 1988b; Finch 1990). Endogenous factors (below in this section), no doubt, help to shape pattern c, but that is less obvious and not well known. Thus, plant physiologists have defined senescence in terms of endogenous processes, while the other groups have defined it statistically or demographically. Although the latter groups would like to find a physiological/molecular basis for senescence, they have not; however, they are gradually moving in the direction of seeing senescence as an active process (Murphy and Partridge 2008) or as endogenous forces of mortality (e.g., necrogenesis below).

Emergence of the Concept of Senescence in Plant Physiology – Leopold (1961) summarizes the growing realization in the plant physiology community that in most plant organs and many (monocarpic) whole plants life is terminated fairly rapidly by active processes under internal control. Thus, senescence was viewed as an endogenous process that causes the death of an organism or organs. This physiological view clearly moves beyond the demographic view of senescence and places an emphasis on causal processes. Presciently, Leopold (1961) also recognizes that organ/organism senescence has a lot in common with more localized cell death processes such as xylem cell differentiation (see Sect. III.F below).

Internal/Correlative Controls of Senescence – Although senescence may be triggered by exogenous signals, senescence (in the physiological sense) is an active, internally controlled process, and that is illustrated by correlative controls (the control of one part of an organism over another). For example, younger growing leaves may cause senescence of older mature leaves, or maturing fruits/seeds may induce senescence of an entire plant including the leaves (see Molisch 1938; Noodén 1980). Studies on hormonal-control systems and genetics also support the idea that senescence is under internal controls (Noodén and Leopold 1988; Woo et al. 2004). Since individual cells or organs are usually capable of living much longer and may even reinstate mitosis if excised and cultured, senescence must be imposed on them when they are left in their normal context (Fig. 13.1; Molisch 1938; Noodén et al. 2004). This applies to polycarpic as well as monocarpic organisms.

A New Terminology: Necrogenic(esis)/Biostatic(stasis) – Obviously, the term senescence is encumbered by its diverse usages, so a different terminology is needed to foster discussion of the processes that cause or prevent death. Once one accepts the idea that there can be internal controls of senescence/degeneration leading to death, it seems reasonable to think in terms of endogenous processes that cause degeneration leading to death (necrogenic) and endogenous processes that maintain life (biostatic) and counteract the necrogenic processes thereby delaying death. Maintenance is more stasis rather than genesis. These terms also have the advantage that their intrinsic meaning is easily recognized. Moreover, they offer a clearer framework for discussion of the molecular/physiological processes involved.

There is evidence for positive (i.e., necrogenic) and negative regulation of senescence from a variety of sources (old and new), but these stories are often complex. Although there are some examples coming out of molecular genetics (see Sect. IV.E below), this has been well-known for a long time. Clear, simple examples of each operating in plants would be the hormones ethylene (necrogenic, Mattoo and Handa 2004) and cytokinins (biostatic, van Staden et al. 1988; Noodén and Letham 1993).

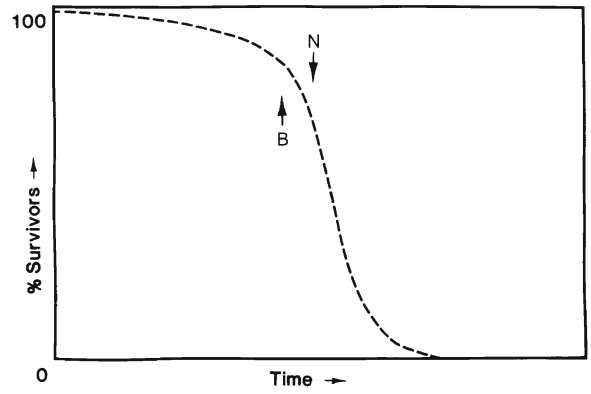

Fig. 13.3. Survival curve (derived from Fig. 13.2) for organisms that are more likely to die in the later years of their life-expectancy period. *B* (biostasis) represents processes that prolong life. *N* (necrogenesis) represents processes that cause death.

Figure 13.3 uses a survivorship curve to show how these processes work at the population level. Necrogenic processes (N) would shift the curve (survival) downward and to the left (shortening longevity), while biostatic processes (B) would shift the curve upward and to the right (lengthening survival). This illustration also helps to relate specific necrogenic and biostatic processes to the demographic view of senescence.

C. Aging: It's Biochemistry and Biological Expression

The Wear and Tear of Life – Given the conspicuous changes that organisms undergo with chronological aging in plants and other organisms, it seems important to elaborate a little further on the nature of these changes to provide a contrast to senescence (in the physiological sense). Sometimes, aging has already been described loosely as "wear and tear". If aging is the passive accrual of lesions with time, then exactly what are these lesions, which are most important and what drives/causes them?

Time-driven Changes in Molecular Conformation – Molecules, especially large organic molecules, are not static over time, and they may undergo spontaneous changes (albeit some are very slow) over time as they age chronologically. Slow as these are, some may be important in dormant seeds, but some may be fast enough to be significant in active cells. Over time, the amino acids in proteins may undergo passive changes including the conversion of L-aspartyl to L-isoaspartyl units (Mudgett et al. 1997), and proteins undergo conformational changes that alter their functionality (Golovina et al. 1997). Likewise, membranes change/rearrange spontaneously, for example the spontaneous migration of phosphatidylserine from the inner plasma (cell) membrane leaflet to the outer thereby randomizing its distribution (O'Brien et al. 1997).

Time-dependent Exogenous Forces – There are also active exogenous and even some endogenous forces that cause age-related deteriorative changes. Significant among the external forces would be the cosmic rays that continuously bombard life on Earth from sources on and off the Earth (Roberts 1988). Cosmic rays are ionizing radiation, and by this action, they cause lesions that cells are generally able to repair; however, sometimes, the cells do not keep up, especially in dry seeds where the repair enzymes cannot function. Of particular importance, ionizing radiation can cause breaks in macromolecules such as DNA and thereby chromosome breaks (Priestley 1986; Roberts 1988). Likewise, the reactive oxygen species in our atmosphere react with many biological molecules (Iriti and Faoro 2008).

Free Radicals as By-products of Living – The essential processes of life themselves may generate damaging free radicals (e.g., reactive oxygen species, ROS) that get loose within a cell and cause all sorts of damage. For example, mitochondria produce ROS (Balaban et al. 2005), but it is of particular importance here that chloroplasts can generate ROS during their normal activities (Zapata et al. 2005). This may cause oxidative damage that could be called aging in leaves (Munne-Bosch and Peñuelas 2003; see Khanna Chopra et al., Chap. 17 in this volume).

Seed Aging – A clear and simple example of aging in plants is the loss of viability over time in seeds whether they are in the soil (seed bank) or in optimized storage conditions for agriculture or gene bank preservation (Noodén and Leopold 1978; Priestley 1986; Noodén 1988a; Roberts 1988). For seeds, membrane damage and loss of membrane integrity seem to be the overriding lesions; however, surviving outgrowths may carry more mutations.

Short-lived (Tachy) Plants – Out in nature, there are many plants (tachyplants) with very short life cycles, and these seem to have "cheap, throwaway" bodies that are not built to last and are designed mainly to produce seeds quickly while conditions are favorable (Begon et al. 2006). In these cases, aging in the passive sense used here may play a significant role. For example, the leaves of short-lived plants such as *Arabidopsis thaliana* seem built to last only a relatively short time, ca 40 days for the main rosette leaves (Noodén and Penney 2001), in contrast to the leaves of many conifers which last 15 or more years (Molisch 1938, p. 100). Even though the Arabidopsis leaves seem to involve "low cost" construction with limited options for life extension, they do show senescence-associated gene expression patterns (Noodén and Penney 2001).

No doubt, aging processes contribute to cell, organ and organism decline and even death in many ways; however, these are difficult to identify in the complex milieu of chronological and developmental changes.

III. The Lexicon of Senescence: A Kaleidoscope of Processes and Nomenclature

A. Overview

The kaleidoscope is an appropriate metaphor here, because many processes seem to be interrelated/work together in the decline and death of organs and organisms. Furthermore, these processes and the acronyms used to designate them seem to get reshuffled in interesting ways both during natural development and conceptually as our understanding deepens.

Many related terms (Table 13.1) have emerged to represent processes that may be related to senescence and degeneration with age as well as various cell elimination processes. Although some were first applied to animal cells, they may also apply to photosynthetic organs/organisms, so they need to be considered here. The processes in this kaleidoscope are connected in various ways. It does seem important to recognize some of these linkages, but this is a work in progress.

B. Variations on the Theme of Senescence

There are many patterns/manifestations of senescence, and these may be designated with various modifiers such as leaf senescence, flower senescence, fruit senescence, monocarpic senescence and so on (Table 13.1). These different patterns of senescence have different functions, and it remains to be determined whether or not they differ mechanistically (see Sects. IV.D, IV.J and V below).

C. Programmed Cell Death (PCD): Apoptosis, Necrosis, etc.

Redefining senescence as a type of PCD – The use of the term PCD has been quite variable, especially with regard to senescence. In order to avoid confusion and unnecessary controversy, van Doorn and Woltering (2004) have made a wide-ranging and thoughtful review of this subject, and they conclude: "The confusion centres on the application of

the terms *senescence* and *PCD*: whether they describe similar events, distinct events or overlapping events." "Senescence in cells is the same as PCD and the two are fully synchronous". Thus, organ/organism senescence (at least in the physiological sense) is programmed (an active, internally-controlled) cell death; however, PCD is often viewed in a more restrictive sense focusing on the death of the individual or small groups of cells, and therefore, not all cases of PCD are seen as senescence (Sect. III.F below). PCD has often been equated with apoptosis (mostly in the animal literature), but it is now recognized that PCD may take many forms. Programmed cell death should be taken to designate what the term implies, internally driven or programmed processes causing cell death, no more, no less. PCD in all its forms is an important facet of development including senescence.

Table 13.1 lists the main forms of PCD, and generally, these are referred to by more specific names, apoptosis being a prime example. Three main cellular patterns of PCD, apoptosis, necrosis (oncosis) and autophagy, can now be defined in terms of cytological and biochemical characteristics (Table 13.1). Judging from cytology, these forms of PCD seem to occur in a wide range of eukaryotes, animals and plants, so we need to look at research with animal cells as well as plants. In spite of the appearance of conservation during evolution (Noodén 2004b), apoptosis appears to differ substantially at the molecular level between animals and plants (Koonin and Aravind 2002), perhaps most notably in terms of the role of the key endoproteases (e.g., caspases vs. metacaspases). Regardless, the biochemical nature (primary components) of the plant PCD processes needs to be better defined. These PCD processes serve a wide variety of functions, and sometimes, it seems that they can substitute for one another depending on conditions.

Although necrosis (oncosis) is often caused by acute trauma or stress (exogenous), it can also be induced by endogenous factors (Proskuryakov et al. 2002), so it also qualifies as programmed cell death. Oncosis (swelling due to electrolyte leakage/loss) is a better descriptor of this cell death pattern; however, necrosis is now more widely used. Apoptosis requires ATP, whereas necrosis does not if caused by trauma (Nicotera et al. 1999).

The nature and role(s) of autophagy (see also Wada and Ishida, Chap. 19) are more complicated, and it may be involved in intraorganism resource recycling more than in senescence (Sect. III.F below). Kroemer et al. (2009) note: " 'Autophagic cell death' is morphologically defined (especially by transmission electron microscopy) as a type of cell death that occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm." "Although the expression 'autophagic cell death' is a linguistic invitation to believe that cell death is executed by autophagy, the term simply describes cell death with autophagy". Nonetheless, autophagy may sometimes function in cell death (Bassham 2009).

No doubt, other forms of cell degeneration leading to death will be found. Indeed, there are some patterns, e. g., PCD types IIIa and IIIb (Noodén 2004b, p. 6) that do not fall neatly into these three patterns. In addition, several esoteric cell death modalities have been described in studies on animal cells (Kroemer et al. 2009), and they may apply to plants. These include excitotoxicity, karyorhexis, mitotic catastrophe, paraptosis and pyronecrosis.

In any case, PCD nomenclature is undergoing reevaluation in both plants and animals, but ultimately, this depends on knowing the causal (primary) primary processes.

D. Other Phenomena Within this Kaleidoscope

Mitotic (Cellular) Senescence – An important set of issues surround the phenomena designated as mitotic (cellular) senescence. In the animal literature, these stem from observations that excised, cultured cells often seem to be limited in the number of mitoses they can undergo (Finch 1990), but

that is generally not true for plant cells (Molisch 1938; Noodén et al. 2004). While it is true that the cells in a plant organ/organism stop dividing early in senescence, this seems to be due to their context rather factors intrinsic to the cells themselves. For example, individual cells or shoots/roots that have ceased growing/mitosis usually can resume when excised and cultured under proper conditions (see correlative controls in Sect. II.B above).

Cessation of mitosis, growth and regeneration may not in itself cause death; however, this cessation is necessary for senescence, i.e., necessary but not sufficient (Noodén 1980, 1988a; Gan 2007). For example, organs such as leaves and whole organisms may continue to live and function robustly for some time after ceasing cell division and growth; however, once senescence is triggered, they usually decline and die quickly. On the other hand, as long as mitosis and regeneration continue, the organ or organism is not going to die. For example, polycarpic organisms undergo a slow decline leading to death, and decreased regeneration may be an integral/causal part of that process (see discussion of clonal organisms below under negligible senescence).

Negligible Senescence? Is this possible? This descriptor is generally applied to clonal organisms (Vaupel et al. 2004), and the validity of its application to unitary organisms is not clear. All plants, including clonal plants, undergo senescence and turnover of their parts. Generally, clonal plants do seem to be able to maintain themselves indefinitely, and this depends on continued growth and replacement of old units (ramets, see Noodén 1988b).

Despite these doubts about negligible senescence at the organ level, organisms specifically clonal organisms may qualify as negligibly senescent. The ramets and their organs may senesce, but the clonal "organism" may escape senescence by continual replacement of the ramets. Here, a distinction needs to be made between unitary organisms, clonal organisms and genets. Unitary organisms (what we normally think of as an organism) have a finite body plan and usually a limited (determinate) growth potential, while clonal organisms are relatively unlimited (indeterminate growth), producing numerous shoots (ramets) which may remain connected or may separate. Once the ramets detach, they are separate (unitary) organisms. These separated parts which share a common genetic identity would still belong to the same genet, just not the same organism. There certainly are some very remarkable examples of very old genets both in nature, e.g., quaking aspen and creosote bush (see Noodén 1988b), which are tens of thousands of years old and cultivars such as Cabernet Sauvignon wine grape which has been repeatedly subcultured from cuttings for over a 1,000 years, apparently without loss of identity (see Noodén et al. 2004). These clones are of interest for their own sake, but these separated fragments (ramets) do not constitute an organism any more than human identical twins are one and the same organism. Furthermore, the ramets of these clones behave more like separate organisms even if they are still attached. Nonetheless, clones may experience declines, even senescence. Some clones such as sugar cane and raspberries may decline, possibly due to low-level pathogen build up. Some clonal plants such as bamboo may grow vegetatively for 30–120 years depending on the species, and then, the whole population of flowers, does senesce and dies (Noodén 1988a).

Negative Senescence? Because the idea of negative senescence seems counterintuitive, it warrants some explanation. The idea that senescence can be reversed is based mainly on demographic/statistical observations, and it remains hypothetical (Vaupel et al. 2004); however, young mature organisms clearly have a lower mortality rate than juveniles (Comfort 1964; Finch 1990). Is this a case of internally-programmed negative senescence, or is it simply a reflection of decreased risk of predation and other life terminating disasters?

E. Relationship Between Senescence and Aging

To what extent is there a linkage between senescence and aging as defined here? It has been widely assumed that aging is necessary to make cells, organs, etc. vulnerable to senescence, and sometimes, the decline in functionality with aging does seem to make them more vulnerable, but senescence and PCD may be imposed on functioning cells organs independent of age. For example, younger leaves may die before older ones in nonsequential senescence of leaves, correlative controls may override age and excised leaves or shoot cuttings often live far longer than their counterparts left intact in their organismal context (Fig. 13.1 and correlative controls and culture of excised parts in Sect. II.B above). The idea that chronological age is the overriding determinant in longevity is simply untenable.

F. Where Do Cellular Death Processes Such as Apoptosis Fit into Organ/Organismal Senescence?

Senescence is a programmed cell death process, but all occurrences of PCD are not necessarily senescence. Seen from the organ and organism perspective, commonality between organ/organism senescence and the cellular death processes such as xylem cell differentiation were recognized long ago (Leopold 1961); however, researchers focusing on these cell death processes often enter this field with a more cell-molecular perspective, and they see these processes primarily as part of a cell-differentiation or a disease-resistance process.

Nonetheless, cellular processes such as apoptosis do have a role in the death of organs and organisms (Sect. III.C above). Various cell death processes seem to be the "end game" in organ senescence (Noodén et al. 1997; Gray 2004; Gan 2007); however, sometimes, that final cell death might be more like necrosis (oncosis) or yet another form of cell death.

Given the reclamation (breakdown and redistribution) of resources that often occurs in senescing organs, one might expect autophagy (self eating) to play an important terminating role in senescence, but that is generally not true. Indeed, the participation of autophagy is quite complicated. For example, mutations blocking autophagy seem to accelerate senescence more than they inhibit it (Krupinska 2007; Bassham 2009). Nonetheless, the accumulation of membrane whorls in senescing cells suggests that autophagy operates in some senescing organs (see Noodén 1988a). The fact that the number of chloroplasts decreases during senescence in some circumstances, but not others (Gepstein 1988; Krupinska 2007) also indicates that a process like autophagy may occur in some senescing cells, but not necessarily in all.

IV. Defining Senescence in Cellular and Molecular Terms

A. Overview

The dramatic deterioration followed by death in many plant organs and whole plants was once considered to be a collapse of internal order; however, the advent of electron microscopy revealed that this process was in fact quite orderly, and a lot of subcellular structure is preserved even in the midst of many degenerative changes (Butler and Simon 1971; Biswal and Biswal 1988). Long before this, senescence of plant organs and monocarpic plants had been demonstrated to be under endogenous control independent of age (Molisch 1938), and later, it was shown to be an active process, i.e., an endogenous program (Noodén and Leopold 1978, 1988; Thimann 1980; Thomas and Stoddart 1980; Gray 2004; Gan 2007). Many questions, even very basic questions about this process, still need to be resolved. Therefore, this section will be presented as a series of questions.

B. Is Senescence Defined by Remobilization/Redistribution of the Resources (Especially Nitrogen) Invested in the Senescing Organs?

This definition certainly has been a common practice. It is also an outgrowth of the old exhaustion death idea (Molisch 1938). Given the amount of degradation, especially in the chloroplasts (which are the major repositories of protein, Mae 2004), and the exodus of the nutrients from senescing leaves, the prominence of the exhaustion idea is not surprising. Nonetheless, depletion generally does not seem to be the cause of death (Sect. VI below; Noodén 1988b; Noodén et al. 2004). For example, petals may lose most of their protein before reaching their functional maturity, and the hypersensitive response including PCD can be imposed on leaves with no reclamation (Heath 2000; Noodén et al. 2004, p. 12). Furthermore, soybean leaves can be spared from death by pod removal even after they have lost most (ca. 90%) of their protein (Okatan et al. 1981). Moreover, fruit-induced senescence occurs in soybean leaves even when nutrient withdrawal is blocked by steam girdling of the petiole (Wood et al. 1986).

C. Is Senescence (Death) Caused by/Defined by Chloroplast Breakdown/Chlorophyll Loss?

The answer appears to be no, but chloroplast parameters are nonetheless very important in photosynthetic tissues.

First, causality – It is implicit above that chloroplast breakdown does not cause death. Evidence comes from numerous observations that many variegated tissues (e.g., variegated leaves) and mutant organisms (e.g., yellow, *xantha*) survive and develop quite well as long as they have an energy supply (Noodén 1988c). Likewise, grafting nonphotosynthetic yellow (*xantha*) mutants onto normal green plants as nurse plants allows the *xantha* segments to grow, bear fruit and undergo monocarpic senescence (Noble et al. 1977).

Second, measurement – Chlorophyll loss and other chloroplast parameters are used extensively to measure senescence (Sect. IV.G below), and they certainly are components of the senescence syndrome (Sect. I.B above). In as much as chloroplast breakdown may be part of a regulatory network, it could mark senescence of photosynthetic tissues with the caveat that some stay-green mutants still senesce (Guiamét et al. 1991; Thomas and Howarth 2000).

Third, the timetable – The decline of photosynthesis and changes in ultrastructure of chloroplasts (e.g., plastoglobule formation) are among the earliest manifestations of the senescence syndrome (Thomas and Stoddart 1980; Biswal and Biswal 1988; Gepstein 1988; Noodén 1988a; Krupinska 2007).

Fourth, the importance – The *decrease in photosynthesis* reflects a decrease in assimilation and that translates into curtailment of yield production (e.g., seeds, fruits, etc.) and biomass. Definitions aside, chloroplast breakdown is the most important facet of the senescence syndrome economically.

D. Is Senescence in Leaves the Same as in Other Organs?

Senescence occurs in leaves, petals and other plant organs (Molisch 1938; Noodén and Leopold 1978, 1988; Thimann 1980; Thomas and Stoddart 1980; Gray 2004; Gan 2007). Is leaf senescence the same as that of other organs; can information be transferred among these different systems? The book "Senescence and Aging in Plants" (Noodén and Leopold 1988) brings together information about these different patterns, on the expectation that there is some commonality while recognizing that sometimes there are some special differences.

Just as different organs may contain different cells with different structures, the ultrastructural changes accompanying senescence may differ (Butler and Simon 1971; Mayak and Halevy 1980; Biswal and Biswal 1988; Mulisch and Krupinska, Chap. 14).

Still, there are some common features, e.g., the mitochondria last until very late.

Comparisons of transcriptomes and other measures of gene expression demonstrate that expression of a very wide range of genes increases (and of some decreases) during senescence (Andersson et al. 2004; Jones 2004; Buchanan-Wollaston et al. 2005; Gregersen and Holm 2007; Price et al. 2008). The expression patterns of these senescence-associated genes (*SAGs*) may differ depending on the organ and the trigger of senescence; however, there are also some similarities. Since some of these *SAGs* can be knocked out by genetic engineering without blocking senescence (e.g., SAG12, Otegui et al. 2005), not all *SAGs* are causal.

E. What Are the Causal/Primary Components of the Senescence Process at the Cell Level?

As discussed above (Sect. I.B), many changes (senescence syndrome) occur during senescence, but very likely, only a few cause death (a causal or primary role), while the others are simply supportive or secondary (a result of, not a cause of senescence). For example, the breakdown of the chloroplast and the processing of released nutrients are not causal changes (Sects. IV.B and IV.C above); however, free radicals escaping from senescing chloroplasts may contribute to leaf senescence (Zapata et al. 2005) as do rising sugar concentrations (Paul and Foyer 2001; Noodén et al. 2004). Likewise, the removal of the protoplast remnants that occurs in dead xylem and some other cells (Morgan and Drew 2004; Obara and Fukuda 2004) is often invoked as causal in cell death; however, this seems more like a postmortum cleanup rather than the death process itself. Senescence generally seems to end up with a cell death process (Sect. III.F above); however, targeted plant cell death programs may take several forms (Vuosku et al. 2009). In any case, collapse of the plasma membrane marks death and seems to be the culmination of senescence (Sect. VI.B below).

Senescence is imposed on chloroplasts and other cell components by their attending nucleus (Noodén 1988c). Why should genes and their expression be implicated as primary drivers of senescence? *First*, it seems important to recognize that even though gene expression (e.g., RNA and protein synthesis) greatly declines during senescence, synthesis of some proteins and RNAs does increase (Brady 1988, also noted above in connection with transcriptomes). *Second*, senescence is not simply a shutdown of protein and RNA synthesis, for selective inhibitors of protein/RNA synthesis and enucleation inhibit senescence (Noodén 1988c). Thus, the protein and RNA synthesis (albeit reduced) that occurs during senescence have vital roles on the necrogenic side. *Third*, numerous mutations have been shown to retard the senescence syndrome (especially parameters related to photosynthesis), and a few have been shown to increase longevity (Noh et al. 2004; Woo et al. 2004).

While the gene expression studies show correlations between the upregulation of many genes and senescence, these simple correlations do not themselves constitute evidence of causality; more is required (Noodén et al. 2004). This problem and its solutions are well illustrated by Koch's postulates, which deal with determining whether or not a particular pathogen (i.e., causal agent) causes a disease. Not only should the presence of the pathogen correlate with disease symptoms, but removal and replacement of the putative pathogen should correlate with the absence and restoration of symptoms. In other words, some experimental testing/probing is required to demonstrate that the gene or process in question is essential for senescence. In the case of senescence, many such tests have been done with hormones (see Noodén and Leopold 1988) and correlative controls (see Molisch 1938; Noodén 1980), and increasingly for gene expression.

Both genetic engineering and screening of mutant phenotypes provide evidence for gene control of senescence, but this picture seems quite incomplete (Noh et al. 2004; Woo et al. 2004). The nature of the genes that act on senescence is very diverse; however, they include transcription factors such

as WRKY53 (Zentgraf et al. 2010), many genes acting on hormone systems (Noh et al. 2004) and others (Woo et al. 2004). Mutants that induce or accelerate senescence are difficult to assess, because they may act by disrupting necessary processes unrelated to senescence. Genes not only induce senescence, but some may actually inhibit it, i.e. negative regulation, biostasis in Sect. II.B above (Zentgraf et al. 2010). Some of these senescence-inhibiting genes are upstream transcription regulators and others control cytokinin synthesis. Likewise, many repair processes would be life-supporting (biostatic).

F. Is Senescence a Single Channel, a Braided Channel or a Network Process?

Is senescence a single channel or a braided stream with splitting and anastomosing channels (Noodén 1988c) or a regulatory network? Are there several routes to the same end result? At this time, given the uncertainty about the nature of the senescence processes, these questions cannot be answered definitively; however, they need to be considered as our understanding of senescence develops.

These questions are complicated by the need to deal with both necrogenic and biostatic processes (Sects. II.B and IV.E above); however, this can be simplified. While biostatic processes are important over the long term, they may not be overriding, at least in the short term, for enucleation and selective inhibitors of protein and RNA synthesis generally inhibit senescence rather than promote it (Noodén 1988c).

Insights leading to answers can come from mutations (Woo et al. 2004). If senescence were a single channel, then one would expect monogenic ("master switch") controls as contrasted to polygenic controls. There is a notable absence of master-switch regulatory genes that control all of senescence and prolong life at the organism or even the leaf level. *d1d2* (possibly ancient duplicated genes) in soybean seem to come close to being monogenic master-switch genes. They produce a stay-green phenotype in soybean and preserve the photosynthetic apparatus, but do not prevent leaf abscission; the turgid green leaves are shed (Guiamét et al. 1991). Likewise, *det2* (a block in brasinosteroid biosynthesis in Arabidopsis) (Fujioka et al. 1997) may prevent senescence/delay leaf death and consequently prolong the life of the plant (Noodén and Penney 2001). Most other senescence mutations seem to act more narrowly and do not produce complete blockage of senescence. Furthermore, most are characterized solely in terms of chlorophyll loss and photosynthetic parameters, which are not causal (see Sects. IV.C above and IV.G below). Even some senescence associated transcription factors (e.g., WRKY53) are not absolutely essential for senescence/death (Zentgraf et al. 2010). The paucity of monogenic master-switch genes suggests that senescence may be a braided channel or a network, and that raises interesting questions about weighting, i.e., which components are more important/have more impact?

Longevity, senescence and even more focused cellular processes such as apoptosis surely have complex (multiple gene) controls. Extensive studies on other processes have demonstrated polygene control of complex and quantitative traits and this has been explored in terms of quantitative trait loci for longevity (Luquez et al. 2006). In other arenas, the idea of control networks is gaining supporting evidence (Costanzo et al. 2010), and studies on yeast are showing the way. The difficulty in assessing the functional weight of any single gene, i.e., how much if any impact any single deletion has, is illustrated in yeast where 80 % of all gene deletions show no visible effect on growth under favorable conditions (Hillenmeyer et al. 2008). In the case of apoptosis, shared protein domains associated with apoptosis suggest interaction networks that may eventually reveal some regulatory networks (Koonin and Aravind 2002), and the same may hold for senescence.

G. Are There Good Markers or Measures of Senescence?

If the biochemical/physiological components of the senescence process can not be identified,

are there any unequivocal markers or measures of the senescence process? The brief answer is no; that requires knowing exactly which genes/processes are causal (Sects. IV.E and IV.F above); however, the end result death can be a definitive measure of the end point. Nonetheless, there are useful parameters, such as chlorophyll loss, for measuring senescence (Noodén 1988a), and they are probably fine as long as their limitations are recognized.

Chlorophyll Loss – Certainly, chlorophyll loss is the most prominent manifestation of the senescence syndrome, and it is the traditional measure of senescence in leaves and other photosynthetic tissues. In fact, it has been so widely used that it is a point of reference, and new measures must be compared with it even though it is not a primary component of senescence (Sect. IV.C above). Furthermore, chlorophyll and other photosynthesis parameters reflect primary productivity, another strong reason to include them. Significantly, many disjuncts may occur including stay-green mutants that disconnect chlorophyll loss from the rest of the senescence syndrome, i.e., senescence can proceed in stay-green mutants (Guiamét et al. 1991; Thomas and Howarth 2000). Breakdown of total protein, most of which is located in the chloroplasts (Mae 2004), falls into the same category (chloroplastic parameters) as chlorophyll breakdown.

Those studying flower/petal senescence have long objected to the use of chlorophyll breakdown to represent senescence, and they have favored wilting and cell leakage (Mayak and Halevy 1980); however, these occur very late, too late, in most leaves (Okatan et al. 1981; Guiamét et al. 1990) to be useful measures of senescence. In fact, wilting and leakage may be better measures of death (Sect. VI.C below).

On the transcriptome and genetic side, senescence-up-regulated genes such as *WRKY53* and many others seem very promising; however, they will not be quick, visible measures like chlorophyll can be. In support of the use of chlorophyll breakdown and other noncausal components of the senescence syndrome, they may be part of the same regulatory network and under the same controls as causal components. This remains to be determined.

Normalizing Data for Comparison – Regardless of the measure used, it is important to normalize the data using an appropriate reference point. Many parameters including chlorophyll, total protein and total RNA (mostly rRNA) decrease precipitously during senescence (Brady 1988), so samples to study the time course of senescence should not be normalized based on these parameters (Noodén 2004b). For example, if mRNAs from different stages of senescence are normalized on the basis of total RNA, they may appear to increase even if they are in fact decreasing on a per cell or fresh weight basis. Identifying regulation patterns and regulatory networks requires accurate identification of up- and down-regulated genes. Most older data normalized on an RNA or protein basis can be reevaluated and reconciled if this problem is understood.

H. Can Senescence Be Defined by Irreversibility and Points of No Return?

Senescence has sometimes been defined as those degenerative changes occurring after the point of no return (reversibility). Irreversibility may sound good, but does it help to identify senescence or is it even a useful measure of senescence? In the case of leaves, reversal of the senescence syndrome can be achieved quite late (ca. 90 % loss of chlorophyll and protein) (Mothes and Baudisch 1958; Thomas and Donnison 2000; Van Doorn and Woltering 2004).

Quite a lot of attention (mostly, but not entirely, with animal cells) has been directed at the first irreversible step or "point of no return" leading to death (Kroemer et al. 2009). This includes massive caspase (endoprotease) activation and several well-defined membrane changes including leakage (Sect. VI.B below). Since these relate to basic properties of eukaryotic cells, they

seem likely to apply to plant cells. These steps (i.e., massive endoprotease activation, loss of $\Delta\Psi_m$, complete permeabilization of the mitochondrial outer membrane or exposure of membrane phosphatidylserine residues) could also be viewed as markers for the processes leading to death. Even though all of these seem to be powerful forces, they may not be lethal, at least not any one operating by itself. For example, cells can recover from the massive endolytic protein cleavage sometimes described as death by a thousand cuts (Vaughan et al. 2002). Likewise, dissipating $\Delta\Psi_m$ with protonophores does not prevent progression to cell death (de Graaf et al. 2004). Furthermore, there is no reason to believe that earlier (reversible) degenerative changes are not part of the senescence process, and the future may well bring techniques for even later reversals. Thus, reversibility seems to be neither a good practical nor a good theoretical criterion for senescence.

I. Nonsenescence Processes – Are All Physiological Declines Senescence?

Not all metabolic/physiological declines represent senescence, and this has been a source of confusion/unnecessary controversy (Noodén 1988a).

Diurnal Rhythms – Most will agree that the diurnal rise and fall of photosynthetic capacity related to diurnal rhythms in leaves (Lumsden and Millar 1998) is not senescence even if some of the biochemistry is similar.

Sink Effects/End-product Feedback Inhibition – End-product feedback inhibition of photosynthesis and other assimilatory processes may be intertwined with leaf and whole plant senescence, but decreases in photosynthesis due to end-product accumulation have sometimes been equated with senescence (see Noodén 1988a, b). End-product inhibition of photosynthesis has been known for a long time (Paul and Foyer 2001). Unfortunately, the decrease in photosynthesis in depodded/desinked soybeans and other plants has been considered senescence even though these plants outlive their intact/pod-bearing counterparts. Normally, foliar chlorophyll would break down, nitrogen would be transferred out and photosynthesis would decrease during pod development; however, pod removal blocks the loss of chlorophyll and nitrogen but not the drop in photosynthesis, and it does not reinstate growth (Okatan et al. 1981). These depodded plants are green and robust in appearance, i.e., they have not senesced. Nonetheless, end-product feedback may also contribute to the decline of photosynthesis in normal senescing leaves where sugar concentrations often rise (Noodén et al. 2004).

J. Is Senescence the Same at the Cell, Organ and Organism Levels?

Probably, both yes and no. This is quite a different question from "Is senescence in leaves the same as in other organs?" in Sect. IV.D above. It is clear that cellular death processes do occur in senescing organs and organisms (Sect. III.F above); however, senescence in multicellular systems also seems to involve some higher-level processes. Just as organs are made up of diverse specialized cells that function coordinately, organisms are composed of diverse organs that must work together in a functioning whole. In other words, in multicellular systems, there are essential functions above the cell and organ levels, and these needed functions, not just the constituent cells, break down during senescence. For example, gas exchange (O_2 release, CO_2 uptake and H_2O loss) in leaves and internal transport in whole plants all decline during senescence (Noodén 1980; Zamski and Schaffer 1996). Likewise, in whole plants, hormone-signaling systems such as production and translocation of the cytokinin antisenescence hormones decline (Van Staden et al. 1988; Noodén and Letham 1993).

Heterogeneity within organisms and organs: It is important to recognize that not all components of these multicellular

systems decline at the same time (see Noodén 1988a, p. 32). For example, in leaves the guard cells and the vascular system last longer, and the photosynthetic tissues, e.g., mesophyll, senesce sooner. This difference is probably related to the need to recover valuable nutrients, e.g., nitrogen, efficiently from the photosynthetic tissues, which are in fact the major depots of these nutrients (Mae 2004).

V. Defining Whole Plant Senescence

Monocarpic and Polycarpic Senescence – Since monocarpic and polycarpic senescence display some significant differences (Sect. II.B above), they need to be discussed separately. This does not exclude the possibility that both patterns share some basic biochemical/physiological components. The survival curves in Fig. 13.2 illustrate the results of these differences very well, i.e., monocarpic plants (Curve b) senesce abruptly/precipitously, while polycarpic plants (Curve c) decline gradually. Nonetheless, both undergo senescence in the demographic sense. Another prominent difference is in the correlative controls which usually control senescence in monocarpic plants and not in polycarpic plants (Sect. II.B above). Numerous older ideas about the limitations on longevity/causes of death in both monocarpic and polycarpic plants such as simple aging, wear and tear and size are discussed elsewhere (Noodén 1980). Many of the considerations/questions applied to senescence at the cellular level (Sect. IV above) also apply at the organismal level. In biochemical/physiological terms, monocarpic plants are much better known, so that seems like a better starting point here.

Global (Organism) vs. Organ Failure – Before getting into the physiological details, it seems important to step back and ask some larger picture questions about whole plant senescence, i.e., do plants die from a global failure or a key organ failure (Rosen 1978)? While leaf senescence is certainly a highly visible and likely a key element in monocarpic senescence, the root system also degenerates and the stem displays visible as well as functional changes (Noodén 1980).

Regarding Organ Failure – If monocarpic plants, e.g., soybean, die because the leaves are failing to supply enough photosynthate, then why do the leaves of senescing plants often undergo increases in their sugar contents, and why do foliar applications of sucrose solutions fail to delay monocarpic senescence (Lindoo and Noodén, unpublished data 1977; Noodén et al. 2004)? Apparently, photosynthate demand generally decreases faster than the supply. Although the longevity of organs and even large segments of plants can be extended by excision and culture (Sect. II.B above), this may result from taking them out of a failing organism, e.g., global failure. Certainly, mutations blocking leaf yellowing or abscission in soybean do not save the plant (Guiamét et al. 1991).

Regarding Global Failure – It has been proposed that whole plants operate as a balanced system somewhat like the just-in-time practice used in manufacturing (Bloom et al. 1985). Indeed, monocarpic plants undergo a fairly universal decline in their functions, although their sequence (relative timing) may vary a bit (Molisch 1938; Noodén 1980, 1988c). Which among these many changes are causal? As in the case of senescing cells (Sect. IV.E above), the answers are not definitive. Although chloroplast breakdown and the loss of photosynthetic activity are certainly important for other reasons, this does not cause cell death (Sect. IV.C above). Likewise, cessation of growth/regeneration does not itself cause death (see mitotic senescence in Sect. III.D above). The long-dominant explanation of monocarpic senescence holds that the developing fruits kill the plant by draining/exhausting its life-sustaining resources (Molisch 1938); however, there is quite a lot of evidence against this idea including accumulation of carbohydrate in senescing leaves (Noodén 1988b; Noodén et al. 2004). Even though it occurs late, there is a widespread breakdown of internal and

external permeability barriers, e.g., (vital dye exclusion, Artis et al. 1985) and failure of the root system to exclude Al^{+3} and Na^{+} (Noodén and Mauk 1987). The wilting that occurs late in monocarpic senescence in many species (Miller 1938; Noodén 1980, 1988b) may also reflect changes in plasma-membrane permeability. Certainly, excessive water loss can cause death (Miller 1938; Hirt and Shinozaki 2004). On the biostasis side, shutting down of life-supporting (biostatic) processes, for example decreased cytokinin production, may lay the foundation for death (van Staden et al. 1988; Noodén and Letham 1993). Thus, it is tempting to speculate that a global failure may cause death in monocarpic plants, but that needs to be clearly established.

Polycarpic Plants present a less clear picture of their senescence in terms of their physiology/molecular biology (see Noodén et al. 2004). Unlike monocarpic plants whose reproductive structures/fruit usually control their senescence, polycarpic plants usually do not have this link to the reproductive structures (Noodén 1988b). Since polycarpic plants do show demographic senescence (Fig. 13.2), it seems likely that there are some underlying biochemical/physiological causes; however, it is not clear how these factors are involved. Many physiological processes decline on a whole plant basis during polycarpic senescence, but decreased growth/regeneration (diminished biostasis) seems to be an important component. It may be that other life-supporting (biostatic) functions are affected when cell/organ replacement lags. It may also be that weakening of their regeneration/repair support systems makes them more vulnerable to stress which can precipitate a fatal crisis. Therefore, it is of particular significance that regeneration/repair is suppressed (Sect. II.B above). Looking at polycarpic senescence in terms of longevity, it certainly is clear that longevity is a genetic characteristic even if it is a complex characteristic (Molisch 1938; Noodén 1988b); however, it is not clear how this is accomplished, e.g., greater stress resistance, more durable construction, better regeneration/repair or something else. Thus, it is easier to identify the biostatic processes (sustained renewal and repair) than the necrogenic processes in polycarpic plants.

VI. Defining Death

A. Overview

Even though senescence is commonly viewed as functional degeneration leading to death, surprisingly little effort has been made to define death or record it when it occurs in senescence studies. Since one of the key characteristics in defining life is the ability of cells/organisms to maintain homeostasis (i.e., maintain a constant, stable internal environment that is separate and different from the ambient environment), it seems reasonable to define death as a collapse of homeostasis (Noodén 1988a). Still, it is necessary to translate this concept into biochemical specifics. The processes that cause death are not synonymous with and do not necessarily define death as opposed to dying. For example, PCD represents processes of dying and leads to death, but the cytological characteristics of PCD do not define the end point, death itself. Nonetheless, knowing precisely what death is should facilitate determination of which facets of the senescence process are actually causal, i.e., reverse engineering.

The death phenomena seem a bit different at the cell and organismal levels, so these need to be considered separately.

B. Cell Death

Maintenance/retention of the valuable cell contents necessary for life is essential for life as is exclusion of ambient interfering chemicals. As noted above (Sects. IV.E and V), electrolyte leakage is a late feature of senescence, and it may reflect the death of the cell. On a practical basis, penetration of nonpermeating dyes such as trypan blue or sulfonfluorescein diacetate (SFDA) (see next section below) does mark massive plasma membrane leakage and the break-

down in this last line of defense for homeostasis. This phenomenon of dye exclusion/penetration is an indicator of cell viability/inviability in studies on aged seeds or cold-stressed leaves (Priestley 1986; Yamori et al. 2006). Thus, the plasma membrane is central to maintaining life, and collapse of its functions seems like the final event marking death (Kroemer et al. 2009).

C. Measuring Cell Death/Viability

Several important broad scope changes in enzyme activity have been used as measures of cell viability and thereby as markers of death. In aged seeds, it is of great economic importance to know whether or not they are still viable (germinable), and very good procedures have been developed for that purpose (Priestley 1986). A widely used method employs phenyltetrazolium chloride (TTC), which is reduced by dehydrogenases to an insoluble, nondiffusible pigment in living cells, while lack of pigment is taken to signify nonviability or death. TTC has some limitations (Yamori et al. 2006); however, a staining procedure involving SFDA which is converted by esterases works very well in leaf cells (Yamori et al. 2006). These broad measures of metabolic activity provide useful measures of cell viability, but they may not define death. Intracellular ATP concentrations and energy change (metabolic indicators) are certainly necessary for many cell processes, but when the supply of ATP is inadequate, cells simply switch from apoptosis to necrosis (Nicotera et al. 1999). Thus, ATP concentrations may likewise not define death. The final step in dying and loss of homeostasis seems to be a breakdown in plasma membrane function/integrity thereby equilibrating the cell contents with the ambient environment, so the permeability measures discussed above seem to be the best.

D. Irreversibility as a Criterion for Cell Death

Irreversibility is widely invoked as a criterion for death as it is for senescence (Sect. IV.H above). Since reconstructing cells from their molecular constituents is approaching reality (Petit 2010), irreversibility simply marks that point beyond which we do not yet know how to reverse senescence or death. Irreversibility is important but not a good criterion for death.

E. Recommendations of the Nomenclature Committee on Cell Death (NCCD): Classification of Cell Death

The NCCD (a committee representing the journal Cell Death and Differentiation) reviews the nomenclature and publications relating to cell death and publishes updates periodically (Kroemer et al. 2009). Although they deal primarily with animal cells, most of these findings likely apply to other eukaryotic cells including plant cells. Their conclusions on "When is a cell 'dead'?" are: "the cell has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes, e.g. PI (propidium iodide) in vitro. Other criteria such as the removal of cell corpses are listed, but they do not define death per se. Phagocytosis of cell remnants is a post-death cleanup process. Furthermore, cell death may occur without formation of apoptotic bodies in necrosis or without DNA cleavage (laddering) or cytochrome c release under many circumstances (Noodén 2004b; Kroemer et al. 2009). Just as enucleation does not cause (but inhibits) senescence (Sect. IV.E above), DNA cleavage, chromosome condensation and elimination of the nucleus do not cause death in developing sieve tube cells (Wang et al. 2008), so these processes may not even be central to senescence or programmed cell death, i.e., not necrogenic. The loss of integrity of the plasma membrane seems to be the critical final step ending life.

F. Organism Death

Organism death is simply not synonymous with cell death; it involves higher level processes. Of course, an organism will die if its constituent cells die; however, an organism is more than the sum of its constituent cells

(e.g., it has emergent properties that are essential). The cells that make up an organism must function coordinately forming a whole-organism system, and failure of these higher-level functions could lead to organismal death. Several aspects of organism death need to be considered.

Three General Considerations – First, does every last cell in an organism need to be dead in order for the organism to be considered dead? No, long ago (Doflein 1919), it was realized that some specialized cells persist in life long after the organism carrying them is dead. *Second*, is irreversibility a useful or even valid criterion for death? The problems with employing irreversibility at the cell level (Sect. IV.H above) also apply at the organism level (Sect. VI.D above). *Third*, in Sect. II.B above, we established that constituent parts of an organism are capable of vastly outliving that organism if they are excised and cultured separately. *In other words*, the parts of an organism die because they are part of a system that is collapsing/dying or they are signaled to die.

The Collapsing System – What are these system properties whose loss leads a whole organism into death? They appear in whole plant senescence (Sect. V above). *Permeability barriers* are essential to homeostasis, and their breakdown can be demonstrated in whole plants (Artis et al. 1985). This breakdown of the exclusion barriers in the whole plant is also reflected in the increased Al^{+3} content of the xylem sap flowing up through a plant during late senescence (Noodén and Mauk 1987). *Electrical conductivity*- In a by-gone era, there was great interest in the electrical properties of plants and their parts. Of particular significance is Bose's observation (1927) that the end of life for a plant is marked by an electrical spasm (a surge in conductivity due to electrolyte leakage?). Does this leakage alone kill the plant or is it part of a broader syndrome? *Water balance and internal transport* stand out as particularly important in organism death. Interestingly, the guard cells remain robust in leaves until quite late in senescence thereby helping to maintain leaf water potential (Ψ_w) up to abscission; however, transpiration (and photosynthesis) decrease sharply before Ψ_w in soybean leaves (Guiamét et al. 1990). It is difficult to appreciate the role of water relations in the death of plants senescing under ample water conditions; however, water deficits could still develop in specific parts as internal transport shuts down. Although soybeans generally shed their leaves before their water potential drops (Guiamét et al. 1990), other plants such as Arabidopsis retain their leaves and they wilt (Hensel et al. 1993). In any case, the senescent plants and remnant parts generally desiccate and often turn brown, so desiccation appears to represent their final demise.

A different way to approach the problem of defining whole plant death is to ask how can this death be measured/determined? Whether or not a plant sheds its leaves during senescence, drying up/desiccation is a common subjective criterion for death, and leaf wilting certainly has been useful as a quantitative death measure in Arabidopsis (Hensel et al. 1993; Noodén and Penney 2001). Nonetheless, the electrical death "spasm" reported by Bose (1927) seems closer to the underlying causes and may still be the best measure.

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Chapter 14

Ultrastructural Analyses of Senescence Associated Dismantling of Chloroplasts Revisited

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Summary

During leaf senescence, chloroplasts are transformed into gerontoplasts involving typical structural changes that have been revealed by electron microscopy for more than 30 years. The structural changes involved in chloroplast-to-gerontoplast transition affect the organization of the thylakoid membrane system which is progressively degraded. In parallel, the number and size of plastoglobules was observed to increase. The internal changes in the structure of chloroplasts occurring during leaf senescence are accompanied by a change from an ellipsoid to a round shape and by a reduction in volume. Recent results on Rubisco degradation involving modern cell biology approaches suggest that plastids during senescence release material including Rubisco and other stromal proteins for degradation outside the organelle. In order to get further insight into the structural changes associated with chloroplast dismantling, we have revisited the

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pertinent literature and furthermore analyzed the ultrastructure of chloroplasts at different stages of barley leaf senescence and under different conditions leading to yellowing of the leaves. Specific changes at the periphery of chloroplasts at certain stages during aging might be related to an exchange of material between chloroplasts and the endoplasmic reticulum. Electron microscopy cannot, however, discriminate between anterograde and retrograde vesicle movements. Electron lucent areas in the matrix of chloroplasts indicate that protein degradation occurs not only outside but also inside the organelle. In many studies it has been observed that the number of plastids per cell declines at late stages of senescence. Our ultrastructural analyses of leaves senescing under field conditions showed that chloroplasts as well as gerontoplasts are surrounded by membranous structures before they are engulfed by the vacuole. Thus, the autophagy pathway appears to be involved in senescence. Many results of electron microscopical analyses of leaf senescence indicate that there exist several mechanisms of chloroplast dismantling. However, further studies by live-cell imaging, immunolabeling and cryo-electron microscopical methods on defined material of plants grown under strictly controlled and comparable conditions will be required for elucidating the mechanisms involved.

I. Introduction

A. Diverse Mechanisms of Chloroplast Dismantling

The dismantling of chloroplasts is the typical feature of leaf senescence. It can be easily monitored by the decline in chlorophyll content of the leaves. With the aim to elucidate the structural changes, accompanying the decline in chlorophylls during senescence, researchers have employed transmission electron microscopy. Most studies have been performed with crop plants such as wheat, rice and soybean, because senescence severely limits crop life. As summarized in reviews of Eilam et al. (1971), Thomson and Platt-Aloia (1987), Biswal and Biswal (1988) and Gepstein (1988), most authors have observed a common pattern in the sequence of senescence-associated changes in cell structure. Chloroplast degeneration was shown to be an early event preceding morphological changes of the nucleus and mitochondria (Thomson and Platt-Aloia 1987). Most studies showed that chloroplasts lose volume and change their shape from being ellipsoid to be round. Plastids with such features were named gerontoplasts by Peter Sitte in 1977 (Sitte 1977). Ultrastructural studies on the formation of gerontoplasts showed, however, inconsistencies with regard to the senescence-associated sequence of events occurring inside plastids, such as changes in the ultrastructure of thylakoids and formation of plastoglobuli. In particular, there is considerable divergence in the literature concerning the pattern of thylakoid membrane degradation during senescence.

Such divergence might be due to the different species used for the analyses and the diverse conditions of plant growth and sample treatment before fixation. Most ultrastructural studies of chloroplast dismantling have been performed with seedlings grown in a controlled light environment or exposed to darkness. In contrast, under field conditions plants are exposed to very high light intensi-

Abbreviations: ATG genes – Autophagy related genes; CLSM – Confocal laser scanning microscopy; cv. – Cultivar; EM – Electron microscopy; ER – Endoplasmic reticulum; GFP – Green fluorescence protein; h D – Hours in darkness; h L – Hours in light; PPV – Precursor protease vesicle(s); PSV – Protein storage vesicle(s); RCB – Rubisco-containing body(s); RCV – Rubisco-containing vesicle(s); rER – Rough endoplasmic reticulum; Rubisco – Ribulose-1,5-bisphosphate carboxylase oxygenase; SAV – Senescence-associated vacuole; TIP – Tonoplast intrinsic protein

ties up to a photon irradiance of 2,000 μmol m^{-2} s^{-1} μE at midday (Humbeck et al. 1996). It is questionable, whether the insights obtained by studying leaf senescence under artificial light of low intensity in climate chambers or even in darkness are applicable to senescence of crop plants under field conditions. Because senescence is affected by diverse environmental factors, plants might use different strategies for dismantling of chloroplasts. The complexity of chloroplast degradation is likely to be even higher, when stress situations leading to premature senescence induced by stress are also considered.

So far, it is not clear whether degradation processes occur exclusively inside the organelle, whether material is released from chloroplasts or whether even entire chloroplasts enter the central vacuole and are degraded there (Krupinska 2006). Here we summarize the current knowledge on senescence-induced degradation of the chloroplast ultrastructure by different pathways including specific vesicles, and complement it with recent results of our own ultrastructural analyses.

To systematically address the question of chloroplast degradation during different conditions, we have used leaves of one plant species only. We have chosen barley leaves, and, in particular, have analyzed natural senescence in flag leaves of the plants grown in the field. To get a more complete picture, we have conducted experiments to examine ultrastructural changes during senescence of leaves treated with senescence promoting hormones, exposure of leaves to stress such as darkness and infection by fungal pathogens involving wounding of the tissue.

B. Drawbacks of Cytological Analyses

Analyses of different senescence-associated processes such as chlorophyll and protein degradation suggested that leaf senescence is a regulated and slowly progressing process. Accordingly, the changes in chloroplast structure paralleling the decline in chlorophyll and protein contents are considered to progress slowly whereby chloroplasts undergo a differentiation into gerontoplasts with specific changes inside the structure of the organelle. The most obvious features paralleling chloroplast-to-gerontoplast transition at the structural level are the decline in the surface of thylakoids and the increase in number and size of plastoglobules (Lichtenthaler 1969; Sitte 1977).

A severe drawback of ultrastructural analyses is, however, their static information making it impossible to determine the dynamics of differentiation processes. The synchronization of plant material for ultrastructural studies during senescence is another major limitation. Cells in a given tissue such as the mesophyll of the leaf are not homogeneous and need to be selected for ultrastructural inspection of chloroplasts. The selection is rather subjective and depends on the aim of the study. Moreover the cells selected in young leaves might not develop into those cells selected in senescing leaves. A methodology showing average pictures from a large number of cells is not available. Microscopic analyses of sections from senescing leaves clearly show that at the cellular level, senescence is not synchronized among the mesophyll cells. For example, when sections from senescing leaves are analyzed by light microscopy, it is evident that cells surrounding the vascular tissue can be still green while cells in the mesophyll between the bundles are yellowing (Niewiadomska et al. 2009). Our recent analyses of leaf senescence in flag leaves collected in the field showed that the cells of the mesophyll tissue located between two bundles are not in the same stage of senescence. Heterogeneity of mesophyll cells with respect to senescence processes has been reported by H. Thomas (1977) also for *Festuca pratensis*. He observed that the chloroplasts in one given cell are all at a similar stage of degeneration, although chloroplasts in neighbouring cells might be rather dissimilar with regard to their ultrastructure. The heterogeneity of senescing tissue was also pointed out by Thomson and Platt-Aloia (1987) in their review on ultrastructure and senescence in

plants. Therefore, it is very important to provide information on the positions of cells analyzed and also on the frequency of special plastid types.

Chemical fixation used for ultrastructural analyses could cause artifactual changes in ultrastructure such as swelling or fusion of membrane-bound compartments, which might even occur more frequently during senescence, when the cellular membranes are disintegrating. As a result cellular components might get translocated, diluted or lost during preparation of specimen.

II. Chloroplast-to-Gerontoplast Transition

A. Characteristic Features of Gerontoplasts

Although chloroplast dismantling during leaf senescence is affected by many internal and external factors and is certainly not mediated by a common mechanism, gerontoplasts of senescent cells in different tissues and under different situations are considered to share certain specific features (reviewed by Biswal et al. 2003). Main characteristics of chloroplast-to-gerontoplast transition involve an increase in size and number of plastoglobules and a change in organization of the thylakoid membrane system which finally breaks down. In most cases, these changes were shown to be accompanied by a reduction in size and a change in shape from ellipsoid to round (Eilam et al. 1971; Mlodzianowski and Ponitka 1973; Wittenbach et al. 1980; Ghosh et al. 2001). Tuquet and Newman (1980) described empty (vacuolar) spaces in the stroma of gerontoplasts. These so-called dilated thylakoids were also frequently observed in senescing leaves of *aurea* mutants by the group of Mercedes Wrischer (Kunst and Wrischer 1984). Furthermore, gerontoplasts are reported to undergo peripheral vesiculation (Tuquet and Newman 1980) resembling the peripheral tubular apparatus described for leaves of the solanaceous genus Cyphomandra (Harris 1978, see also Wise 2006). Harris (1978) observed a decrease in the tubular structures developed from thylakoids during senescence of Cyphomandra. These structures are branched and could be involved in isolation of parts of the stroma which then might be released into the cytoplasm (see Sect. V). Thylakoid coiling together with cup-shaped stacking has also been observed to be associated with thylakoid degradation in senescing leaves of *aurea* mutants (Wrischer et al. 2009).

Gerontoplasts share several features with chromoplasts which also develop from chloroplasts. Both have a reduced thylakoid membrane system and accumulate carotenoids (Tuquet and Newman 1980; Thomson and Platt-Aloia 1987). Gerontoplasts in contrast to chromoplasts were, however, never observed to divide (Matile 1992; Zavaleta-Mancera et al. 1999).

B. Plastoglobules: Number, Content and Dynamics

A characteristic feature of gerontoplasts was reported to be the occurrence of numerous large plastoglobules (see also Lichtenthaler, Chap. 15). When we compare reports on different plant species grown under different conditions, plastoglobules seem to accumulate, in particular, in high numbers in chloroplasts when the plants were grown in the field (Table 14.1). Recent studies using high pressure freezing and immunogold labeling confirmed that plastoglobules in chloroplasts are permanently coupled to thylakoid membranes and are involved in various biosynthetic processes (Austin et al. 2006). In another biochemical study, it has been shown that they possess enzymes involved in biosynthesis of tocopherols (Vidi et al. 2006), which are known to accumulate during light exposure of plants. This observation indicates that plastoglobules also have functions in biosynthesis and in restructuring of the thylakoids in response to changes in light environment. Plastoglobule formation is, however, not strictly dependent on light and has been also observed in degenerating etioplasts (Rascio et al. 1986).

Table 14.1. Ultrastructural changes in the thylakoid membrane system during leaf senescence

Species	Material	Light conditions	Observations	References
Cumumis sativa	Attached and detached cotyledons		Gradual disappearance of thylakoids	Butler (1967)
Nicotiana rustica	Leaves of 6 months old plants	15 h L/9 h D	Breakdown of stroma thylakoids	Ljubešić (1968)
Triticum aestivum L.	Cut leaves, dark incubation	16 h L/8 h D, growth chamber	Intergranal thylakoids disintegrate before degeneration of grana	Mittelhäuser and Van Stevenick (1971)
Petrosilium sativum	Leaves collected in autumn	Dark incubation	Breakdown of stroma thylakoids, late disintegration of grana no reversibility	Mlodzianowski and Ponitka (1973)
Cichorium intybus L.	Leaves from flower stalks in the second year of vegetation	Cut leaves kept at 2 lux light intensity for 12 days	Unstacking of grana, formation of long unstacked thylakoids	Mlodzianowski and Mlozianowska (1973)
Populus deltoides	Specimen cut between the veins	Leaves collected in nature	Differentiation in grana and stroma thylakoids gets lost	Hernandez and Schaedle (1973)
Glycine max	Cotyledons	Grown on vermiculite supplied with nutrients	Stroma lamellae degenerated	Huber and Newman (1976)
Festuca pratensis Huds	Fully expanded leaves from plants grown in a 16 h photoperiod	Dark incubation of cut leaves	Degeneration of grana and stroma thylakoids	Thomas (1977)
Oryza sativa	Flag leaves	Field	Breakdown of thylakoids, decrease in number of grana	Chonan et al. (1977)
Triticum aestivum L.	Primary foliage leaves	12 hL/12 h D, growth chamber comparison of natural senescing leaves and dark incubation of cut leaves	*Natural senescence*: preferential loss of stroma lamellae; grana get narrow and finally resemble a series of appressed membrane vesicles without intergrana lamellae *Dark-induced senescence*: higher stability of membranes	Hurkman (1979)
Glycine max			Enlargement of grana; reduction in stroma thylakoids	Tuquet and Newman (1980)
Glycine max	Collected leaves	Rows in the field	Grana stacks remained, stroma thylakoids decreased	Wittenbach et al. (1980)
Nicotiana tabacum	Fully expanded attached leaves	Glasshouse	Disorganization of thylakoids and formation of plastosomes	Harris and Schaefer (1981)
Triticum aestivum L.	Primary foliage leaves	Growth chamber 250 μE transfer to darkness	Grana stack preserved	Wittenbach et al. (1982)
Oryza sativa	Leaf segments	Outdoor growth, pots	Large grana (20–30 thylakoids)	Hashimoto et al. (1989)

(continued)

Table 14.1. (continued)

Species	Material	Light conditions	Observations	References
Triticum aestivum	Flag leaves, 2 mm squared segments from the center	Hydroponic culture, light/dark regime; exposure to high temperature	Increased thylakoid lumen volume, decreased area of appressed thylakoids	Xu et al. (1995) Kutik (1998)
Nicotinana tabacum	*O. vires*: leaf segments at different positions of leaf	Growth chamber, light/dark regime	Thylakoid dilation and breakage; stroma thylakoids seem to degrade before grana	Simeonova et al. (2000)
Ornitholagum virens	*N. tabacum:* leaves in three stages			
Brassica napus	Cotelydons	Growth chamber 16 h photoperiod (140 μmol m^{-2} s^{-1})	Decrease in granal stacking	Ghosh et al. (2001)
Cucumis sativus	Chloroplasts isolated from cotyledons	Growth chamber (40–50 μE)	Formation of extended grana like structures	Prakash et al. (2001)
Hordeum vulgare L.	Primary foliage leaves, segment 1.5 cm below the tip	16 h L/8 h D, 90 μE	Light:disintegration of grana earlier than of stroma thylakoids, darkness: grana well preserved	Spundova et al. (2003)
Hordeum vulgare L.	Second leaves of 10- and 20-day old seedlings	16 h L(40 W × m^{-2})/8 h D	Degradation of thylakoid membranes	Kolodziejek et al. (2003)
Zea mays L.		growth chamber		
Hordeum vulgare L.	Second leaves	16 h L(40 W × m^{-2})/8 h D	Disrupted thylakoids and damaged envelope	Kolodziejek et al. (2006)
Zea mays L.		Growth chamber		
Oryza sativa hybrid variety	Flag leaves, middle part without midrib	Field	Swelling of all thylakoids	Zhang et al. (2010)
Hordeum vulgare L. *cv. Carina*	Flag leaf blades, segment of middle part	Field	Grana stacks remain without interconnections	Krupinska et al. (2012)
Hordeum vulgare L. *cv. Lomerit*	Flag leaf blades segment of middle part	Field	Long single or pairwise thylakoids, grana disappear	Krupinska (2011)

By ultrastructural studies with different plants, Lichtenthaler proposed the existence of two types of plant species with respect to the number and size of plastoglobules that accumulate in their gerontoplasts (Lichtenthaler and Sprey 1966; Lichtenthaler 1968). The first type represented by annual plants such as spinach and barley entails an increase in the size of plastoglobules without obvious increase in number. The second type represented by perennial plants such as Tradescantia and Billbergia has a larger number of plastoglobules with almost unchanged size. In some plants such as soybean, however, both an increase in number and size of the plastoglobules was observed (Tuquet and Newman 1980). Although in some plants the sizes of plastoglobules do not change, a general increase in plastoglobule size has been proposed as a marker of senescence (Lichtenthaler 1969).

Sizes of plastoglobules observed in mature chloroplasts range from 80 to 120 nm. Diameters measured for plastoglobules in gerontoplasts range from 200 to 300 nm with extreme examples having sizes up to 5 μm in diameter. Large plastoglobules in the range of 3–5 μm have been observed in senescent leaves of Gingko, Fagus and Ficus (Lichtenthaler and Weinert 1970). In contrast to the small plastoglobules detected in chloroplasts before senescence, which are clearly separated from each other, the large plastoglobules in senescent cells tend to be attached to each other. Based on this observation, it has been proposed that plastoglobules might fuse at a late stage of development (Lichtenthaler 1969; Mittelhäuser and Van Stevenick 1971; Ghosh et al. 2001).

The ultrastructure of plastoglobules changes during chloroplast development. The large plastoglobules of gerontoplasts were observed to be wrinkled while the small plastoglobules of chloroplasts were spherical and showed a smooth surface when analyzed by scanning electron microscopy (Tuquet and Newman 1980). Often the electron density of plastoglobules was observed to decline with age, and plastoglobules were reported even to be translucent. Such translucent globules in previous reports were named plastosomes, because the authors aimed at discriminating them clearly from the small spherical and electron dense plastoglobules observed in chloroplasts (Harris 1978; Harris and Schaefer 1981). Accordingly, the chemical composition of plastoglobules was shown to change during senescence and chromoplast development. The contents of carotenoids and quinones were observed to increase in chromoplasts (Lichtenthaler 1969, 1970). By comparing plastoglobules in leaves with different carotenoid content, Lichtenthaler (1970) suggested that the decrease in electron density might be caused by an increase in the level of carotenoids. Therefore the carotenoid content could at least partly determine the degree of osmiophily. It has been shown further that the plastoglobules are reservoirs for excess lamellar lipids which are not decomposed after the breakdown of thylakoids (Lichtenthaler and Weinert 1970). Later studies revealed that plastoglobules in senescent leaves contain xanthophyll esters which are formed during breakdown of carotenoids (Tevini and Steinmüller 1985). They also contain tocopherols which are known to accumulate in senescing leaves (Lichtenthaler 1966; Chrost et al. 1999; Dertinger et al. 2003; Falk et al. 2003). It is therefore possible that plastoglobules in gerontoplasts have a function in both biosynthesis and storage of tocopherols.

Chloroplasts redifferentiated from gerontoplasts during regreening of leaves were shown to include large plastoglobules typical for gerontoplasts (Tuquet and Newman 1980; Zavaleta-Mancera et al. 1999b). While some were observed to be perfectly round shaped and smooth, others were observed to have irregular shape and a wrinkled surface as also shown for those observed in gerontoplasts (Tuquet and Newman 1980). These observations indicate that most material stored in plastoglobules of gerontoplasts during regreening is not reused for enlargement of the thylakoid membrane system.

In a study with senescing bean leaves, an accumulation of lipid-protein particles was

reported (Ghosh et al. 1994). Although these are rather small (10–320 nm in diameter), they were described to morphologically resemble plastoglobules. Biochemical analyses showed that these particles contain thylakoid membrane proteins and catabolites. The authors (Ghosh et al. 1994) proposed that the blebbing of the particles might be a way to remove potentially destabilizing catabolites from the thylakoids.

C. Changes in Organization of the Thylakoid Membrane System

Thylakoid membranes are the sites of the light reactions in photosynthesis. In higher plants they are differentiated into grana stacks and unstacked stroma thylakoids which are all interconnected. Most ultrastructural studies on leaf senescence reported that grana thylakoids persist over a longer time than stroma thylakoids (Table 14.1). In some studies it has, however, been observed that all thylakoids got swollen and were degraded simultaneously. This could be due to the different conditions of plant growth and treatments to induce senescence processes. For example, during senescence of wheat leaves under natural conditions a preferential loss of stroma thylakoids was observed while during dark induced senescence all thylakoids were affected (Hurkman 1979). Treatment with senescence-inducing hormones such as jasmonic acid and salicylic acid can lead to a severe disintegration of the thylakoid membrane system. These hormones are known to accumulate in senescing leaves (Morris et al. 2000; He et al. 2005) and might promote different processes of the senescence syndrome. Analyses with barley primary foliage leaves incubated in the presence of 100 μM salicylic acid revealed that this compound has a dramatic impact on the thylakoid membrane structure (Uzunova and Popova 2000). It appeared to cause swelling of grana thylakoids, coagulation of stroma, and an increase in chloroplast volume.

The variations in protein and lipid compositions of the different regions of the thylakoid membrane system are known as lateral heterogeneity (Anderson and Andersson 1982; Anderson 1999). Because most of the chlorophyll is associated with the light harvesting complex attached to photosystem II in grana thylakoids, changes in the chlorophyll a/b ratio occurring during leaf senescence can be used as an indicator of the underlying process of chloroplast degradation. If chloroplasts are digested completely before they are transformed into gerontoplasts, no change in the chlorophyll a/b ratio is expected to occur. If, however, chloroplasts undergo changes in the thylakoid membrane system, these should be accompanied by specific changes in the chlorophyll a/b ratio. In most studies on leaf senescence, stroma thylakoids were reported to be degraded prior to grana thylakoids resulting in a decrease of the chlorophyll a/b ratio. Such changes were reported for leaves of trees showing autumnal senescence (Wolf 1956) and also for crop plants such as rice (Kura-Hotta et al. 1990). Most of these studies used 80 % acetone for extraction of chlorophylls. Because under these hydrophilic conditions part of hydrophobic chlorophyll a might not get extracted, the values obtained for chlorophyll a/b might be too low and need correction by conversion factors (Porra et al. 1989). Nevertheless, in most cases chlorophyll a is degraded faster than chlorophyll b.

In accordance with a reduced chlorophyll a/b ratio measured in senescent leaves, gerontoplasts were observed to have grana stacks without interconnecting stroma thylakoids (Kura-Hotta et al. 1990). We also found such gerontoplasts in flag leaves collected in a field of the spring barley variety cv. Carina. However, when we analyzed gerontoplast formation in flag leaves of the modern high-yielding variety cv. Lomerit, we found gerontoplasts with decreased grana stacks, which finally had only long single or pairwise thylakoids. These structural changes were accompanied by a dramatic increase in the chlorophyll a/b ratio (Krupinska et al. 2012). To distinguish this type of gerontoplast from the gerontoplast having preserved

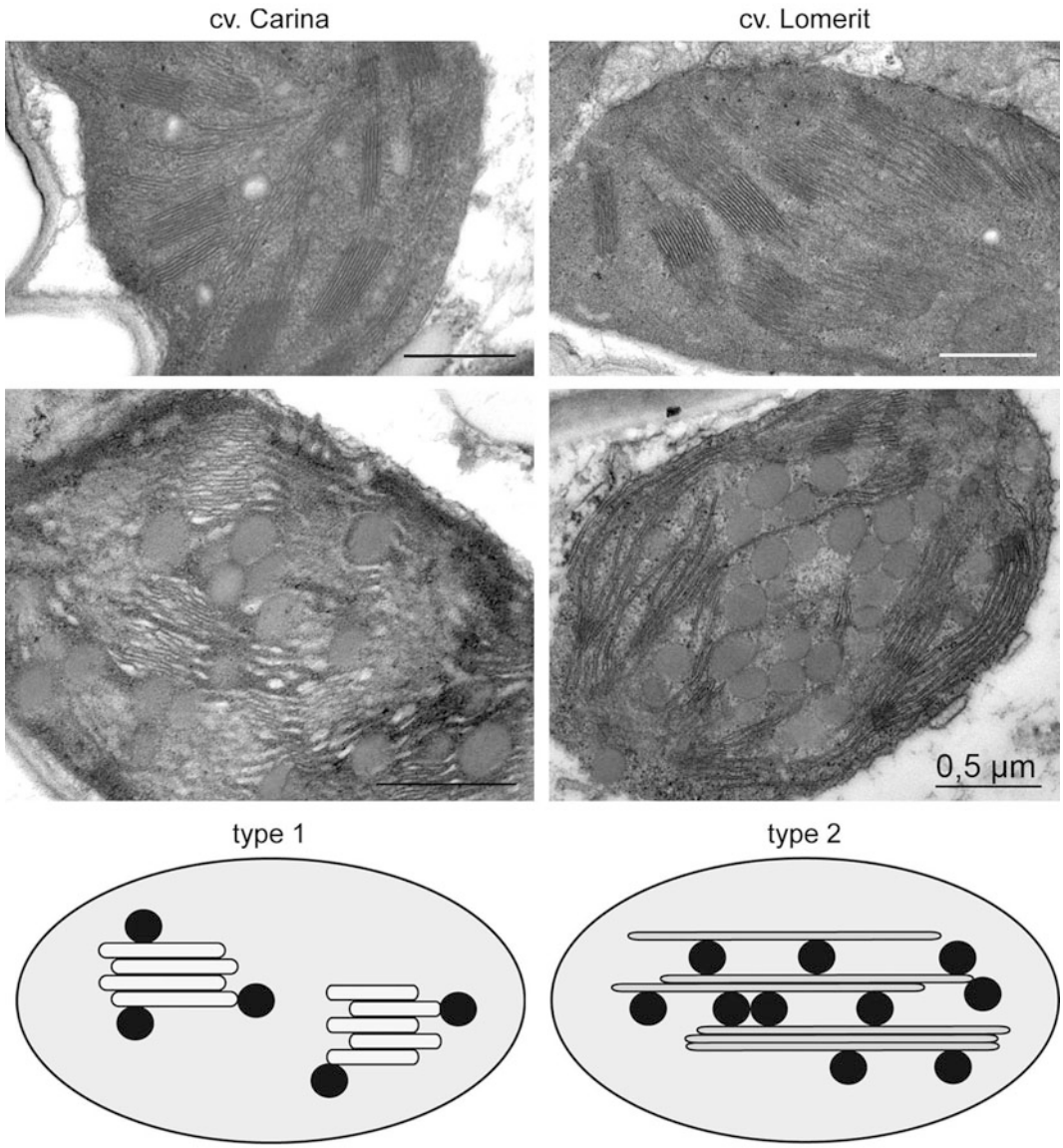

Fig. 14.1. Ultrastructure of chloroplasts and gerontoplasts in mesophyll cells of flag leaves from the barley cultivars Carina (*left*) and Lomerit (*right*), respectively. Chloroplasts before senescence (*top*) and gerontoplasts (*below*) from leaves having lost about 50 % of chlorophylls are compared. *Bars* indicate 0.5 µm. The differences in ultrastructure of gerontoplasts detected in cv. Carina (*type 1*) and cv. Lomerit (*type 2*) are highlighted by a schematic drawing. The *black* discs represent plastoglobules. (The figure is reproduced from Krupinska et al. 2012).

grana stacks, we named it type 2 gerontoplast (Fig. 14.1).

Different strategies of thylakoid membrane degradation were observed when leaves were exposed to treatments leading to senescence like degeneration of chloroplasts (Fig. 14.2). When barley leaves were exposed to darkness a preferential degradation of stroma thylakoids was observed (Fig. 14.2a). In contrast to type 1 gerontoplasts formed during

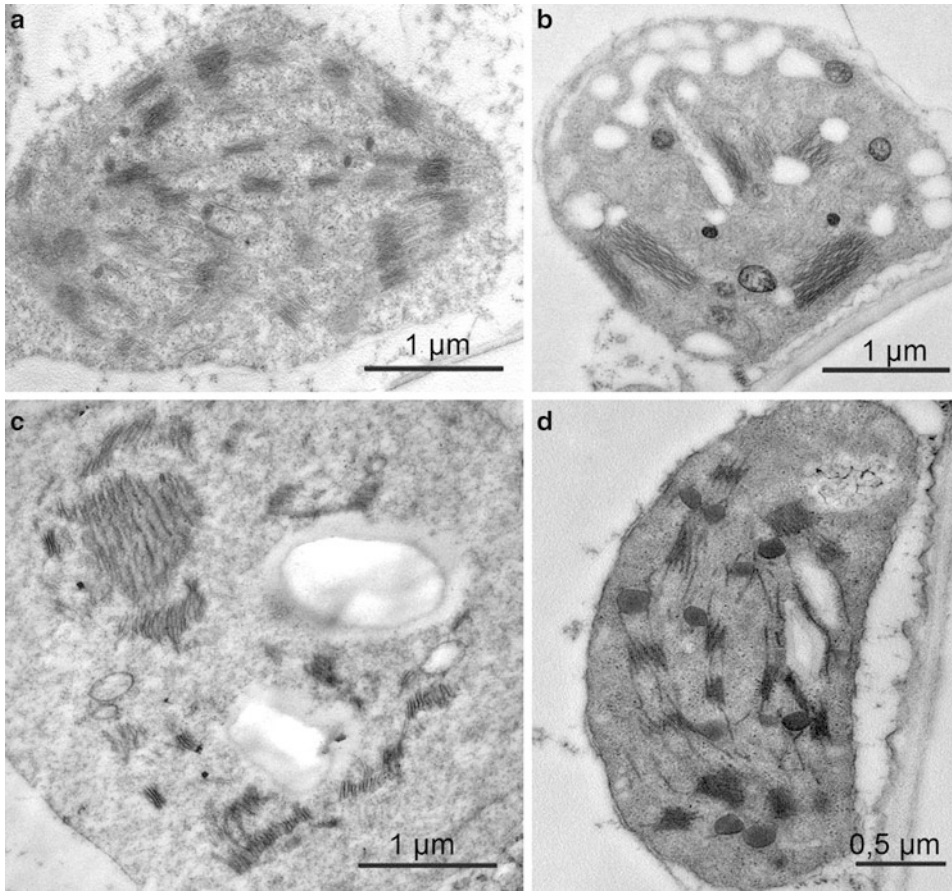

Fig. 14.2. Ultrastructure of degrading chloroplasts observed in leaves of the barley cultivar Carina after different treatments. (**a, b**) Gerontoplast observed in primary leaves induced to senescence by transfer to darkness for 2 days (a) or 10 days (b). (**c**) Ultrastructure after flotation of leaf segments for 3 days on water including 45 µM jasmonic acid as well as 2 mM salicylic acid. (**d**) Ultrastructure after flotation of leaf segments for 3 days on 45 µM jasmonic acid.

development dependent senescence, in gerontoplasts formed during darkness the height of grana stacks seems not to increase and plastoglobules do not enlarge. The pattern of thylakoid membrane degradation is also affected by treatment with hormones known to induce senescence-like processes such as salicylic acid and jasmonic acid. For example, application of jasmonate simultaneously with salicylic acid to barley leaf segments induced specific disorders in organization of grana stacks without degradation of stroma thylakoids (Fig. 14.2b).

Different ways of chloroplast dismantling appear to occur also in *aurea* mutants of different vascular plants (Fulgosi et al., Chap. 26). Leaves of these mutants are green and have a fully developed thylakoid membrane system with large grana stacks when grown in low light. When leaves were exposed to high light intensity they turned yellow and formed gerontoplast-like plastids. The structure of these gerontoplasts was shown to depend on age and on the light conditions to which the leaves were exposed to (Kunst and Wrischer 1984). In young yellow leaves of *Ligustrum*

ovalifolium a high chlorophyll a/b ratio was observed to coincide with plastids having longitudal arrays of dilated thylakoids (Kunst and Wrischer 1984).

An enhanced chlorophyll a/b ratio was also observed when barley leaves underwent senescence in continuous light (Spundova et al. 2003). The authors discussed that preferential degradation of grana stacks in the light could be a means to avoid excess energy associated stress under a condition of advancing degradation of Rubisco. In our opinion a photon irradiance of 90 µmol m^{-2} s^{-1} as used in this study (Table 14.1) has, however, rather low intensity compared to plants grown in the field. We rather propose that this change in the Chl a/b ratio might be an intrinsic feature of the barley variety cv. Akcent.

Most nitrogen in chloroplasts is in Rubisco and its degradation is a prerequisite for efficient nitrogen remobilization to the growing leaves, seeds and fruits. Crop varieties forming type 2 gerontoplasts might have a higher rate of nitrogen recycling, because they simultaneously degrade proteins located in the grana membranes such as light harvesting complexes, which have bound up to 30 % of the nitrogen and so are the second most important nitrogen source in a senescing leaf.

III. The Chloroplast Periphery (Periplastic Space) During Gerontoplast Development

Proteomics of chloroplasts revealed that about 10 % of the organelle precursor proteins don't have an N-terminal transit peptide as required for import by the canonical import apparatus consisting of TOC and TIC complexes (Ling et al., Chap. 12), but are synthesized in the endoplasmic reticulum and are most likely transported to the organelle through vesicles (Villarejo et al. 2005; Radhamony and Theg 2006). Possibly this way of protein delivery to the organelle makes use of the lipid trafficking pathway by vesicles established between extra-plastidic membranes and chloroplast envelope membranes (Benning et al. 2006). These findings indicate close connections between the endomembrane system and plastids. Indeed, several early ultrastructural investigations reported on the existence of close interconnections between the two compartments. Although vascular plants don't possess a chloroplast ER as described for algae developed by secondary endosymbiosis, ER cisternae were shown to form partial sheaths around plastids of vascular plants (Gibbs 1981). Furthermore ER membranes and the outer envelope membrane of plastids were observed to have temporal continuities during certain periods of development (Gibbs 1981; Whatley et al. 1991). It has been observed that during periods of physical connection between the ER and plastids, development of the plastids proceeds synchronously (Whatley et al. 1991). From ultrastructural studies on the fern *Pteris vittata*, Crotty and Ledbetter (1973) suggested direct functional channels between the perichloroplast space and smooth ER cisternae which may serve for transport of substances in both directions. Whatley et al. (1991) found by freeze-fracture studies on young primary leaves of *Phaseolus vulgaris* that ER cisternae regularly form a sheath around the plastids, and that in this case the ER membrane becomes continuous with the outer envelope membrane of chloroplasts.

Cisternae of the endoplasmic reticulum closely associated with chloroplasts were frequently observed in mesophyll cells of our sections prepared from barley leaves. When different stages of chloroplast-to-gerontoplast transition were compared with respect to ultrastructure of the plastid periphery, it became obvious that the structure of endomembranes surrounding the organelle undergoes typical changes (Fig. 14.3). In case of young chloroplasts with tightly appressed thylakoids, ER cisternae in the vicinity of the organelles are clearly separated and typically have attached ribosomes facing the cytoplasm. In adult chloroplasts with partly swollen thylakoids, the periphery

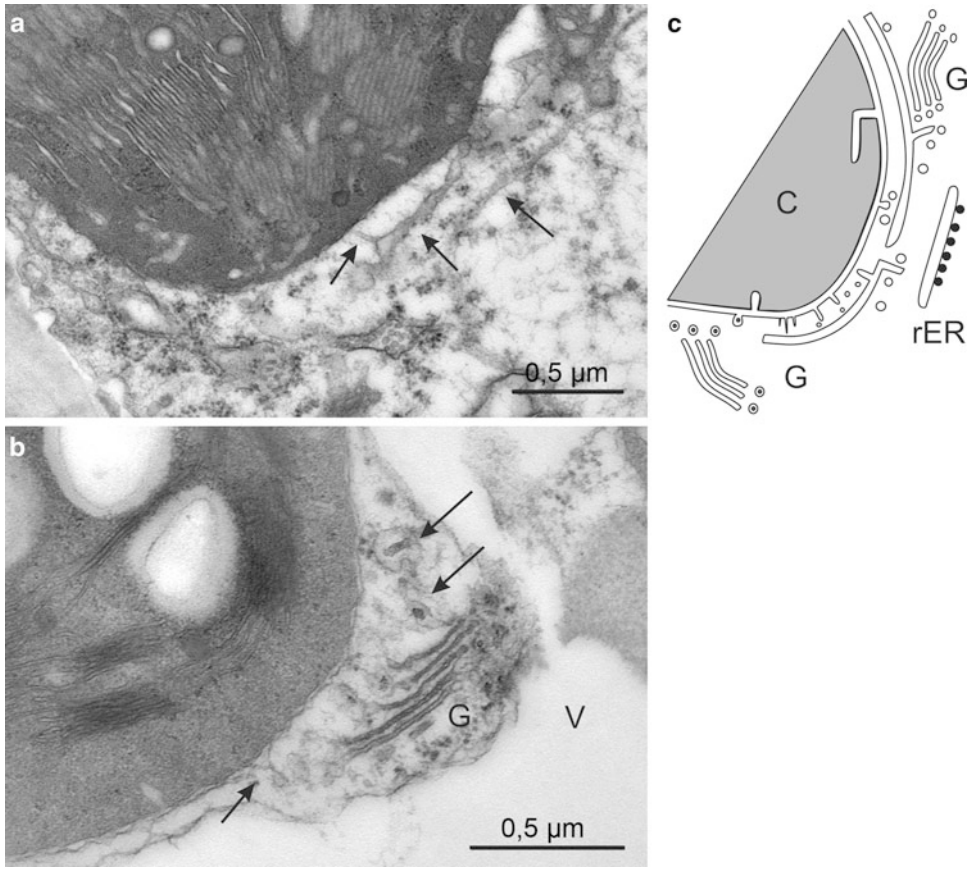

Fig. 14.3. Ultrastructure of the periplastidic space of mature and senescing chloroplasts in barley leaves. (**a**) Chloroplast with surrounding smooth cisternae (*arrows*) observed in an adult barley flag leaf grown in the field. (**b**) Chloroplast observed in a barley primary leaf piece 40 h after wounding (removal of the epidermis). Vesicles (*arrows*) appear to shuttle between the chloroplast outer membrane and a Golgi structure (G). *V* vacuole. (**c**) Schematic summary of membranous structures observed in and around early senescing chloroplasts (C). *G* Golgi apparatus, *rER* rough endoplasmic reticulum.

of the organelle appears to be reorganized with small protrusions emanating from the chloroplast surface and with many small vesicles. A subfraction of the vesicles, accumulating between the surface of plastids on one hand and the smooth ER cisternae and narrow tubules on the other hand, appears to be coated (Fig. 14.3). It seems that the chloroplasts are caged in a network of a tubular/cisternal membrane system. Furthermore, we found an intense Golgi activity in the vicinity of the chloroplast envelope. This typical appearance of the plastid periphery was seen not only in mesophyll cells of flag leaves collected in the fields, but also in the mesophyll of primary foliage leaves after either wounding or infection with *Pyrenophora teres*.

Physical contacts between the ER in the plastid periphery and the inside of the organelle are expected to increase during senescence, when the outer envelope gets damaged (Kolodziejek et al. 2006). During this stage the endomembrane network might deliver enzymes involved in chloroplast degradation. Considering that vesicles need to fuse with plastids for delivery of organelle proteins and lipids, such transport is very likely.

So far, no senescence specific protease with an N-terminal transit peptide for entering plastids by the canonical import pathway (Ling et al., Chap. 12) has been identified. It is more likely that the enzymes required for bulk degradation of chloroplast constituents during senescence are synthesized in the ER and transported to plastids via vesicles. Activity of such hydrolases could be responsible for the empty spaces detected in gerontoplasts (Fig. 14.7). Indeed, in a recent proteome analysis of soluble proteins in pea chloroplasts, an enzyme belonging to the family of cysteine proteases has been identified (Bayer et al. 2011).

IV. Partial Organelle Degradation by Blebbing and Vesicle Formation

A. Release of Osmiophilic Globules

During chloroplast-to-gerontoplast transition the release of osmiophilic material from plastids to the cytoplasm was frequently observed. It is likely that this material derives from plastoglobules increasing in abundance and size during leaf senescence (Table 14.2). This osmiophilic material in some cases was shown to be attached to the outer envelope of plastids (Guiamet et al. 1999). In most reports the globules released from plastids during the phase of senescence were described to be enriched in lipids resulting from the degradation of thylakoid membranes. By light microscopy of senescing broccoli florets, globules containing chlorophylls were detected in the cytoplasm (Terai et al. 2000). Images obtained by scanning electron microscopy showed that these small globules might derive from large globules, which inside the chloroplast give rise to numerous small vesicles expelled into the cytoplasm (Terai et al. 2000).

At the final stage of chloroplast dismantling plastoglobuli-like structures were found to be released into the cytoplasm (Butler 1967; Mlodzianowski and Mlozianowska 1973; Hurkman 1979; Guiamet et al. 1999). The release into the cytoplasm was also reported for the large translucent globules named plastosomes (Harris and Arnott 1973).

In barley, the formation of translucent globules and the release of osmiophilic droplets have not only been found in senescing leaves, but could be observed more frequently in leaves treated with hormones inducing senescence-like processes. When barley leaf segments were incubated under continuous illumination for up to 3 days with jasmonic acid and salicylic acid, severe alterations of the chloroplast ultrastructure were observed (Fig. 14.4). These include a reduction, up to a total loss, of stroma thylakoids, destacking of the residual thylakoids, accumulation of protein fibrils, formation of vesicles in the stroma as well as a massive release of electron dense material into the cytoplasm (Fig. 14.4). The release of plastoglobules and related structures to the cytoplasm might be due to a perforation of the chloroplast envelope. The release of plastoglobules into the cytoplasm was only observed when leaves were exposed to light. When senescence was induced by darkness, plastoglobules were reported to remain associated to the thylakoids (Hurkman 1979).

B. Degradation of Chloroplasts Might Involve Anterograde and Retrograde Vesicle Flows

The major purpose of leaf senescence is the remobilization of valuable compounds formed by degradation of chloroplast constituents. The major protein to be degraded is the stroma-located Rubisco, making up about 70 % of the soluble protein located in chloroplasts. Biochemical analyses indicated that Rubisco is degraded by cysteine proteases, which are usually found in vacuoles. The dominant scenario currently discussed for Rubisco degradation therefore is its degradation outside of plastids. The current models propose that Rubisco is transported out of plastids by Rubisco containing bodies (RCB) (Chiba et al. 2003) or Rubisco containing vesicles (RCV) (Prins et al. 2008). RCB are bound by a double membrane and have a

Table 14.2. Properties and dynamics of plastoglobules accumulating in gerontoplasts

Species	Material and growth conditions	Properties of plastoglobules	Release into cytoplasm	References
Phaseolus vulgaris	Leaves collected according to colour; greenhouse	Large globules surrounded by remnants of the thylakoid system	Not reported	Barton (1966)
Cucumis sativa	Attached and detached cotyledons	Large osmiophilic globules accumulating	Yes	Butler (1967)
Triticum aestivum L.	Cut leaves, dark incubation after growth of plants at 16 h photoperiod in growth chamber	Plastoglobuli of diameter of 0.1 μm, increase in size with age; small globules coalesce to form larger bodies	Yes: large lipid bodies in cytoplasm and vacuole	Mittelhäuser and Van Stevenick (1971)
Cichorium intybus	Cut leaf petioles from field in late autumn 12 d in semidark place	Increase in number and osmiophilicity	Not reported	
Petrosilium sativum	Leaves collected in autumn dark incubation (light deprivation)	Increase in number and size	Osmiophilic masses on plastid surfaces	Mlodzianowski and Ponitka (1973)
Cichorium intybus L.	Leaves from flower stalks in the second year of vegetation; cut leaves kept at two lux light intensity for 12 days (light deprivation)	Young leaves: plastoglobuli had diameter of 150 nm; plastoglobuli not more numerous, but larger with a maximal size of 350 nm	Yes	Mlodzianowski and Mlozianowska (1973)
Nicotiana tabacum	Leaves collected form stalks of greenhouse plants, no shade	Alveolate suborganelles, named plastosomes	Yes	Harris and Arnott (1973)
Populus deltoides	Specimen cut between the veins; leaves collected in nature	Very few large plastoglobuli	Not reported	Hernandez and Schaedle (1973)
Populus tremula L.	Leaves infected by a rust fungus; tree in October	Plastoglobuli present in young leaves; increase in volume of plastoglobuli	Not reported	Mlodzianowski (1975)
Glycine max	Cotyledons of seedlings grown on vermiculite with nutrient supply	Osmiophilic globuli increase in number and size; appearing at places where stroma lamellae were located before	Not reported	Huber and Newman (1976)
Festuca pratensis Huds.	Fully expanded leaves from plants grown in a 16 h photoperiod; then dark incubation	Increase in number and size		Thomas (1977)
Oryza sativa	Flag leaves from fields	Increase in number and size	Not reported	Chonan et al. (1977)
Triticum aestivum L.	Primary foliage leaves, basipetal comparisons 12 hL/12 h D, growth chamber comparison of natural senescing leaves and dark incubation of cut leaves	L/D: osmiophilic globuli were present at young stage, but were enlarged in senescent leaves, globules and membrane vesicles can be released into cytoplasm Darkness: Osmiophilic globules remained associated to the thylakoids	Yes, when grown in L/D	Hurkman (1979)

Glycine max				Tuquet and Newman (1980)
Glycine max L.	Collected leaves from plant rows in the field	The size of osmiophilic granules increased		Wittenbach et al. (1980)
Glycine max L.	Grown in vermiculite under continuous light	Increase in size and number; large plastoglobuli (190–400 nm) with very light contrast accumulate in the stroma; surface appeared wrinkled; some run together to form larger ones	Not reported	Tuquet and Newman (1980)
Nicotiana tabacum	Fully expanded attached leaves of glasshouse grown plants	Transient increase in plastoglobuli (no membrane, no internal structure); accumulation of plastosomes (seemingly limiting membrane and internal structure)		Harris and Schaefer (1981)
Triticum aestivum L.	Sections as well as isolated plastids from protoplasts of primary foliage leaves, growth chamber, 250 μE, transfer to darkness	Not reported	Not reported	Wittenbach et al. (1982)
Oryza sativa	Leaf segments of plants grown outdoor	Plastoglobuli were present in young leaves; increased with age in number and size (0.1 up to 0.25 μm)	Not reported	Hashimoto et al. (1989)
Triticum aestivum	Flag leaves, 2 mm squared segments from the center of hydroponic culture, light/dark regime; exposure to high temperature	Increase in size and number, in particular at high temperature	Not reported	Xu et al. (1995)
Nicotiana rustica L.	Leaf segments taken between veins; plants grown in a glasshouse	Decrease in number, but increase in size (from 150–450 nm to 300–800 nm)	Yes: clumps of osmiophilic material in the cytoplasm	Zavaleta-Mancera et al. (1999b)
Ornitholagum virens *Nicotinana tabacum*	*O. virens:* segments from three positions of a leaf*; N. tabacum:* leaves of different developmental stages; plants from growth chamber, light/dark regime	*O. virens:* slight increase in number *N. tabacum:* enormous increase in number		Simeonova et al. (2000)
Brassica napus	Cotyledons, Growth chamber, 16 h photoperiod (140 μmol m^{-2} s^{-1})	Aggregation/enlargement of plastoglobuli; few large globuli in gerontoplasts	Not reported	Ghosh et al. (2001)

(continued)

Table 14.2. (continued)

Species	Material and growth conditions	Properties of plastoglobuli	Release into cytoplasm	References
Hordeum vulgare L.	Primary foliage leaves, segment 1.5 cm below the tip; 16 h L/8 h D, 90 μE	Translucent plastoglobuli	Not reported	Spundova et al. (2003)
Hordeum vulgare L.	Second leaves; 16 h L	Increase in number and size of plastoglobuli	Not reported	Kolodziejek et al. (2006)
Zea mays L.	Growth chamber			
Oryza sativa hybrid variety	Flag leaves, middle part without midrib, field	Hybrid line had smaller and fewer plastoglobuli than parent lines; more and larger plastoglobuli in maternal line than in parental line	Not reported	Zhang et al. (2010)
Hordeum vulgare L. *cv. Carina*	Flag leaf blades, segment of middle part, field	Plastoglobuli increased in size, were highly osmiophilic and were arranged adjacent to grana stacks	Not detected	Krupinska (2011)
Hordeum vulgare L. *cv. Lomerit*	Flag leaf blades segment of middle part field	Plastoglobuli of low osmiophilicity attached to single or pairwise thylakoids	Not detected	Krupinska (2011)

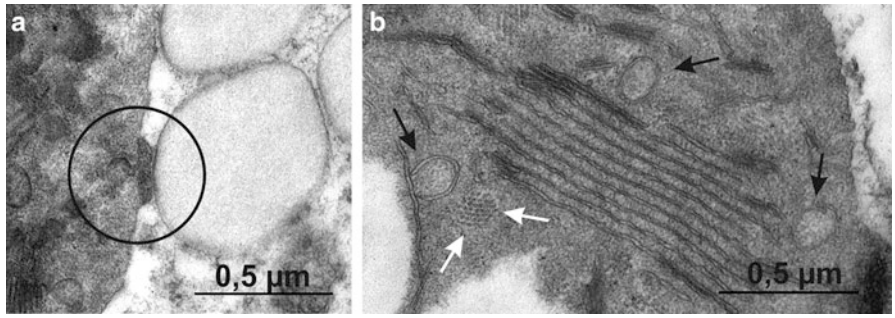

Fig. 14.4. Ultrastructure of chloroplasts in mesophyll cells of barley leaf segments incubated for 2 days in 2 mM salicylic acid after one day of treatment with 45 µM jasmonic acid. (**a**) Release of a plastoglobule into the cytoplasm (*encircled*). (**b**) Cup- and ring-shaped membrane structures (*black arrows*) and protein fibrils (*white arrows*) inside the chloroplast.

diameter of 0.4–1.2 µm. The presence of additional membranes around the chloroplast membranes of RCB is interpreted to be of autophagosomal origin. There are no reports whether RCB are acidic or contain proteolytic enzymes. RCB and the autophagosomal pathway are presented by Ishida and Wada in this volume (Chap. 19). RCB or autophagosomes including RCB are suggested to transport Rubisco and other stromal proteins finally to the vacuole for degradation by cysteine proteases.

Other structures proposed to be involved in Rubisco degradation are the senescence-associated vacuoles (SAV) which were shown to have a lower pH than the major vacuole (Otegui et al. 2005). The current knowledge on SAV is described in detail in the chapter of Costa et al. in this volume (Chap. 18). In a number of different senescing plant tissues, vesicles with a comparable size to SAV have been identified and were named ricinosomes or precursor protease vesicles (PPV), which contain large amounts of a 45 kDa cysteine endoprotease precursor having a C-terminal motif consisting of the amino acids KDEL (Schmid et al. 1999). Electron microscopical and biochemical studies suggest that they bud off from the endoplasmic reticulum. However, in contrast to SAV, ricinosomes are not acidic, but the proteases identified in ricinosomes were observed to be activated by release of protons from acidic vacuoles (Schmid et al. 1999; Greenwood et al. 2005). The intracellular transport of a vacuolar cysteine protease with the same ER-retention signal (KDEL) has been analyzed during protein remobilization of germinating seeds of mung beans (Toyooka et al. 2001). Thereby, proteases in ER-derived vesicles (KV) that bypass the Golgi apparatus and fuse with protein storage vacuoles (PSV) have been detected.

The origin of SAV and their relationship with RCB/RCV is far from being clear. If cysteine proteases likely to be included in SAV are indeed involved in degradation of Rubisco, several scenarios are possible. One scenario for bringing together proteases and substrate would be a fusion of SAVs with the RCB/RCV. Another scenario would be the fusion of SAV with chloroplasts resulting in the delivery of the enzymes for Rubisco degradation inside the organelle. In the latter case, Rubisco would be degraded inside the plastid. Such a model is not consistent with the export of Rubisco from the stroma, and it is also not consistent with the finding of Rubisco in SAV (Martínez et al. 2008). The origin of SAV remains to be demonstrated. It is possible that SAV are the result of the fusion of chloroplast derived vesicles with small vacuoles. This could be a prevacuole as reported for Chlamydomonas (Park et al. 1999). For demonstration, the characterization of the SAV-residing proteases as well as detailed ultrastructural and immunogold labeling studies of carefully fixed material (preferably by high pressure freezing) would

be required. RCB are reported to directly bud off from the chloroplast envelope or are shed from the tip of stromules (Chiba et al. 2003). Optical or ultrathin sections, however, are not sufficient to demonstrate the existence of separate chloroplast vesicles in the cytoplasm. Our own ultrastructural studies suggest that chloroplasts under many conditions including senescence can form long protrusions/stromules. In order to rule out the possibility that sections through these structures may be mistaken for as RCB or RCV, 3-dimensional reconstructions by CLSM or EM tomography analyses are necessary.

Considering that there is an intensive vesicle flow from the endomembrane system to plastids, it is also likely that conversely vesicles are formed at the envelope membrane and fuse with ER-derived cisternae or with either the small SAV or the central vacuole. Juan Guiamet's group recently showed that SAV also contain proteins of stromal thylakoids (Costa et al., Chap. 18). This observation is not consistent with an anterograde transport of SAV to the plastids. Rather this finding is consistent with a release of SAV from plastids. This could mean that SAV, similar to RCB/RCV, are involved in export of plastid proteins. In contrast to RCB/RCV, they obviously carry thylakoid membrane proteins and chlorophyll catabolites.

Several other observations point towards a role of vesicles in transport of stromal components to lytic compartments. In Chlamydomonas, protrusions of the outer membrane of the envelope have been shown to contain stromal proteins (Park et al. 1999). The protrusions detach as vesicles and were suggested to fuse with lysosome-like structures formed from the Golgi apparatus. Subsequently, their content is delivered to the vacuole and is degraded (Park et al. 1999). Such protrusions could be similar to stromules mainly observed with young plastids of higher plants (Gray, Chap. 9). Stromules were observed to disintegrate into vesicles whereby stromal material could be released from chloroplasts (Gunning 2005).

In barley flag leaves undergoing natural senescence, many small vesicles were observed in close vicinity to the outer chloroplast envelope. These vesicles often appear to be in close contact with vesicles clearly belonging to the Golgi apparatus or smooth ER cisternae (Fig. 14.3). Ultrastructural analyses cannot, however, distinguish between the vesicles budding off the envelope and those vesicles fusing with the envelope. The positions of the vesicles are in accordance with trafficking either between the endomembrane system and plastids or between the endomembrane system and the central vacuole. The vesicles have a diameter of about 50 nm and are enclosed by a single membrane. Many of them have an electron-dense globular content. Similar vesicles accumulate in mesophyll cells of primary leaves analyzed 40 h after removal of the epidermal cell layer, but they have not been observed in barley after any other treatment. This is surprising, considering that this type of wounding also occurs during infection of leaves by pathogens. When we analyzed chloroplasts in the mesophyll of barley leaves after infection with the fungal pathogen *Pyrenophora teres*, we found, however, only double-membrane bound vesicles that seem to bud off from the chloroplasts (Fig. 14.5a). These vesicles might carry chloroplast proteins destined for degradation in the vacuolar compartments, as it has been described for RCBs or other vesicles emanating from the outer chloroplast membrane in Chlamydomonas (Park et al. 1999). In senescing flag leaves of barley, vesicles resembling RCB could be identified. Their shapes and positions, however, suggest that at least some of them represent cross-sections of stromules (Fig. 14.5b).

In late-senescing mesophyll cells of barley leaves, the plastids were found to be attached to the vacuole, and the limiting membranes of the two compartments are seemingly ruptured (Fig. 14.6a). These plastids are often observed to release vesicles (Fig. 14.6b). It is, however, not clear whether these vesicles are involved in a regulated transport of plastid components to the vacuole, or whether the vesiculation is due to

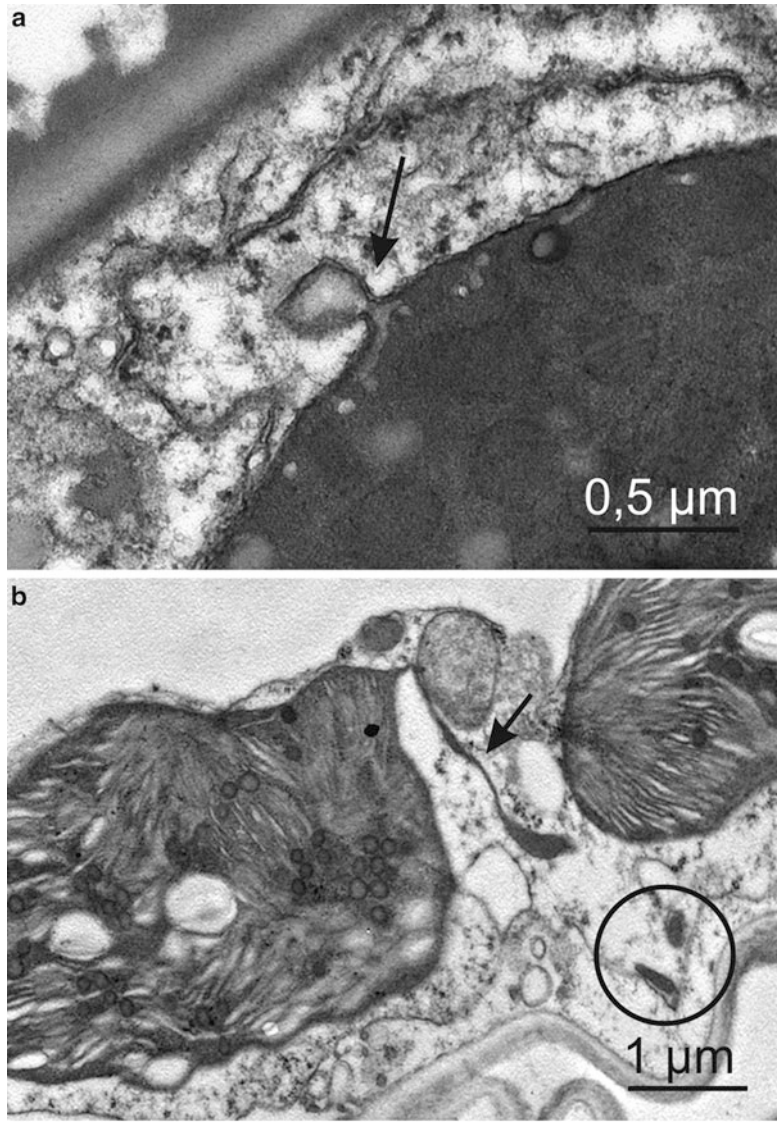

Fig. 14.5. Formation of vesicles and protrusions emerging from chloroplasts. (**a**) Chloroplast after infection of barley leaves with *Pyrenophora teres*. Vesicle (*arrow*) surrounded by two membranes pinching off from the chloroplast envelope. (**b**) Chloroplast with a stromule-like protrusion in a senescent barley flag leaf. *Encircled* are structures resembling RCB that, however, most likely represent cross-sections of similar protrusions.

severe damage of plastids by their contact to the vacuolar content.

There is no doubt that vesicles are involved in degradation of chloroplasts. Regulated vesicle formation has been reported for bacteria as well as for mitochondria (Neuspiel et al. 2008; Andrade-Navarro et al. 2009). It is therefore quite probable that vesicles from chloroplasts are formed in a controlled manner, although the molecular mechanism of vesicle formation remains to be elucidated. In order to understand the significance of RCB and SAV and their relationship, their properties and subcellular localizations remain to be determined with the same senescent leaf material.

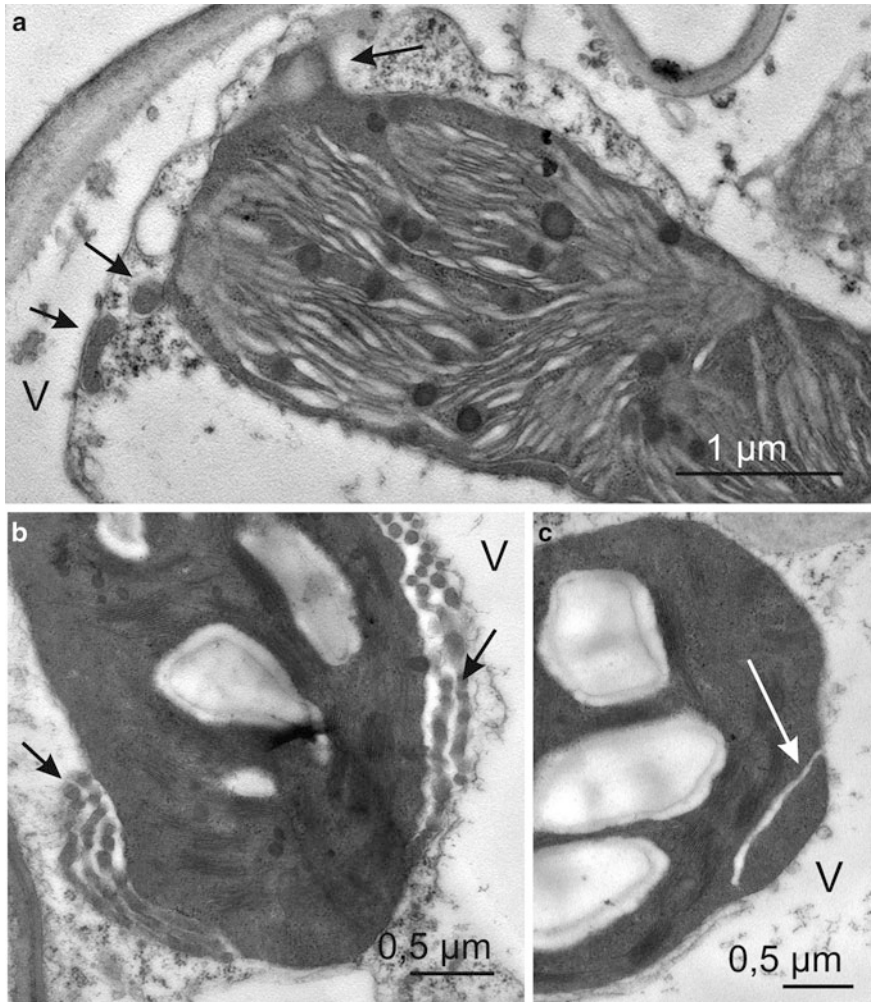

Fig. 14.6. Vesicle formation (*arrows*) from gerontoplasts which are located adjacent to the vacuole (V). (**a**) Section of a senescing barley flag leaf collected in the field after loss of more than 50 % of chlorophyll. *Arrows* point at sites of vesicle formation, (**b, c**) Chloroplasts observed in leaf segments of barley 30 h after wounding. They are surrounded by additional membranes. In (**b**), the chloroplast gives rise to many vesicles (*arrows*), whereas in (**c**), a portion of the chloroplast seems to bud off (*arrow*).

Although in recent years evidence for the degradation of chloroplast components outside the organelle has accumulated, another scenario is also possible. Kunst and Wrischer (1984) have observed empty spaces in chloroplasts of senescing leaves of *aurea* mutants which are hypersensitive to light. When we incubated barley leaf segments after removal of the epidermal cell layer on water, we observed empty spaces indicating intrinsic degradation of parts of the plastid stroma (Fig. 14.7). We suppose that under these particular conditions compounds are produced which favor degradation processes inside the organelle. It is likely that such processes also occur under natural conditions, but then they are likely not to be as dominant as under the extreme conditions leaves are exposed to during our experiments. Our ultrastruc-

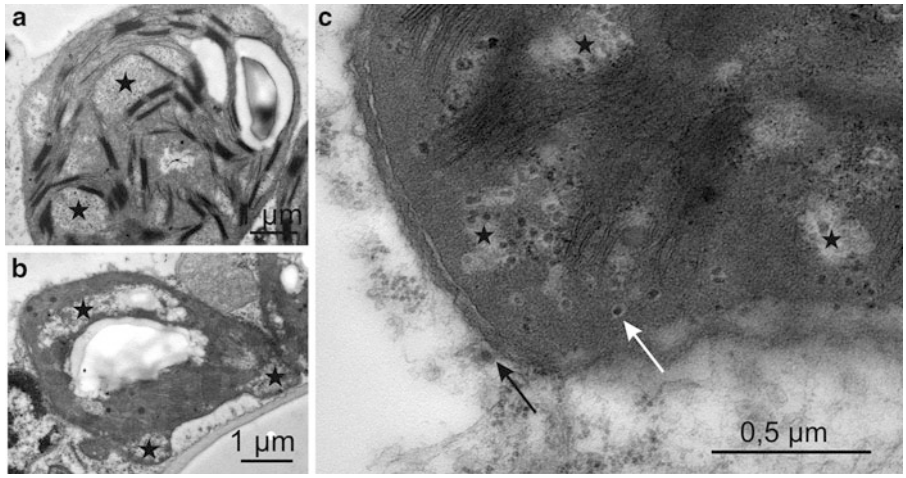

Fig. 14.7. Electron-lucent areas (*stars*) in degrading chloroplasts observed in leaf segments of barley 24 h (**a**), 30 h (**c**) and 40 h (**b**) after wounding. The *arrows* in (**c**) point at small globules, one of which is membrane-bound and situated outside the chloroplast directly at the outer chloroplast envelope (*black arrow*), whereas many globules are obvious inside the chloroplast and surrounded by an electron-lucent "halo" (*white arrow*).

tural observations suggest that at least some of the vesicles at the chloroplast periphery might not carry plastid components to the vacuole but deliver material potentially containing degrading enzymes into the chloroplast.

Mlodziansowski and Siwecki (1975) described starch degradation in Populus leaves infected by fungi. They also reported on the occurrence of electron-dense granules associated with starch degradation. We have seen similar granules around starch granules to be degraded and also much smaller granules in the stroma in barley chloroplasts disintegrating after wounding by removal of the epidermal cell layer (Fig. 14.7).

Considering that plastid proteins do not need to have N-terminal extensions for import into the organelle, but could also derive from the ER, the delivery of degrading enzymes into the organelle is likely. Gärtner and Nagl (1980) demonstrated acid phosphatase activity in senescing plastids and therefore called them plastolysomes. Recently, a cysteine protease has been identified in the proteome of the stroma of chloroplasts of pea (Bayer et al. 2011) clearly demonstrating that cysteine proteases can be imported into the organelle.

V. Digestion of Entire Organelles by the Vacuole

In a study on photosynthesis during senescence of wheat flag leaves it has been detected that the photosynthetic activity is not declining when it is calculated per chlorophyll rather than per leaf area (Camp et al. 1982). This result indicates that chloroplasts in the mesophyll of leaves efficiently function until a very late stage of senescence. The decline in chlorophyll content is rather due to a loss of entire chloroplasts than to a smooth transformation of chloroplasts into gerontoplasts (Camp et al. 1982). This interpretation is in accordance with ultrastructural observations on dying cells surrounded by intact cells with functional chloroplasts as reported for Festuca (Thomas 1977).

Furthermore, the result of Camp et al. (1982) is in accordance with a senescence associated decrease in the number of plastids per cell. The decline in the plastid population

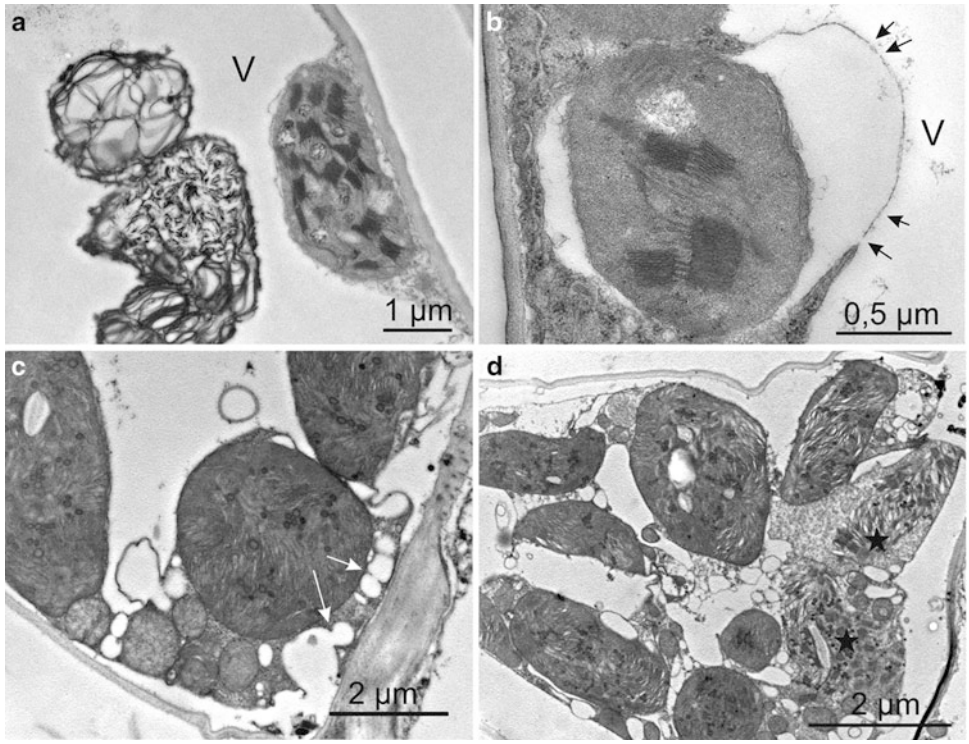

Fig. 14.8. Autophagy-like processes observed in senescent mesophyll cells of barley leaves. (**a**) Chloroplasts in a senescent leaf after 2 days of darkness. Two degenerated chloroplasts inside the vacuole (*V*) and a chloroplast in the remaining cytoplasm at the cell periphery can be observed. (**b**) Chloroplast in a leaf segment treated with 2 mM of salicylic acid after one day of treatment with 45 µM jasmonic acid. It is surrounded by an additional membrane layer which separates it from the tonoplast. The two membranes between chloroplast and central vacuole (*V*) appear to be perforated (*arrows*). (**c, d**) Senescent mesophyll cell in a flag leaf of the barley cultivar Lomerit collected in the field when 50 % of chlorophyll was lost. (**c**) The chloroplasts are surrounded by swollen cisternae which in places appear to fuse with each other (*arrow*). (**d**) A network of membrane cisternae separates portions of cytoplasm with single or multiple chloroplasts. Some portions contain degraded chloroplasts (*stars*).

has been described as a rather late event in development-dependent leaf senescence (Kura-Hotta et al. 1990) and was also observed late during senescence induced by darkness (Wittenbach et al. 1982) as well as during senescence induced by starvation (Mae et al. 1984; Ono et al. 1995). A study on wheat leaves showed that the decline in plastid number might occur in two phases. While 20 % of the chloroplasts were observed to disappear in parallel with the decline in chlorophyll content, many more chloroplasts disappeared at the final stage of senescence (Ono et al. 1995). The senescence-associated decline in chlorophyll content of a leaf is, however, not solely due to the loss of entire chloroplasts. Wada et al. (2009) showed that the decrease in the number of chloroplasts was accompanied by the decrease in the volume of chloroplasts during senescence of individually darkened leaves of Arabidopsis. Their observations suggest that both chloroplasts and gerontoplasts can be eliminated as entire organelles. Our own ultrastructural analyses on senescence of barley leaves collected in the field suggest that the formation of gerontoplasts precedes the final degradation of the organelle in the vacuole (Krupinska et al. 2012) (Fig. 14.8). Gerontoplast remnants have also been detected in vacuoles in studies with senescent primary foliage leaves of wheat (Wittenbach et al. 1982)

and were also reported to occur in French bean leaves (Minamikawa et al. 2001).

It has been proposed that the entire plastid might be engulfed in the central vacuole by a process resembling autophagy. This process is characterized by an invagination of the tonoplast membrane that finally surrounds the organelle (Matile 1975, 1997). Based on the results of Saito et al. (2002) describing a bulb structure in chloroplasts of Arabidopsis cotyledon leaves, Wada and Ishida in this volume (Chap. 19) postulated a phagocytosis-like engulfment of the chloroplasts by the central vacuole. However, such a mechanism still remains to be shown. Rather it seems that chloroplasts get surrounded by smooth cisternae and vesicular structures before they might get engulfed by the vacuole (Fig. 14.8).

The smooth cisternae and tubules surrounding chloroplasts might correspond to isolation membranes (phagophores) which engulf portions of the cytoplasm to form the double membrane autophagosomes (Ohsumi 2001). Similar structures surrounding plastids have been observed in endosperm and suspensor tissue (Wredle et al. 2001) and have also been proposed to be involved in the degeneration of mitochondria during senescence of mung bean cotyledons (Toyooka et al. 2001). The occurrence of membranes isolating organelles from the cytoplasm suggests the involvement of autophagy in degradation of chloroplasts/gerontoplasts. In accordance with this idea, identified whole chloroplasts in the vacuole of senescing mesophyll cells of Arabidopsis leaves, whereas they could not be observed in corresponding cells of mutants impaired in autophagy. The investigations on mutants deficient in autophagy revealed that autophagy is not only required for engulfment of the entire plastid into the vacuole but also for delivery of RCB/RCV to the vacuole (Wada et al. 2009; Wada and Ishida, Chap. 19).

We occasionally observed plastids surrounded by additional membranes in the vacuole both during early senescence of barley flag leaves collected in the field as well as after treatment of leaves with salicylic acid and jasmonic acid (Fig. 14.8). As senescence of the leaves progressed, more often clustered plastids and cytoplasm were observed to be degraded (Fig. 14.8). The clusters of plastids appeared to be surrounded by vacuolar structures which may represent autophagosomal compartments, vacuolar invaginations or evaginations (Fig. 14.9). The group of H. Ishida observed autophagy of RCB and whole chloroplasts under nutrient limiting conditions (Ishida et al. 2008; Wada et al. 2009b). Saito et al. (2002) observed vacuolar subregions called “bulb” in chloroplasts analyzed in cotyledons of Arabidopsis. The bulbs were observed to emit fluorescence in cells expressing a *γ-TIP:GFP* fusion construct indicating their origin from vacuolar membrane invaginations. Further characterization of these compartments by antibodies and pH measurements may help to clarify origin and fate of these bulbs.

Caging of chloroplasts inside an acidic compartment might be a mechanism for protection of the cell from toxic substances deriving from the leaky chloroplasts. Wada and Ishida (Chap. 19) conclude from a number of reports that one function of whole chloroplast autophagy is the quality control of organelles. In mutants impaired in autophagy, senescence-like processes were observed to be accelerated by cell death mechanisms which are negatively regulated by autophagy (Yoshimoto et al. 2009).

The observation that the caging of chloroplasts also occurs just after they have been released from wounded cells (C. Desel, unpublished) suggests that the process is very quick and therefore might be due to fusion of the existing tubular-cisternal network around the chloroplast. The signal for fusion is expected to be immediately delivered by the enclosed chloroplast.

Taken together, all these observations suggest that the close structural association between chloroplasts and ER is indicative of a functional association of the two compartments. During senescence, the ER cisternae surrounding the chloroplast might sense the state of the organelle, build up a barrier around it, and finally give rise to lytic compartments involved in degradation

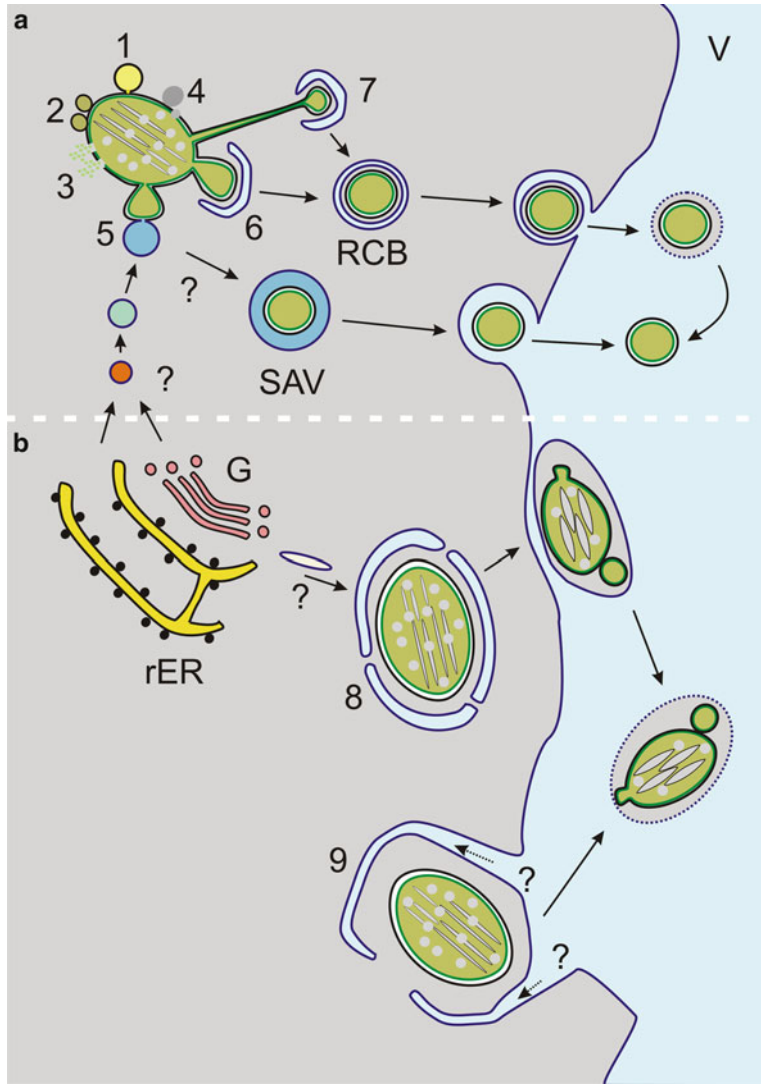

Fig. 14.9. Model summarizing hypothetic ways of chloroplast dismantling. The *arrows* indicate putative alternative routes; *question marks* point at events that still remain to be elucidated. (**a**) Involvement of vesicles in release and degradation of chloroplast material. (*1*) Fusion of a protease containing vesicle with a chloroplast. (*2*) Release of chloroplast content by vesicles. (*3*) Release of chloroplast content through the perforated envelope. (*4*) Release of plastoglobules through the chloroplast envelope. (*5*) Hypothetical formation of a SAV by fusion of an acidic, protease-containing vesicle (which probably originated from endoplasmic reticulum (*rER*) and Golgi-apparatus (*G*)) with a vesicle budding from the chloroplast. The outer membrane of the SAV fuses with the tonoplast and releases its content into the vacuole. (*6, 7*) Hypothetical formation of Rubisco-containing vesicles (RCB). (*6*) An autophagosome encloses a vesicle budding off from the chloroplast. (*7*) Enclosure of a stromule-derived vesicle by an autophagosome. This RCB surrounded by three membranes is engulfed by the vacuole (*V*), where first the outer membrane becomes degraded. (**b**) Digestion of whole chloroplasts. (*8, 9*) Two alternative routes of chloroplast transfer into the vacuole. (*8*) Enclosure of a chloroplast by autophagosomal cisternae. These fuse and release a membrane-bound chloroplast into the vacuole. (*9*) Branches of the central vacuole enclose one or several chloroplasts with surrounding cytoplasm (and other organelles). Inside the vacuole, the membrane around the engulfed material becomes degraded.

of the organelles. Recent investigations with optical scissors revealed strong attractive forces at membrane contact sites between ER and surface of plastids which vary during development (Andersson et al. 2007; see also Andersson, Chap. 8).

VI. Conclusions and Open Questions to Be Addressed by Ultrastructural Analyses

Based on the ultrastructural information on chloroplast dismantling that we have collected from the literature and supplemented by our own results on barley leaves, we propose new ideas on chloroplast dismantling, which we present in a scheme (Fig. 14.9). The data presented in this review demonstrate that chloroplasts are dismantled by several pathways. Their prevalence depends on the conditions inside and outside of the plant or leaf such as natural senescence, darkness, starvation, stress. The chloroplast-to-gerontoplast transition in the first phase of senescence includes relatively slow degradation processes that alter the morphology and composition of the organelle but retain its integrity and regeneration capacity until its function is severely disturbed. Nutrient recycling at this stage might include a vesicle trafficking between plastids and the endomembrane system and vice versa. At later stages of senescence, other types of vesicles, namely SAV or RCB, seem to be involved in chloroplast degradation (Fig. 14.9a). They could selectively or unselectively transport material out of, or into, the organelle. SAV and RCB appear to carry Rubisco into the central vacuole; many details on their properties, origin, development and fate remain to be determined. Entire chloroplasts might become engulfed into the central vacuole either after enclosure by autophagosomes or by direct uptake (Fig. 14.9b). Labeling of tonoplast marker proteins by specific antibodies could help to clarify the origin of the membranes surrounding chloroplasts at this stage.

In our opinion, the significance of the endomembrane network surrounding adult and senescing chloroplasts has so far been underestimated with regard to senescence-associated chloroplast dismantling. Although vesicles emanating from chloroplasts were considered in models of chloroplast dismantling (Matile 1992; Krupinska 2006), the involvement of vesicles fusing with the chloroplast envelope has been so far neglected. Detailed ultrastructural and immunogold-labeling studies with antibodies specific for compartment-specific proteins and for hydrolases supposed to enter the plastid from the ER might help to clarify the nature and function of the tubular-cisternal compartment around the chloroplasts. Because the vesicles and tubules involved in senescence-associated chloroplast dismantling are labile structures occurring transiently, future studies should be preferably done with material fixed under conditions of high-pressure.

Although ultrastructural analyses have been performed on senescing leaves for several decades, they are still important tools to get insights into the sequence of events leading to leaf senescence. In senescence research, such studies are expected to give rise to new ideas and hypothesis, when they include Arabidopsis mutants and transgenic lines showing altered senescence. To get a clear conception of the processes in living cells, ultrastructural analyses need to be combined with high resolution fluorescence microscopy using specific markers to follow the route of the chloroplast components and the other cellular compartments.

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Chapter 15

Plastoglobuli, Thylakoids, Chloroplast Structure and Development of Plastids

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Summary

The overview provides basic information on the appearance and biosynthesis of thylakoids and osmiophilic plastoglobuli and their association with chloroplast development and senescence. The light-induced formation of sun chloroplasts at high irradiation with a different thylakoid arrangement, grana stacking and plastoglobuli content as compared to shade chloroplasts at low irradiation is reviewed. During the light-induced biosynthesis of

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thylakoids from etioplasts of dark-grown seedlings the osmiophilic plastoglobuli disappear. Young chloroplasts are actually free of osmiophilic plastoglobuli. With increasing age of chloroplasts osmiophilic plastoglobuli appear again and are either fairly frequent at a small diameter or show up in a lower number with rather large size. In senescing chloroplasts and in their final form, gerontoplasts, thylakoids and chlorophylls are successively broken down with formation of large plastoglobuli. In addition to the plastoglobuli of chloroplasts, the occurrence and role of plastoglobuli during the development of chlorophyll-free plastid forms, such as proplastids, leucoplasts, and chromoplasts are presented. The main function of plastoglobuli as stores for plastidic lipids, such as plastoquinone-9, plastoquinol-9 and α-tocopherol and in certain plastid stages also other lipids is discussed. Recent observations suggest that plastoglobuli contain on their outer surface certain functional chloroplast proteins participating in biosynthesis and the channeling of lipid molecules.

I. Thylakoids and Osmiophilic Plastoglobuli as Structural Elements of Chloroplasts

Chloroplasts with their photochemically active thylakoids are essential organelles of all green plant tissues, e.g. leaves, green fruits and stems. They accomplish the overall process of photosynthesis, i.e. the light-induced transformation of inorganic CO_2 into energy-rich organic compounds such as sugars, starch, glycero-lipids and isoprenoid lipids (e.g. carotenoids, surplus α-tocopherol and plastoquinone-9). They also possess the competence for *de novo* fatty acid biosynthesis (Stumpf 1984) and their own, non-mevalonate pathway for isopentenyl diphosphate (IPP) and isoprenoid biosynthesis, the DOXP/MEP pathway (Lichtenthaler 1999, 2000, 2010). Thylakoids as photosynthetic biomembranes perform the photosynthetic light and associated electron transport reactions, including photolysis of water, that lead to the formation of ATP (photophosporylation) and the reducing agent NADPH, which function as coenzymes in the assimilation of CO_2 to sugar phosphates in the chloroplast stroma. This process is known as carbon reduction cycle, Calvin cycle or Calvin-Benson cycle (see e.g. Lichtenthaler 2007).

Chloroplasts are encircled by the chloroplast envelope that consists of two biomembranes, the outer and inner envelope biomembranes. The inner chloroplast matrix, the stroma, is characterized by two major structural elements: (1) the photochemically active thylakoids and (2) the osmiophilic, lipid-containing plastoglobuli of the chloroplast stroma. In addition, at high incident light condition chloroplasts, e.g. in sun exposed leaves, a third structural component shows up: starch grains. The latter are, however, not found at low light condition or in darkened plants, whereas thylakoids and osmiophilic plastoglobuli are regular chloroplast structures (see reviews Lichtenthaler 1966, 1968, 2007). The thylakoid biomembranes containing the photosynthetic pigments (Lichtenthaler and Park 1963; Lichtenthaler and Calvin 1964) bound to various chlorophyll-carotenoid protein complexes (Thornber 1975; Lichtenthaler et al. 1982a, b; Bennett 1983; Lichtenthaler and Babani 2004) are arranged in particular ways whereby one can differentiate between free, unstacked stroma thylakoids and grana regions (Lichtenthaler and Babani 2004; Lichtenthaler et al. 1984; Lichtenthaler 2007), where the circular grana thylakoids are stacked or appressed to each other forming either broad and high grana stacks (shade chloroplasts) or narrow and low grana stacks (sun chloroplasts) as shown in Fig. 15.1.

Abbreviations: α-T – α-tocopherol; Chl a/b – Ratio chlorophyll; LHCPs – Light-harvesting chlorophyll proteins; P – Osmiophilic plastoglobuli; PQ-9 and PQ-9•H_2 – Plastoquinone-9 (oxidized and reduced form); PTB – Protubular body; st – Starch

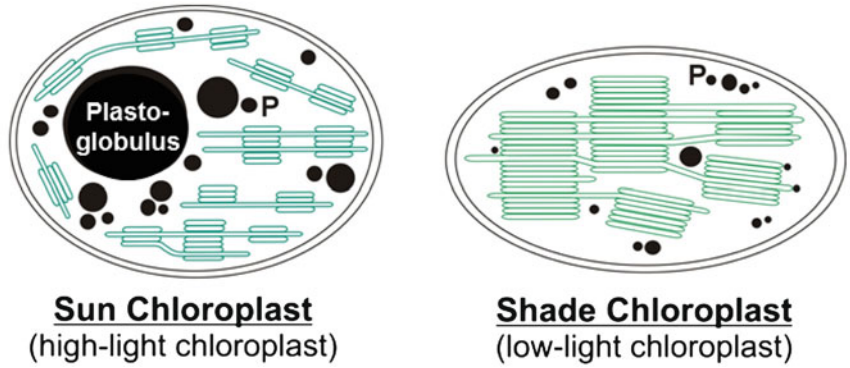

Fig. 15.1. Differences in the fine structure of sun-type and shade-type chloroplasts with regard to size and content of plastoglobuli as well as frequency, width and stacking degree of thylakoids. Sun-type chloroplasts usually contain one or several large starch grains which are not shown here. *P* osmiophilic plastoglobuli. (The scheme is based on the results of Lichtenthaler 1981; Lichtenthaler et al. 1981a, 1982b, 1984.)

Another way to address this particular thylakoid arrangement is to differentiate between appressed and exposed (non-appressed) thylakoid biomembranes. Sun chloroplasts are found in sun leaves and leaves of herbaceous plants grown at high-light conditions, whereas shade-type chloroplasts are found in shade leaves of trees and in plants grown at low light conditions.

A very essential structural element of chloroplasts is the possession of osmiophilic plastoglobuli that function as an extrathylakoidal storage site for excess isoprenoid lipids, such as α-tocopherol (vitamin E) and plastoquinone-9 (mainly present in the reduced plastoquinol form), traces of xanthophylls, but are practically free of chlorophyll, ß-carotene and phylloquinone K1 (Lichtenthaler 1964; Lichtenthaler and Sprey 1966; Grumbach and Lichtenthaler 1974; Lichtenthaler 2007). These 'osmiophilic globuli' as regular components of the chloroplast stroma have been termed "*osmiophilic plastoglobuli*" by Lichtenthaler and Sprey (1966) who isolated and analyzed plastoglobuli of several plants (*Billbergia, Eucharis, Tradescantia*). These 'osmiophilic globuli' were first seen and isolated in Melvin Calvin's laboratory in Berkeley by Park and Pon (1961) when they fractionated sonicated spinach chloroplasts and fixed the yellow, low density lipid layer of the 145,000 × g supernatant with OsO_4. They initially termed the osmium-stained spherical objects 'osmiophyllic granules' and stated that they corresponded "in size and staining properties to the osmiophilic granules observed in thin sections of osmium-stained chloroplasts in the intact leaf". In 1962, I started to work in the same laboratory and continued the studies of Park and Pon (1961) analyzing the lipoquinone and carotenoid composition of whole chloroplasts, isolated thylakoid membranes and 'quantasome aggregates' (Lichtenthaler and Calvin 1964). When analyzing the particular lipid fraction at the top of the centrifuge tube (145,000 × g supernatant) containing these 'osmiophilic globuli', I found that they contained high amounts of plastoquinol-9 (part of which was present in the oxidized form plastoquinone-9), α-tocopherol, trace amounts of xanthophylls, but no chlorophyll (see Lichtenthaler 1964). This finding is also, yet briefly mentioned in the paper by Lichtenthaler and Calvin (1964). In an independent research, yet also stimulated by the paper of Park and Pon (1961), two English laboratories started to work on the isolation of 'osmiophilic globules' of chloroplasts. Bailey and Whyborn (1963) isolated osmiophilic globules from spinach beet chloroplasts and found plastoquinone-9,

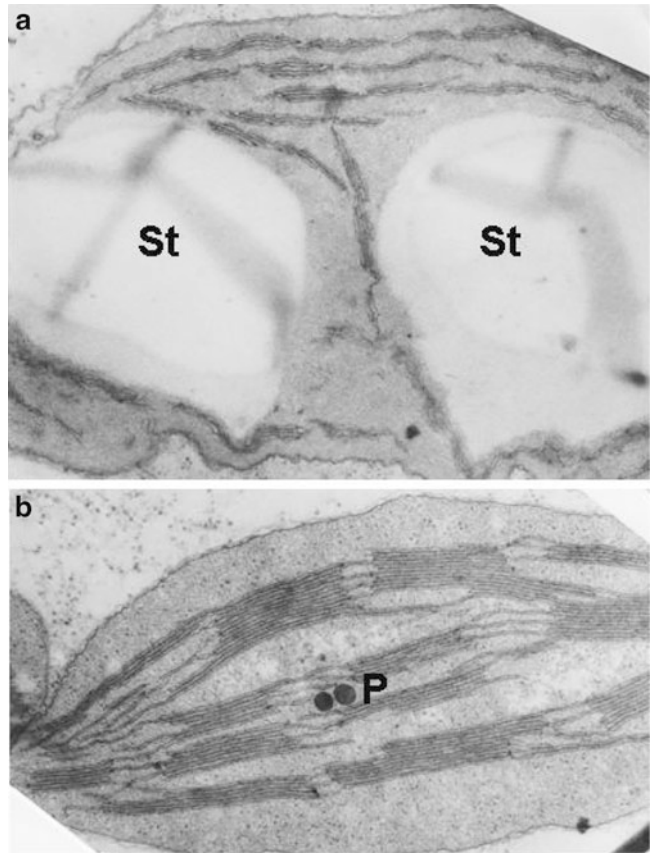

Fig. 15.2. (**a**) Sun (high-light) chloroplast with two starch grains (*St*) from the leaf of a high-light grown radish seedling and (**b**) shade (low-light) chloroplast of a young primary leaf of radish seedlings (*Raphanus sativus* L.) grown under low-light growth conditions. Fixation: glutardialdehyde + OsO_4. *P* Plastoglobuli.

a trace of chlorophylls but no ß-carotene, whereas Greenwood et al. (1963) isolated osmiophilic globules from chloroplasts of *Vicia faba* seedlings indicating that the globules contained plastoquinone-9, two galactolipids, and possibly less polar lipids, but were devoid of ß-carotene and chlorophyll.

The two isoprenoid compounds of plastoglobuli, plastoquinone-9 and α-tocopherol, that are regular essential components of thylakoids are particularly accumulated in excess amounts in chloroplasts of sun-exposed plants, sun leaves and plants grown under high-light condition and are stored in plastoglobuli (as reviewed by Lichtenthaler 2007). In contrast, chloroplasts in shade leaves and leaves of low-light plants exhibit only very low amounts of extrathylakoidally stored α-tocopherol and plastoquinone-9. As a consequence, sun-chloroplasts in sun and high light leaves possess a higher number and a larger size of osmiophilic plastoglobuli as compared to shade chloroplasts with only few small plastoglobuli (Fig. 15.1). α-Tocopherol and plastoquinol-9 are strong reducing agents that perfectly reduce OsO_4, and this determines the osmiophilic character of plastoglobuli (see also Lichtenthaler and Sprey 1966). An electron-micrograph of a typical sun and shade chloroplast from the leaves of high-light and low-light grown radish seedlings is shown in Fig. 15.2. The sun or high-light chloroplast exhibits large starch grains and on most

micrographs also several medium size plastoglobuli, which – although present – are rare in the shade chloroplast.

II. Differences in Pigment Composition, Ultrastructure and Photosynthetic Rates of Sun and Shade Chloroplasts

There exist considerable differences between sun and shade chloroplasts not only in their ultrastructure, but also in their quantitative composition of photosynthetic pigments, electron carriers, and photosynthetic rates (Boardman 1977; Lichtenthaler et al. 1981a; Meier and Lichtenthaler 1981; Wild et al. 1986; Givnish 1988; Anderson et al.,1995). Under high irradiance (sun leaves and high-light leaves) sun chloroplasts exhibit much higher rates of photosynthetic CO_2 assimilation on a leaf area basis and on a chlorophyll basis as compared to shade leaves or leaves from low light plants (as reviewed by Lichtenthaler and Babani 2004). Sun leaves that receive much more incident light as compared to shade leaves, consequently invest predominantly in higher rates of photosynthetic quantum conversion, whereas shade leaves and shade chloroplasts invest in a large pigment antenna in order to overcome the general shortage of light. As a consequence, there are essential differences in the composition of pigments and chlorophyll-carotenoid protein complexes. Thus, sun chloroplasts exhibit higher values for the ratio chlorophyll a/b, a much lower level of light-harvesting chlorophyll a/b proteins (LHCII), and, accordingly, a lower stacking degree of thylakoids (lower amounts of appressed membranes) than shade chloroplasts (Lichtenthaler et al. 1981a, 1982b, 1984). In addition, they possess a higher level of the chlorophyll a/ß-carotene protein complex CPa (including CP47, CP43 and the PS II reaction center complex), and the ß-carotene containing pigment-protein complexes (CPI and CPIa), which also comprise the reaction centers of PS I. Detailed information on these individual pigment protein complexes are given in the following publications (Thornber 1975; Lichtenthaler et al. 1982a; Bennett 1983; Green and Durnford 1996; Nelson and Yocum 2006). Some of the major differences in the characteristics of sun and shade chloroplasts are contrasted in Table 15.1.

The weight ratio chlorophyll a/b of chloroplasts from sun ranges from 2.9 to 3.8; in contrast, those of shade leaves and shade chloroplasts exhibit values of 2.3–2.8. Moreover, with respect to the chlorophyll a+b levels, sun leaves and high-light chloroplasts are characterized by a higher proportion of total carotenoids. Consequently, the higher carotenoid content of sun leaves on a chlorophyll basis is documented by significantly lower values for the weight ratio of chlorophylls/carotenoids (a+b)/(x+c) ranging from 3.6 to 4.6 as compared to shade leaves with significantly higher values (range: 4.9–7.0). Such pigment values and ratios are given for sun and shade leaves of beech in Table 15.2. Additional examples are found in Lichtenthaler and Babani (2004); Lichtenthaler (2007) and Sarijeva et al. (2007). The photosynthetic pigments, chlorophylls *a* and *b* as well as total carotenoids x+c, had simultaneously been determined spectrophotometrically (Lichtenthaler 1987) in the same leaf pigment extract solution, and the individual carotenoids were differentiated via HPLC (Schindler and Lichtenthaler 1996).

Sun leaves of beech possess on a total carotenoid basis a higher percentage of ß-carotene (36%), xanthophyll cycle carotenoids (violaxanthin+zeaxanthin+antheraxanthin) (19%), as compared to shade leaves, which exhibit only a percentage of 26 and 12, respectively (Table 15.2). These xanthophylls perform the high light induced xanthophyll cycle (photoreduction of violaxanthin to zeaxanthin) (Yamamoto et al. 1962; Yamamoto 1985; Demmig-Adams and Adams 1992, 1996; Schindler and Lichtenthaler 1996; also cf. Lichtenthaler

Table 15.1. Some characteristic, structural differences between sun and shade chloroplasts of leaves with respect to thylakoid and plastoglobuli frequency as well as differences in the level of total plastoquinone-9 (oxidized and reduced form: PQ-9 and PQ-9•H_2), α-tocopherol (α-T) and light-harvesting Chl a/b proteins (LHCPs) are provided. The differences in the ratio of appressed to exposed thylakoid biomembranes are indicated as well. Higher values of individual parameters either in sun or shade chloroplasts are high lighted in bold print. In the case of Fagus chloroplasts electromicrographs of sun and shade leaves and in the case of Raphanus, Triticum and *Zea mays* chloroplast electromicrographs of seedlings grown at high-light and low-light conditions were investigated (Based on Lichtenthaler 1981; Lichtenthaler et al. 1981a, 1984)

Sun chloroplasts	Shade chloroplasts
Low thylakoid amounts (per chloroplast section)	**High thylakoid amounts** (per chloroplast section)
Narrow grana stacks (width: 0.2–0.26 µm)	**Broad grana stacks** (width: 0.33–0.5 µm)
Few thylakoids per granum	**High grana stacks**
Lower stacking degree (%)	**High stacking degree** (%)
Fagus: 57 ± 6	**82** ± 6
Raphanus: 55 ± 5	**64** ± 4
Triticum: 54 ± 5	**73** ± 3
Zea mays: 55 ± 3	**77** ± 3
Appressed thylakoids (low level)	**Appressed thylakoids** (high level)
Appressed/exposed thylakoids	**Appressed/exposed thylakoids**
Fagus: 1.3	**4.7**
Raphanus: 1.2	**1.8**
Triticum: 1.2	**2.7**
Zea mays: 1.2	**3.3**
Low levels of LHCPs	**High levels of LHCPs**
Numerous and large plastoglobuli	Few small plastoglobuli
High amounts of excess α-T	Low α-T levels
High level of excess plastoquinone-9 (PQ-9 + PQ-9•H_2)	No excess plastoquinone-9 (PQ-9 + PQ-9•H_2)
Large starch grains	No starch
High photosynthetic rates	Low photosynthetic rates

2007) which helps to protect the photosynthetic apparatus against photoinhibition and photo-oxidation. The higher level of ß-carotene in sun leaves is also indicated by the lower value of 8.8 for the ratio a/c (chlorophyll *a* to ß-carotene), and the weight ratio of xanthophylls/ß-carotene (x/c) of 1.8 as compared to shade leaves with values of 14.5 and 2.8, respectively (Table 15.2). ß-carotene is exclusively found in the reaction center chlorophyll *a*-pigment-proteins CPI and CPa of both the photosystems, PSI and PSII, which are more frequent on a chlorophyll basis in sun-type chloroplasts. In contrast, the lower proportion of lutein and neoxanthin in sun leaves and sun chloroplasts is due to the fact that both these xanthophylls are specifically bound to the light-harvesting chlorophyll a/b proteins (LHCPs) (Lichtenthaler et al. 1982a) that are less frequent in chloroplasts of sun leaves (Lichtenthaler et al. 1982b).

III. Biosynthesis of Thylakoids from Etioplasts

A. Etioplast Structure

When seedlings are grown in the dark the proplastids of primary leaves or cotyledons are enlarged and develop into etioplasts (Solymosi and Aronsson, Chap. 4), which are characterized by well-structured, crystalline-like prolamellar bodies ('Heitz-Leyon-crystals') and numerous small osmiophilic plastoglobuli as has been shown in detail in barley plants by Sprey and Lichtenthaler (1966). That the light-induced thylakoid genesis proceeds via vesicle addition to primary thylakoids and that prolameller bodies are used for thylakoid biosynthesis had already been realized by Menke (1961) and Schnepf (1964). The function of osmiophilic plastoglobuli in this process was, however,

Table 15.2. Differences in pigment levels (of chlorophylls; carotenoids) and in pigment weight ratios between sun and shade leaves of beech (*Fagus sylvatica* L.)[a]

	Sun	Shade
Pigment levels (mg m^{-2})		
Chlorophyll *a*+*b*	508	378
Chlorophyll a	389	275
Chlorophyll b	119	103
Carotenoids x+c	123	72
ß-Carotene (c)	44	19
Xanthophylls (x)	79	53
Pigment ratios		
Chl *a*/*b*	**3.27**	2.67
Chl *a*/ß-carotene, *a*/c	**8.8**	14.5
x/c	**1.8**	2.8
(*a*+*b*)/(x+c)	**4.14**	5.25
% Composition of carotenoids		
ß-Carotene	**36**	26
Lutein	**38**	52
Neoxanthin	**7**	10
v+an+z	**19**	12
Zeaxanthin (z)	**11**	0
Antheraxanthin (an)	**3**	1
Violaxanthin (v)	**5**	11

[a]The pigment levels of fully developed leaves are given in mg m^{-2} leaf area. For better comparison the pigment ratios and percentage carotenoid composition of sun leaves are shown in bold print. Mean values of six determinations on a sunny day in July. Standard deviation < 7% (pigment levels) and < 4% (pigment ratios). The differences are significant $p < 0.01$. v+an+z is the sum of the xanthophyll cycle carotenoids (violaxanthin + antheraxanthin + zeaxanthin, respectively), *x* xanthophylls and *c* ß-carotene. The ratio (*a*+*b*)/(x+c) is the weight ratio of total chlorophylls *a*+*b* to total carotenoids x+c. The photosynthetic pigments, Chls *a* and *b* as well as total carotenoids x+c, were determined spectrophotometrically (Lichtenthaler 1987; Lichtenthaler and Buschmann 2001) and the individual carotenoids via HPLC (Schindler and Lichtenthaler 1996). The ß-carotene fraction shown here contains minor amounts (usually < 5%) of α-carotene

pointed out only by Sprey and Lichtenthaler (1966) (see also Lichtenthaler 1967). In etioplasts the electron-dense plastoglobuli (diameter 40–90 nm) are mostly arranged in groups that are located in the etioplast stroma around or in between the readily growing prolamellar bodies. In etiolated primary leaves of dark grown barley seedlings the number of plastoglobuli considerably increases from day 5 to day 10 paralleling the enlargement of prolamellar bodies (Fig. 15.3a). Plastoglobuli are visible only when the leaf tissues were fixed either with OsO_4 or with glutardialdehyde + OsO_4. When, as in early electron microscopic investigations in the late 1950s, $KMnO_4$ was applied as fixans, the plastoglobuli did not show up.

B. Thylakoid Formation

Upon illumination of etiolated leaf tissue the process of thylakoid formation starts. This is indicated by a rapid disappearance of prolamellar bodies and osmiophilic plastoglobuli. In fact, the material of both structural elements is used up and transformed to the first thylakoids (Sprey and Lichtenthaler 1966). In the electron micrographs one detects early longitudinal thylakoid structures with many vesicles that attach to the thylakoid ends and prolongate the thylakoids (Fig. 15.3b, c). In this developmental stage the osmiophilic plastoglobuli have practically disappeared (Sprey and Lichtenthaler 1966). During this light-induced phase of thylakoid biosynthesis apparently all lipids deposited in the plastoglobuli in the dark are used for the construction of the lipid bilayer of active thylakoids and their pigment protein complexes. In fact, we found that during the first hours of illumination of dark grown seedlings those carotenoids (lutein, violaxanthin) and prenylquinones (plastoquinol-9, plastoquinone-9, α-tocopherol) which are present in plastoglobuli are not accumulated, whereas ß-carotene as well as the naphthoquinone phylloquinone K1 are accumulated at high rates (Lichtenthaler 1969d). This observation is an essential additional indication that the plastoglobuli lipids of etioplasts are consumed in the biosynthesis of the first thylakoids.

C. Young Chloroplasts

After about one day of illumination the etioplasts have been fully converted to young completely functional chloroplasts with the typical grana and stroma thylakoid structures. However, plastoglobuli are not present at this

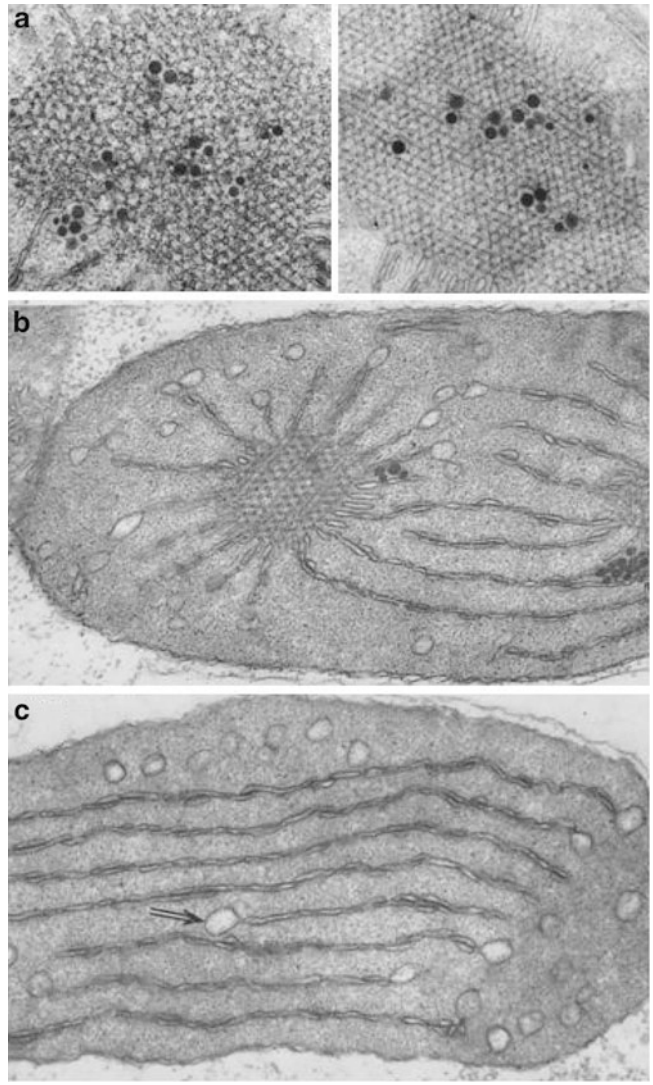

Fig. 15.3. Light-induced biosynthesis of photochemically active thylakoids from prolamellar bodies and plastoglobuli of etioplasts in primary leaves of etiolated barley (*Hordeum vulgare* L.) seedlings (**a**–**c**). Prolamellar bodies and plastoglobuli successively disappear with the appearance of the primary thylakoids. The latter are elongated by fusion with flattening vesicles (see *arrow* in (**c**)). (Based on Sprey and Lichtenthaler 1966.)

stage as shown for young chloroplasts of barley and radish (Fig. 15.4). We have proven this fact for various other plants as well, and it was checked via serial cuts through the same chloroplast. Only several weeks after greening of the leaves and chloroplast formation do single osmiophilic plastoglobuli show up again. Their size and number are increasing with time, especially at high light conditions and in sun-exposed leaves.

IV. Plastoglobuli in Older Chloroplasts and Gerontoplasts

In older chloroplasts plastoglobuli show up and can be rather numerous and/or reach larger sizes. This happens especially in chloroplasts of green leaves that are several years old. Concerning the size and frequency of osmiophilic plastoglobuli in leaf chloroplasts, there seem to exist two strategies in

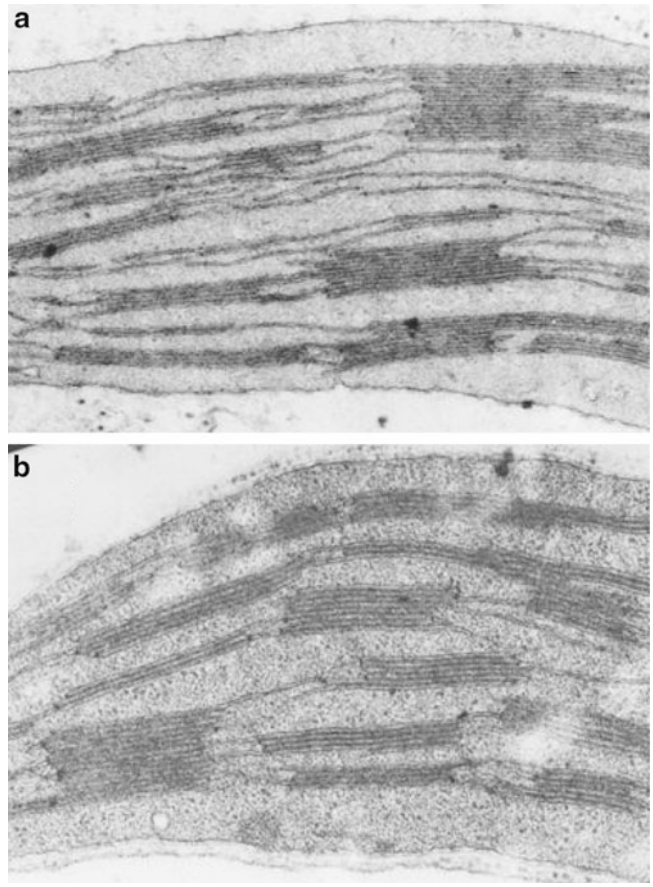

Fig. 15.4. Young leaf chloroplasts (4 days after light-induced thylakoid formation) with stroma and grana thylakoids lacking osmiophilic plastoglobuli. (**a**) From radish (*Raphanus sativus* L.). (**b**) From barley (*Hordeum vulgare* L.).

annual plants: (1) in spinach and other herbaceous plants the number of osmiophilic plastoglobuli increases in older leaves (before the autumnal senescence) up to several hundred per chloroplast at a rather small diameter in the range of 0.070–0.2 μm (Lichtenthaler 1969a), and (2) in older sun-exposed leaves of the deciduous trees beech and oak fewer but considerably larger plastoglobuli (diameter range: 0.2–1.5 μm) are formed (Lichtenthaler 1968). Numerous as well as larger plastoglobuli were found in the perennial leaves of Billbergia (cf. Fig. 15.7b), in Hoya and in the stem of Cereus (cf. Fig. 15.5). In addition, in green Ficus leaves of several years only few yet particularly large plastoglobuli with a diameter range of 0.3–3.0 μm (Fig. 15.6) and single, even larger ones (Fig. 15.7c) were detected (Lichtenthaler and Weinert 1970). Such large plastoglobuli in Ficus chloroplasts had first been observed by Falk (1960) and were termed "magnoglobuli".

Concerning chloroplast breakdown and gerontoplast formation one has to differentiate between (1) an early stage that still contains some thylakoids and (2) a later stage where thylakoids, chlorophylls and most of the stroma protein are already broken down. This is found, e.g., in degenerating chloroplasts of spinach where initially many plastoglobuli are formed with a somewhat larger size than previously in green chloroplasts (Lichtenthaler 1969b). However, when the thylakoids and stroma proteins are almost fully broken down, the osmiophilic plastoglobuli

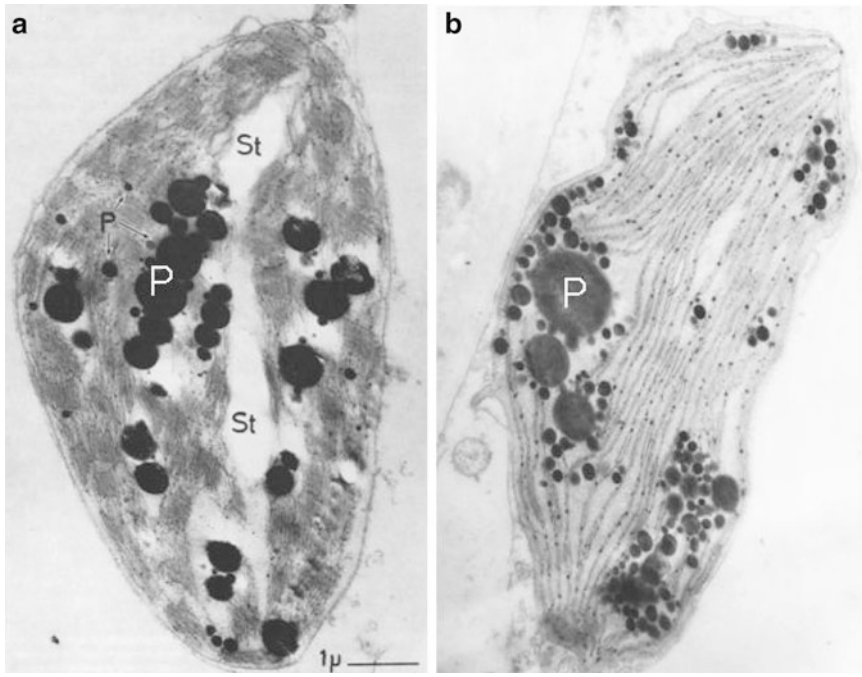

Fig. 15.5. Chloroplasts with numerous osmiophilic plastoglobuli in green plant tissue that is several years old. (**a**) From leaves of *Hoya carnosa* R.Br, and (**b**) from the green stem of *Cereus peruvianus* (L.) Mill. *P* Plastoglobuli, *St* Starch. Fixation OsO_4. (Based on Lichtenthaler and Peveling 1966 and 1967a; Lichtenthaler 1969e, modified.)

flow together to form a few large plastoglobuli (Fig. 15.8) (Lichtenthaler 1969b). A similar situation is found in chloroplasts of the perennial leaves of *Ficus* where the osmiophilic plastoglobuli are present in fewer numbers but larger sizes (cf. Fig. 15.6) as compared to spinach. Here as well, the plastoglobuli will form one large osmiophilic plastoglobulus in the final gerontoplast stage (Lichtenthaler and Weinert 1970).

During natural or induced chloroplast degeneration, an increase of plastoglobuli numbers had already been described by Toyoma and Ueda (1965) and Thompson et al. (1964) as well as an increase of the plastoglobuli size (Lamprecht 1961). Moreover, an increased appearance of osmiophilic plastoglobuli had also been found in an artificial chlorosis induced by treatment with thio-urazil (Heslop-Harrison 1962) and in the yellowish xantha-3-mutants of barley (v. Wettstein 1957). An excellent review of the fate, activities and ultrastructural changes of chloroplasts during leaf senescence including observations on plastoglobuli has been given by Krupinska (2007) (see also Mulisch and Krupinska, Chap. 14).

V. Lipid Composition of Plastoglobuli in Different Plastid Forms

A. Chloroplasts

The osmiophilic plastoglobuli are regular globular chloroplast structures primarily containing high excess amounts of plastoquinone-9 (PQ-9), plastoquinol-9 (PQ-$9H_2$) and α-tocopherol (α-T) (Lichtenthaler 1964, 1968, 2007; Lichtenthaler and Sprey 1966) that cannot be deposited in the thylakoids. In plastoglobuli from older spinach leaves we found besides PQ-9 and PQ-$9H_2$ and α-T also considerable amounts of a second

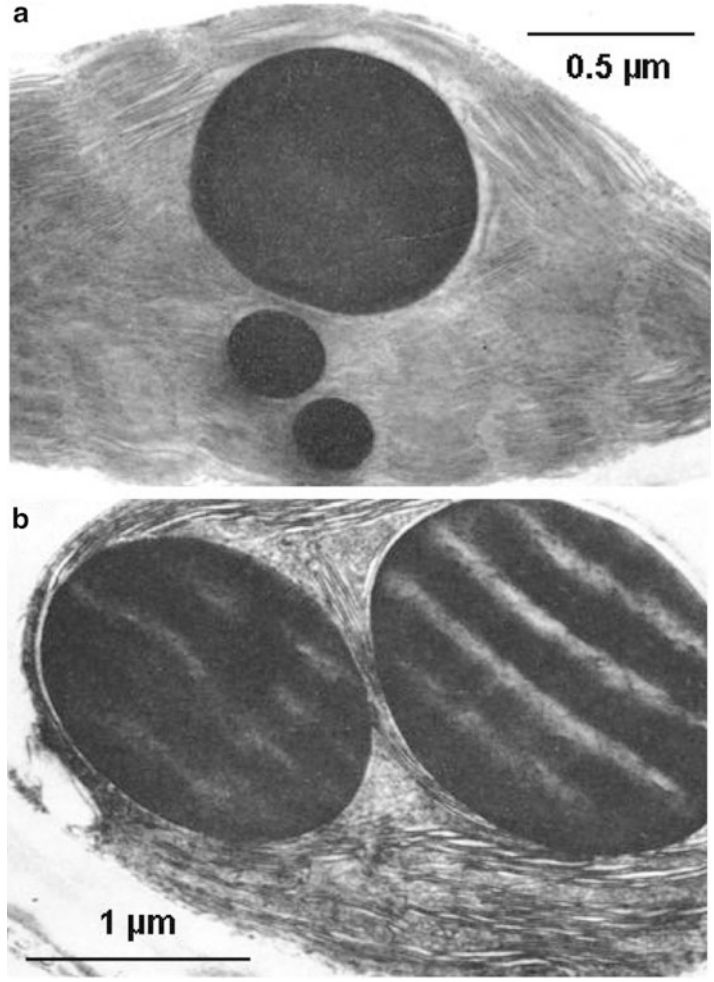

Fig. 15.6. (**a**) Large osmiophilic plastoglobuli ("magnoglobuli") in a chloroplast of a 5-year old green leaf of *Ficus elastica* Roxb. and (**b**) large plastoglobuli in a gerontoplast of a yellow-green *Ficus* leaf. Fixation: OsO_4, postfixation with lead citrate. (Based on Lichtenthaler and Weinert 1970.)

plastoquinone (PQ-B) which is not present in the thylakoid fraction or only in trace amounts (Lichtenthaler 2007). The reduced prenylquinones (PQ-$9H_2$ and α-T) determine the osmiophilic character of plastoglobuli. However, the osmiophilic plastoglobuli of chloroplasts, when purified from small thylakoid membrane fragments by a second centrifugation step, do not contain chlorophylls or phylloquinone K1 compounds which are exclusively bound to thylakoids. Carotenoids (xanthophylls) may be present in trace amounts; yet these might come from a contamination of the plastoglobuli fraction with chloroplast envelope membranes which are known to contain violaxanthin and neoxanthin (Lichtenthaler et al. 1981b). However, ß-carotene is not found in isolated purified plastoglobuli of functional leaf chloroplasts. In this respect we investigated plastoglobuli isolated from chloroplasts of green leaves of spinach, barley, radish (*Raphanus sativus*), *Ficus* and *Billbergia*. Besides plastoquinone-9, plastoquinol-9 and α-tocopherol which are continuously accumulated in plastoglobuli with

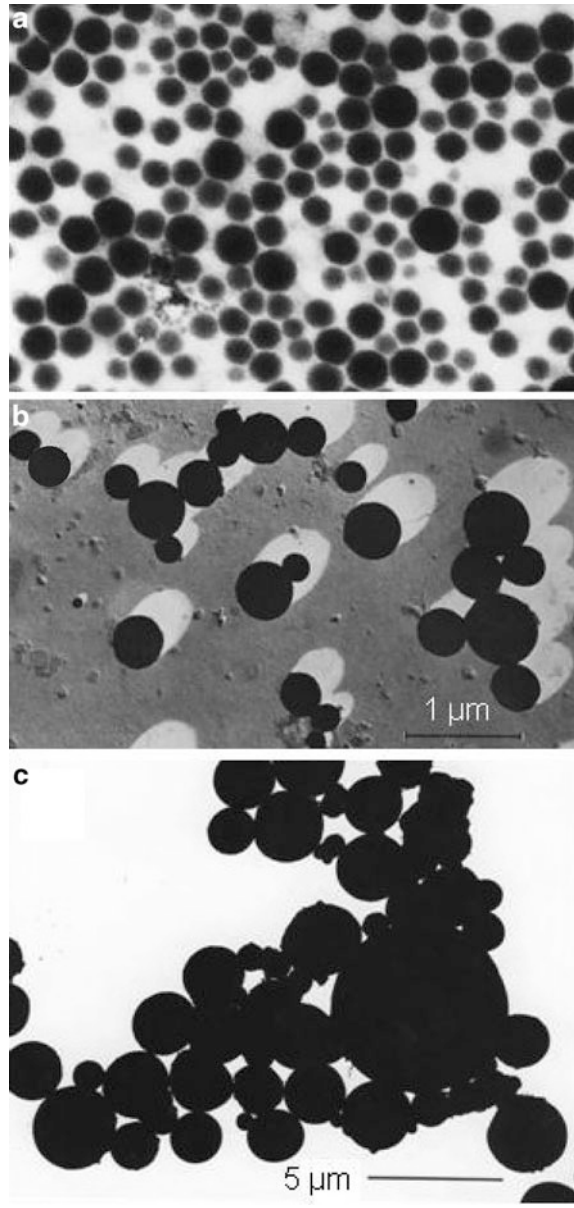

Fig. 15.7. Osmiophilic plastoglobuli isolated from chloroplasts of *green* leaves (fixation OsO_4). (**a**) Small plastoglobuli (diameter: 0.06–0.13 µm) from spinach, (**b**) from leaves of *Billbergia forgetiana* Sander with chromium shadowing, and (**c**) very large plastoglobuli from *Ficus elastica* chloroplasts of leaves of several years old. (Based on Lichtenthaler and Sprey 1966; Lichtenthaler 1968, 1969a; Lichtenthaler and Weinert 1970.)

increasing age of leaf-chloroplasts (see Lichtenthaler 2007), plastoglobuli tend to contain some galactolipids and small amounts of triacyl-glycerols (neutral lipids) that we found in isolated plastoglobuli fractions of Ficus and Tilia chloroplasts (Lichtenthaler, unpublished), with only traces of phospholipids. Phospholipids and galactolipids, however, appear to be stored only temporarily in plastoglobuli of functional chloroplasts. Whether plastoglobuli store larger amounts of neutral lipids, such as triglycerides, still

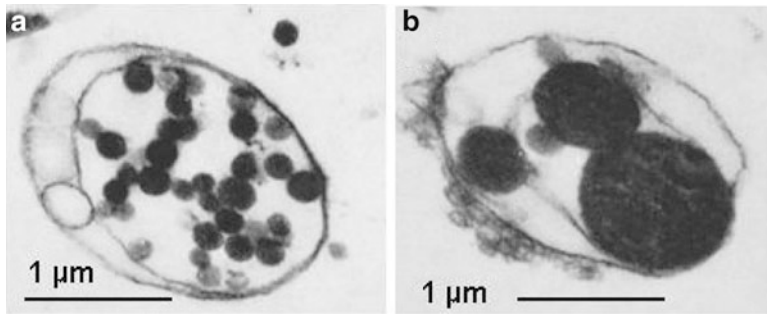

Fig. 15.8. Degeneration of chloroplasts in senescing yellow spinach (*Spinacea oleracea* L.) leaves, i.e. formation of gerontoplasts (**a**) at an early stage with many smaller plastoglobuli and (**b**) at a later stage with few larger plastoglobuli. Fixation: OSO_4. (Based on Lichtenthaler 1969b.)

remains an open question. Relatively high amounts of triacylglycerols (neutral lipids) had earlier been described for an isolated beech plastoglobuli fraction (Tevini and Steinmüller 1985), yet the occurrence of more than minor amounts of triglycerides in plastoglobuli of beech chloroplasts is not certain. The problem is, that in this investigation the chloroplast and plastoglobuli fractions isolated from beech sun leaves were contaminated by large cytosolic lipid droplets (lipid bodies) containing neutral triglycerides. These lipid bodies are about the size of chloroplasts and co-sediment with chloroplasts. For this reason they show up in the isolated plastoglobuli fraction and bring with them large amounts of triacyl-glycerides. However, translucent plastoglobuli isolated from chloroplasts of wheat seedlings, treated with the herbicide norfluorazone, contained considerable amounts of triacyl-glycerides and phytoene as well as distinct amounts of phytofluene, plastoquinone-9 and α-tocopherol (Dahlin and Ryberg 1986).

B. Senescing Chloroplasts and Gerontoplasts

During autumnal or stress-induced senescence of leaves, thylakoids and their chlorophyll-carotenoid-proteins are successively broken down. Chlorophylls and most of ß-carotene are degraded, whereas the major part of xanthophylls (lutein, violaxanthin, neoxanthin) is preserved. Parallel to the thylakoid breakdown the number and size of osmiophilic plastoglobuli of senescing spinach chloroplasts initially increase, but at the final stage of chloroplast degeneration the osmiophilic plastoglobuli decrease by forming few and large plastoglobuli (Lichtenthaler 1969b). The formation of few and very large plastoglobuli has also been shown in the gerontoplasts of senescing yellow-green leaves of *Ficus elastica* (Lichtenthaler and Weinert 1970). In both cases, the isolated plastoglobuli contained carotenoids and galactolipids and to some extent also phospholipids.

C. Chromoplasts

By contrast, plastoglobuli of typical carotenoid-rich chromoplasts possess, besides α-tocopherol, plastoquinol-9 and plastoquinone-9, carotenoids and secondary carotenoids. This has been shown for plastoglobuli isolated from chromoplasts of the yellow petals of common broom flowers (*Sarothamnus scoparius* = *Cytisus scoparius)* (Lichtenthaler 1970a), and also in plastoglobuli isolated from chromoplasts of tulip flowers that turn their petals from green to yellow (Lichtenthaler 1970b). Carotenoids are less osmiophilic than

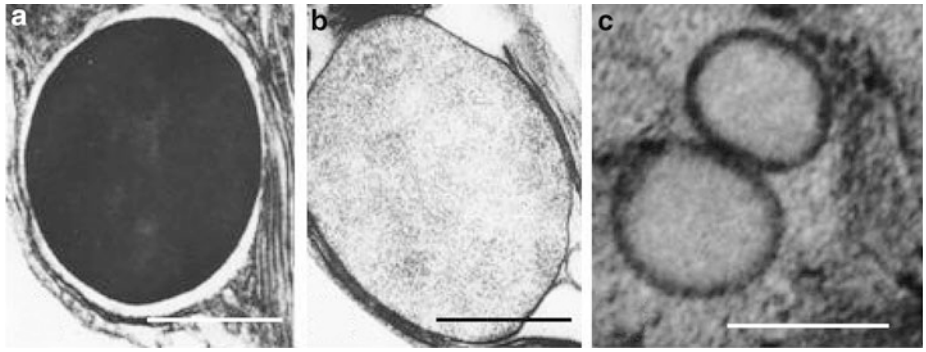

Fig. 15.9. Plastoglobuli in chloroplasts of a green leaf of *Billbergia forgetiana* Sander: (**a**) fixed with OsO_4, (**b**) fixed with glutardialdehyde and $KMnO_4$ (surrounded by a "membrane" and a greytone interior), (**c**) two translucent plastoglobuli of a sun chloroplast from the leaf of a radish plant grown at high-light conditions (fixation with glutardialdehyde and postfixed with OsO_4). The *bar* in (**a**) and (**b**) marks 0.5 µm and in (**c**) 0.2 µm. (Based on Lichtenthaler and Sprey 1966.)

plastoquinol-9 and α–tocopherol. Carotenoids and secondary carotenoids accumulate in developing chromoplasts at much higher rates as compared to plastoquinol-9 and α–tocopherol (Lichtenthaler 1969c). As a consequence, the osmiophilic character of plastoglobuli that increase in number during chromoplast development (Sect. VII.E) is generally lower than that of chloroplast plastoglobuli that are practically free of carotenoids.

D. Etioplasts

Also in a plastoglobuli fraction isolated from thylakoid-free barley etioplasts, which contain, besides the prolamellar bodies, very many small plastoglobuli, we found carotenoids (primarily lutein and violaxanthin), in addition to plastoquinone-9, plastoquinol-9 and α-tocopherol. ß-Carotene which is present in etioplasts in trace amounts only was not observed in the isolated plastoglobuli of etioplasts. Some other lipid material, e.g. galactolipids and traces of phospholipids, could be detected. However, we did not find any particular protein. Protochlorophyllide-binding-proteins were definitely not present in the plastoglobuli fraction.

VI. Appearance of Plastoglobuli on Electron Micrographs

The osmiophilic plastoglobuli show up on electron micrographs (transmission electron microscope) when the leaf or other green plant tissue is fixed by OsO_4 or by a combination of glutardialdehyde with OsO_4. They appear as round globular lipid droplets that are osmiophilic due to their high content of the reducing agents plastoquinol-9 and α-tocopherol which readily reduce OsO_4. Since OsO_4 is volatile, it easily penetrates plastoglobuli and is reduced inside plastoglobuli and on their surface as well. This then results in homogeneously fixed osmiophilic plastoglobuli as shown in Fig. 15.9a for a plastoglobulus of a Billbergia chloroplast. This plastoglobulus is surrounded by a hyaline phase of the chloroplast stroma. It is neither in close or full contact with thylakoids, nor is the plastoglobulus surrounded by a membrane or half membrane. The ends of stroma thylakoids can, however, touch the plastoglobulus. In fact, osmiophilic plastoglobuli are only visible when the leaf tissues had been fixed either with OsO_4 or with glutardialdehyde + OsO_4.

The situation is, however, different when other fixatives are applied. When $KMnO_4$ is

taken as fixative for small leaf pieces, as had often been done in early electron microscopic investigations in the late 1950s, thylakoids and the stroma are contrasted, but the plastoglobuli do not show up. Instead "star-shaped bodies" can be seen, especially at sites of medium-sized plastoglobuli, where the $KMnO_4$ had been reduced at the outside of the plastoglobulus and was deposited at the site of its reduction. The problem is that the water soluble $KMnO_4$ cannot penetrate the lipid interior of the plastoglobuli and is only reduced and deposited on the outer surface of the plastoglobuli.

With glutardialdehyde as sole fixative plastoglobuli do not show up. The lipids of plastoglobuli are not fixed and their lipids are extracted during the dewatering of the small fixed leaf piece with alcohol solutions before embedding it in Epon. The situation is again different when glutardialdehyde is used as fixans together with $KMnO_4$. Then the plastoglobuli of Billbergia appear surrounded by a "membrane" and their interior is structured by a grey tone (Fig. 15.9b). This plastoglobulus "membrane" is definitely not a biomembrane per se; it looks more like a deposition membrane or half membrane. The formation of such a deposition is to be expected at places where the stroma proteins are in direct contact with lipid material, such as the lipids stored in a plastoglobulus. Again, there the plastoglobulus is not surrounded by thylakoids, although the ends of stroma thylakoids may touch the plastoglobulus surrounded by a half membrane.

Under certain conditions when small leaf pieces are fixed with glutardialdehyde and OsO_4, plastoglobuli are not homogenously fixed in their interior but only on their outer part as shown in Fig. 15.9c. This happens often in leaves of plants grown at high-light conditions or in sun-exposed leaves, e.g. sun leaves of trees. Inhomogenously stained plastoglobuli with changes of electron opacity have also been noticed by various authors as indicated below. Then the plastoglobuli are termed "translucent" or "electron-translucent" plastoglobuli. It seems that in such cases there may not have been enough OsO_4 available inside the chloroplast to fully fix the interior lipids of the plastoglobuli. Then the non-fixed lipids of the plastoglobuli are dissolved in the alcohol solutions applied to the small fixed leaf pieces for dehydration and the plastoglobuli appear translucent on the electron micrograph of ultrathin cuts of fixed and epon-embedded leaf pieces. It is quite possible that in such cases the OsO_4 is predominantly reduced by other substances, e.g. those found in leaf vacuoles, and that its concentration is too low to fully fix the osmiophilic plastoglobuli. Another explanation would be that in such cases the plastoglobuli contain more neutral lipid material that does not readily react or reduce OsO_4.

Many of such translucent plastoglobuli have been found in desiccoplasts, a special chloroplast form that survives in the fully desiccated stage of leaves of poikilochlorophyllous desiccation tolerant plants (Tuba et al. 1993; Tuba and Lichtenthaler 2011). Electron-translucent plastoglobuli are also detected when plants and their green leaves or needles are treated with gases, such as O_3 and SO_2 (Holopainen et al. 1992; Sutinen 1987). In addition, dark stained plastoglobuli were detected in needles of Norway spruce growing in industrial areas (Wulff et al. 1996). Moreover, inhomogenously stained, more or less translucent plastoglobuli have been detected particularly in older needles of certain Norway spruces growing in the Austrian Alps of the "Zillertal" (Zelling and Gailhofer 1989). Translucent, non-osmiophilic plastoglobuli were also found in wheat seedlings treated with the herbicide norflurazon that blocks carotenoid biosynthesis and leads to the accumulation of high amounts of phytoene and phytofluene. In these plants both carotenoid precursors were stored together with neutral lipids as well as plastoquinone 9 and α-tocopherol in translucent plastoglobuli (Dahlin and Ryberg 1986). Electron-translucent plastoglobuli have also been observed in chloroplasts of senescent leaves or during induced senescence, e.g. by ethylene fumigation

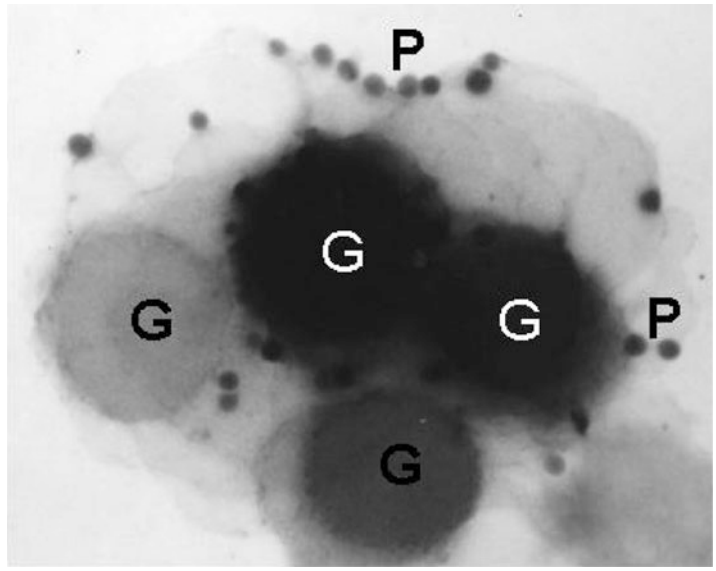

Fig. 15.10. Part of a single chloroplast of spinach leaves showing grana stacks of different height surrounded by osmiophilic plastoglobuli. The average diameter of the grana disks amounts to ca. 0.3 μm. Fixation: OsO_4. *G* grana, *P* plastoglobuli. (Based on Lichtenthaler 1969a.)

(Heath 1974). During the decline of photosynthesis in cotton, large electron-translucent plastoglobuli are prominent components of the chloroplast stroma (Pettigrew et al. 2000). In summary, electron-translucent plastoglobuli can apparently show up during (1) normal chloroplast development blocked by herbicides, (2) senescence of plants and chloroplasts and (3) particular stress constraints, such as treatment with air pollution gases and high irradiance stress conditions.

That plastoglobuli are spherical bodies, i.e. real globuli, can be seen when they are fixed with OsO_4 after their isolation by ultracentrifugation from broken chloroplasts as is shown in Fig. 15.7. Their globular character is demonstrated after shadowing of isolated and OsO_4 fixed plastoglobuli with chromium (Fig. 15.7b). It can be seen on an isolated chloroplast preparation which was directly fixed with OsO_4 (Fig. 15.10). Plastoglobuli are not directly surrounded by thylakoids but are found outside the grana stacks surrounding these or being located as single plastglobulus in the thylakoid-free stroma parts.

VII. Plastoglobuli and the Development of Plastids

Plastoglobuli are found in nearly all stages of plastid development from proplastids to leucoplasts, chromoplasts, amyloplasts, etioplasts, chloroplasts and gerontoplasts. Their size and frequency, however, are quite different and depend on the differentiation stage. This is summarized in the scheme of plastid development (Fig. 15.11).

A. Proplastids

Single or a few plastoglobuli show up in the small proplastids, the plastids in their structurally most reduced form, and are present in the meristems of roots, sprouts, seeds, developing leaves, such as primordia of primary leaves (wheat or barley, etc.) and in cotyledons of dicot seedlings. They do not possess internal biomembranes. In such meristematic tissue one can often differentiate proplastids from developing mitochondria by their

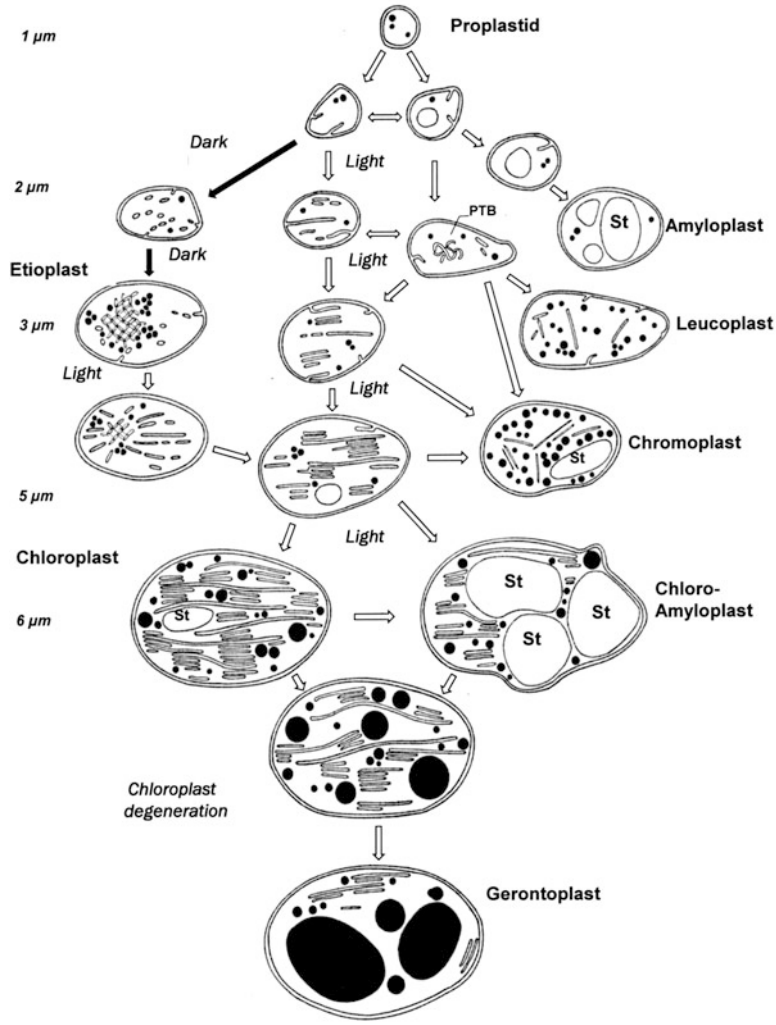

Fig. 15.11. Scheme of plastid and chloroplast development in plant tissues showing the size and frequency of osmiophilic plastoglobuli and also the light-induced thylakoid biosynthesis. The average longitudinal diameter of plastid stages and chloroplasts (in µm) is indicated on the *left* side of the figure. *PTB* protubular body of pro-chloroplasts; *St* starch. (Based on Lichtenthaler 1966, 1968, 1969b, c; Lichtenthaler and Meier 1984; Lichtenthaler and Weinert 1970.)

possession of one or two plastoglobuli. In the light proplastids develop into young chloroplasts via the intermediate stages when at first a few starch grains and initiation of invagination of biomembranes from the inner envelope of the proplastid are detectable. This is followed by a stage where the starch is used up and a protubular body shows up that precedes the formation of the first photochemically active thylakoids. This stepwise process of differentiation of proplastids to young chloroplasts proceeds apparently very fast and is normally not being detected. Examples of such transitional stages of pro-chloroplasts are shown in Fig. 15.12. Such pro-chloroplasts with protubular bodies have first been observed by Whatley (1977), and they are quickly transferred to young chloroplasts. They are rather frequent in cases when the normal light-induced chloroplast development is retarded or suppressed by chemicals, such as sethoxydim that blocks *de novo*

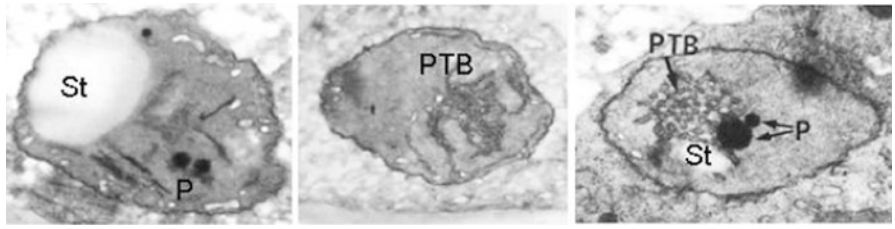

Fig. 15.12. Three proplastids in light-grown barley seedlings (*Hordeum vulgare* L.) at the transition stage to young chloroplasts (so called pro-chloroplasts) with demonstration of the protubular body (PTB) that precedes the formation of the first thylakoids. *P* plastoglobuli, *St* starch. (Based on Lichtenthaler and Meier 1984 and unpublished.)

Table 15.3. Size of osmiophilic plastoglobuli in the different developmental stages of chloroplasts and various other plastid forms. The individual diameter range is indicated in μm (Based on Lichtenthaler 1967, 1968, 1969a, b, e; Lichtenthaler and Sprey 1966; Lichtenthaler and Weinert 1970; Lichtenthaler and Peveling 1967b; Sprey and Lichtenthaler 1966)

Proplastids	0.040–0.130
Etioplasts	0.040–0.120
Leucoplasts	0.050–0.160
Chromoplasts	0.050–0.300
Chloroplasts (3–5 months old)	0.050–0.200
Older chloroplasts	0.100–2.000
Gerontoplasts	0.300–5.000

fatty acid biosynthesis in plastids (Lichtenthaler and Meier 1984). In all stages from proplastids to young chloroplasts few and small osmiophilic plastoglobuli (diameter range: 0.040–0.130 μm) are usually found on electron micrographs (Table 15.3).

B. Etioplasts

During dark growth conditions etioplasts are formed, that contain numerous osmiophilic plastoglobuli (diameter range: 0.040–0.120 μm) with increasing age. During the light-induced biosynthesis of thylakoids and transformation of etioplasts into functional chloroplasts the osmiophilic plastoglobuli disappear, as mentioned above (Sprey and Lichtenthaler 1966). Thus, fully differentiated, yet very young chloroplasts are free of plastoglobuli. However, a few weeks after completing the phase of thylakoid formation, osmiophilic plastoglobuli re-appear which is paralleled by the accumulation of extrathylakoidal amounts of plastoquinol-9 and α-tocopherol which are stored in the plastoglobuli (see review by Lichtenthaler 2007).

C. Chloroplasts

With increasing age of leaves, especially in sun-exposed annual leaves and in leaves of several years of age, osmiophilic plastoglobuli in chloroplasts show up, usually gain larger diameters and can become also more numerous as mentioned above in Sect. IV. Their size is initially relatively small (diameter range: 0.070–0.200 μm), but in older chloroplasts they can have larger diameters up to 1 or several μm. In certain perennial plants, where leaves (but not stems or roots) function as starch deposits, and more generally under high-light growth conditions and in sun-exposed leaves, chloroplasts are transformed to chloro-amyloplasts and function as starch reservoirs. This and also the formation of even larger plastoglobuli in the final stage of gerontoplast development are indicated in Fig. 15.11 as well as in Table 15.3.

D. Amyloplasts and Leucoplasts

Amyloplasts and leucoplasts develop from proplastids that first go through a stage with a few small starch grains. In the case of amyloplasts, the number and size of starch grains continuously increase together with the size of the plastid to finally yield the more spheri-

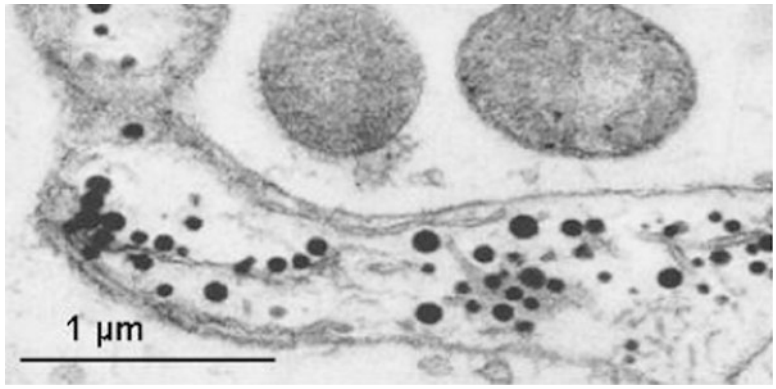

Fig. 15.13. Leucoplast of the mesophyll of white scales of the onion bulbus (*Allium cepa* L.) with many small plastoglobuli. Fixation: OsO_4. (Based on Lichtenthaler and Peveling 1967b.)

cally rounded amyloplast whose main function is the storage of starch, but it still contains a few plastoglobuli (Fig. 15.11). The development of leucoplasts starts the same way, yet the initial starch grains disappear with the enlargement of plastids to usually form a longitudinal leucoplast, e.g. in white onion scales, which possess few or many small plastoglobuli as shown in Fig. 15.13.

E. Chromoplasts

The yellow to orange-red chromoplasts in flower petals and fruit scales usually go through the stage of a young chloroplast that loses its thylakoids and chlorophylls whereby in most cases high amounts of carotenoids and secondary carotenoids accumulate together with plastoquinol-9 and α-tocopherol, although at lower levels (Lichtenthaler 1969c). During the process of *de novo* biosynthesis and accumulation of isoprenoids (carotenoids, prenylquinones), numerous osmiophilic plastoglobuli appear which store these isoprenoid compounds. The chromoplast is enlarged and may even contain small starch grains. Examples of such chromoplasts (Fig. 15.14) are found in the yellow flower petals of common broom (*Sarothamnus scoparius*) (Lichtenthaler 1970a), yellow tulip petals (Fig. 15.14a, b) and in the orange colored floral bracts as well as the fruit scales of *Physalis alkekengi* (Lichtenthaler 1969c). Such processes also occur in green bell pepper fruits (*Capsicum annum*) that turn orange-red and accumulate enormous amounts of carotenoids, and show a considerable increase in the number of osmiophilic plastoglobuli. However, some secondary carotenoids, occurring only in chromoplasts of red *Capsicum annum* fruit scales, do not appear in the many osmiophilic plastoglobuli, but rather accumulate in protubular structures that show up in electron micrographs next to the plastoglobuli (Fig. 15.14c).

VIII. Function of Plastoglobuli

Osmiophilic plastoglobuli occur in all stages of chloroplasts (except for very young chloroplasts during or shortly after the phase of thylakoid formation), and all other plastid forms, although in different frequency and size. They are of lipid nature in their interior, and this is no milieu for deposition of proteins. The essential plastoglobuli function is that of an extrathylakoidal deposit or reservoir for excess chloroplast lipids that cannot be stored in the thylakoids. This especially applies to the prenylquinone derivatives plastoquinone-9, plastoquinol-9 and α-tocopherol that continuously accumulate as photosynthetic products in chloroplasts at high-light conditions as reviewed by Lichtenthaler (2007). When the normal thylakoid biosynthesis is

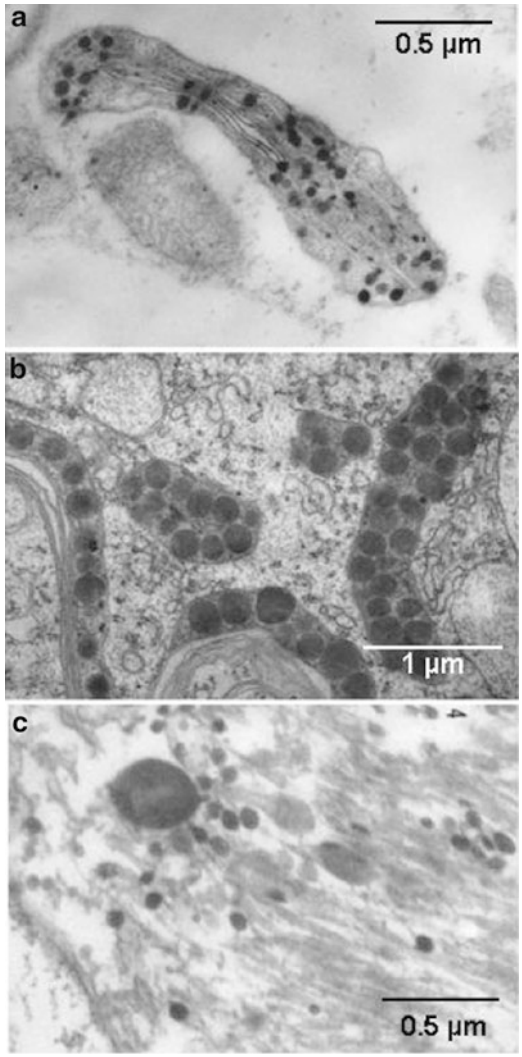

Fig. 15.14. (**a**) Young chloroplast of Tulipa petals at the transitional stage to a chromoplast with degenerating thylakoids and various small plastoglobuli. (**b**) Chromoplasts of yellow flower petals of Tulipa with larger plastoglobuli. (**c**) Chromoplast with various plastoglobuli and protubular structures of a fully red sweet pepper fruit (*Capsicum annuum*). Fixation: OsO_4. (Based on Lichtenthaler 1969c, 1970b.)

disturbed by herbicides or special other treatments, triacyl-glycerides may also accumulate and be as well deposited in plastoglobuli, resulting in the formation of translucent plastoglobuli as mentioned above. Plastoglobuli function as lipid stores also in other plastid forms. This has especially been shown for chromoplasts where plastoglobuli usually store secondary carotenoids, carotenoids and prenylquinone derivatives that accumulate during chromoplast formation (cf. Lichtenthaler 1969c). This function of plastoglobuli as plastidic lipid reservoirs has been undisputed for many decades since their first description as regular components of chloroplasts by Lichtenthaler and Sprey (1966). During their isolation from broken chloroplasts, chromoplasts or gerontoplasts, e.g. by centrifugation, plastoglobuli always behaved like pure lipid bodies and did not show up in particular biomembrane fractions. In some preparations, we found traces of small thylakoid fragments and also very low amounts of small envelope membrane fragments. This lipoprotein material was reduced or removed when the plastoglobuli were re-centrifuged.

Due to their high plastoquinol-9 content, plastoglobuli represent an enormous pool of reducing equivalents and may have a direct physiological function by protecting thylakoids, their photosystems and the entire photosynthetic apparatus from photoinhibition and photo-oxidation. In thylakoids, the total plastoquinone-9 pool (quinone and quinol form) is mainly present in its oxidized form, whereas in plastoglobuli it is mainly in the reduced form plastoquinol-9, as quantified in spinach (61%), beech (88%) and *Ficus* (81%) (Grumbach and Lichtenthaler 1974). In fact, in these plants we found that the plastoquinol-9 pool in plastoglobuli was partially photo-oxidized during the light-induced onset of photosynthesis (Grumbach and Lichtenthaler 1974), a process that was partially reversed during darkness. The photo-oxidation of the plastoquinone-9 pool occurred in parallel to the photo-reduction of violaxanthin to zeaxanthin, i.e. the performance of the xanthophyll cycle. This indicated that during illumination, an electron flow occurred from plastoglobuli to thylakoids, which was fully or partially reversed in the dark. It is of high interest in this respect that the formation of plastoglobuli with a high content of plastoquinol-9 occurred almost exclusively in sun chloroplasts and sun leaves which also possess a high level of

xanthophyll cycle carotenoids and a high performance of the xanthophyll cycle. Moreover, in another study on beech sun leaves that have a high plastoglobuli-bound surplus content of total plastoquinone-9 (in its oxidized and reduced form) (Lichtenthaler 2007), we found the percentage of its reduced form being considerably higher in the dark and on cloudy days (80–90%) as compared to days with full bright sunshine (45–55%). These observations point to an interesting regulatory function of the plastoquinol-9 pool of plastoglobuli in the photosynthetic process and the performance of the xanthophyll cycle.

Already in 1966 we had shown with a special fixation technique, using glutardialdehyde and $KMnO_4$, that plastoglobuli had an outer membrane-type margin (Fig. 15.9b) appearing to us as a thin deposition membrane, that was apparently a 'half membrane' but not a real biomembrane (Lichtenthaler and Sprey 1966). Our interpretation was that this boundary layer became visible because $KMnO_4$, as a water soluble molecule, cannot penetrate the lipid interior of plastoglobuli and was reduced only on the surface of the plastoglobuli. More recently it has been proposed that plastoglobuli may be surrounded by a lipid monolayer studded with proteins (Kessler et al. 1999; Smith et al. 2000), which would explain the membrane boundary of the Billbergia plastoglobulus described earlier (Fig. 15.7b).

Since about the mid 1990s several papers have been published with new ideas on plastoglobuli composition and function. Suddenly, plastoglobuli are regarded as lipoprotein particles (with proteins or lipoproteins on their surface) (Austin et al. 2006; Bréhélin et al. 2007; Bréhélin and Kessler 2008). Some of these lipid-associated proteins have been termed plastoglobulins (Kessler et al. 1999). They comprise plastid-lipid associated-proteins (PAP), such as fibrillin, plastoglobulin 1, carotene globule protein, 32- and 34-kD drought-induced chloroplast stress proteins and the enzyme tocopherol cyclase, involved in tocopherol biosynthesis (Ytterberg et al. 2006). Thus, it appears that plastoglobuli in chloroplasts and chromoplasts might participate in the biosynthesis of isoprenoid lipids (carotenoids, prenylquinone derivatives) and possibly other lipids, and at the same time also have a function in their storage (Austin et al. 2006). Moreover, in the same paper evidence is given that plastoglobuli are possibly permanently coupled to thylakoid membranes and perhaps are connected to each other by an extension of their outer half-lipid lipoprotein layer (e.g. Austin et al. 2006; Bréhélin and Kessler 2008; Grennan 2008). This would explain the fact that plastoglobuli, after their isolation, do not flow together to form a few large plastoglobuli because their interior lipids are separated from each other by such a lipoprotein boundary membrane. In addition, the arrangement of osmiophilic plastoglobuli in groups around the end of grana thylakoids of spinach (Fig. 15.10) would be in agreement with the view that they are somehow coupled to thylakoids. Moreover, the fact that during chloroplast degeneration in the final stage of gerontoplasts, the numerous plastoglobuli finally merge into one or two large plastoglobuli might indicate that the lipoprotein boundary matrix around the plastoglobuli was then destroyed, thus permitting the merging of plastoglobuli and their lipids.

XI. Concluding Remarks

The development of chloroplasts starts from small proplastids that in green leaves transform into young functional chloroplasts characterized by the possession of stroma and grana thylakoids, but without any plastoglobuli. Young chloroplasts can adapt to the intensity of the incident light and form either sun chloroplasts or shade chloroplasts, which differ in the arrangement of thylakoids and the content of starch and osmiophilic plastoglobuli. In the dark, proplastids develop into etioplasts with prolamellar bodies and numerous osmiophilic plastoglobuli. Both structural elements are used up in the light-induced biosynthesis of thylakoids. Older chloroplasts can contain either numerous

plastoglobuli or a few large ones. During chloroplast degeneration and gerontoplast formation, the plastoglobuli finally merge to form very few and very large plastoglobuli. Plastoglobuli are also characteristic to different plastid forms. Osmiophilic plastoglobuli that function as extra-thylakoidal reservoirs for specific plastid lipids are found in different size and frequency in all plastid forms, such as proplastids, etioplasts, older chloroplasts and gerontoplasts as well as in leucoplasts and chromoplasts. The only exception are the young chloroplasts, which are free of plastoglobuli just after thylakoid formation and do not show accumulation of extrathylakoidal surplus lipids. All the data available so far indicate that the interior of osmiophilic plastogobuli is of pure lipid nature. The proteins due to their aqueous character are not found within the plastoglobuli. This does, however, not exclude the possibility that in chloroplasts certain functional stroma proteins specifically bind to the surface of plastoglobuli (if they are present).

The fact that plastoglobuli as lipid reservoirs in chloroplasts are somehow coupled to thylakoids and to each other via their surface proteins, which may function in the biosynthesis of lipids and possibly also in an active channeling of lipid molecules and lipid breakdown (Austin et al. 2006; Bréhélin et al. 2007), is an interesting additional aspect for understanding plastoglobuli function. This is the starting point for new research and many questions need to be addressed in the future. One essential point is the question why young chloroplasts (immediately after the phase of thylakoid formation) do not have any plastoglobuli. Are the particular enzymes and proteins, later found on their surface, not yet present in young chloroplasts at this stage? Future research will give new insights into the dynamic and versatile functions of osmiophilic plastoglobuli in plastids.

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Chapter 16

The Pathway of Chlorophyll Degradation: Catabolites, Enzymes and Pathway Regulation

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Summary

During leaf senescence and fruit ripening, chlorophyll is broken down to colorless linear tetrapyrroles, which are stored in the vacuoles of degreened cells. The pathway of chlorophyll degradation that is active in these developmental processes is fairly well known regarding its biochemistry and cell biology. It comprises at least six enzymatic and one non-enzymatic reaction and the chemical structures of several intermediary and final chlorophyll catabolites have been elucidated. In the last few years, genes coding for a number of chlorophyll catabolic enzymes have been characterized and mutants in these genes have been analyzed. This includes pheophorbide *a* oxygenase (PAO), the key enzyme of the pathway, which is responsible for

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opening of the chlorine macrocycle present in chlorophyll, thereby providing the characteristic structural basis of all further downstream breakdown products. The pathway is therefore nowadays termed the 'PAO pathway'. This review summarizes information on the structures of chlorophyll breakdown products and the reactions involved in their formation. In addition cell biological and regulatory aspects of the PAO pathway are discussed.

I. Introduction

Evolution of advanced life forms on Earth would probably not have occurred without the evolution of oxygenic photosynthesis some three billion years ago (Xiong and Bauer 2002). It allowed for cellular energy production through access to both indefinitely available H_2O as a source of electrons and solar radiation as energy source. Chlorophyll (Chl) is essential for the absorption of sun light and thus for the conversion of solar energy to chemical energy during photosynthesis. Heterotrophic organisms depend on this source of energy. Besides its importance in photosynthesis, green-colored Chl appears to have positive effects on human psychological well-being (Pretty et al. 2007), and in urban environments, human health benefits from green infrastructure and urban green space systems (Tzoulas et al. 2007).

However, under adverse conditions, such as during stress or after the application of herbicides, the photosynthetic apparatus of plants can be overexcited. In these cases, Chl can act as a strong photosensitizer, generating reactive oxygen species (ROS) which in turn can cause cell damage and death (Apel and Hirt 2004). Likewise, defects in heme and Chl biosynthesis, which partly share the same pathway, as well as in Chl degradation, have been shown to result in cytotoxic effects, which are caused by the accumulation of photodynamic metabolic intermediates (Hu et al. 1998; Pružinská et al. 2003; Mochizuki et al. 2010).

These toxic effects explain the requirement for a tight regulation of Chl metabolism, well known in the case of Chl biosynthesis. Here transcriptional and posttranslational mechanisms as well as complex product feedback control at the level of aminolevulinic acid exist to prevent accumulation of biosynthesis intermediates (Cornah et al. 2003; Tanaka and Tanaka 2006, 2007). There is also evidence that Chl breakdown is subject to different levels of regulatory mechanisms with the aim of limiting the occurrence of photodynamic breakdown intermediates (Hörtensteiner 2006; Park et al. 2007).

It is believed that plants degrade Chl throughout their lifespan. Thus, breakdown occurs not only during leaf senescence and fruit ripening when Chl is massively degraded within short periods of time, but also as a response to many biotic and abiotic stress events and during post-harvest. Even at steady state, Chl turns over at a certain rate. Whether the mechanism of Chl breakdown under all these conditions is the same, remains unknown. To date, the best characterized mechanism is the 'PAO pathway' of Chl breakdown occurring during leaf senescence, and there is convincing evidence that Chl degradation in ripening fruits is (largely) identical. The pathway is named after a key enzyme, *p*heophorbide *a* *o*xygenase (PAO), which accounts for the open-tetrapyrrolic

Abbreviations: ABC – ATP binding cassette; ACD – Accelerated cell death; Chl – Chlorophyll; Chlide – Chlorophyllide; CLH – Chlorophyllase; Fd – Ferredoxin; *h*FCC – Hypermodified fluorescent Chl catabolite; HMR – Hydroxymethyl Chl reductase; LHC – Light harvesting complex; MCS – Metal chelating substance; *m*FCC – Modified fluorescent Chl catabolite; NCC – Nonfluorescent Chl catabolite; NOL – NYC1-like; NYC – Non yellow coloring; NYE – Non yellowing; PAO – Pheophorbide *a* oxygenase; pFCC – Primary fluorescent Chl catabolite; Pheide – Pheophorbide; Phein – Pheophytin; PPH – Pheophytinase; PS – Photosystem; RCC – Red chl catabolite; RCCR – Red Chl catabolite reductase; ROS – Reactive oxygen species

backbone structure of several key Chl catabolites found in senescent leaves and fruits. The first identification and structure determination of a nonfluorescent catabolite (NCC) from barley (*Hordeum vulgare*) in 1999 (Kräutler et al. 1991) marked a milestone in deciphering the fate of Chl, which before had been termed a biological enigma (Hendry et al. 1987).

The PAO pathway can be divided into two parts, i.e. (early) reactions affecting colored intermediates ending with the formation of a primary fluorescent Chl catabolite (*p*FCC) and (late) *p*FCC-modifying reactions, typically ending with the isomerization of modified FCCs (*m*FCCs) to their respective NCCs. These two parts of the pathway are also spatially separated in the cell: formation of *p*FCC occurs in plastids whereas subsequent modification and isomerization are localized in cytosol and vacuole, respectively.

This review summarizes our current knowledge of the PAO pathway of Chl breakdown during leaf senescence, including Chl catabolites, Chl catabolic reactions and their subcellular localization and pathway regulation. In addition, it aims to provide an overview of Chl turnover at the steady state, and finally discusses Chl breakdown in the context of stress responses.

II. Chlorophyll Turnover at Steady State

Labeling experiments using either radiolabeled CO_2 or aminolevulinic acid have provided evidence that Chl turns over at the steady state in leaves, i.e. without net loss of overall Chl amount. The half-life of Chl has been calculated as between a few hours and several days (Stobart and Hendry 1984; Hendry and Stobart 1986), but interestingly, turnover seems mainly to affect Chl *a* (Feierabend and Dehne 1996; Beisel et al. 2010), thus correlating with the rapid turnover of the D1 polypeptide of photosystem II (PSII) (Melis 1999). Furthermore, long-term highlight acclimation accelerated rates of Chl turnover, indicating that under these conditions turnover also affected Chl bound to light harvesting complex (LHC) proteins of PSII (LHCII) (Beisel et al. 2010), the abundance of which decreases during high light acclimation (Ballottari et al. 2007).

Although Chl turnover occurs at steady state levels, the mechanism of turnover is largely unknown. ^{13}C-labeling experiments in Synechocystis followed by pigment analysis using MALDI-TOF mass spectrometry indicated that Chl turnover involves hydrolysis of the phytol chain of Chl to yield chlorophyllide (Chlide) and re-esterification of Chlide to Chl (Vavilin and Vermaas 2007). As in plants, turnover in the cyanobacterium mainly was associated with PSII and was increased at high light intensities. Thus, Chl seems to be not degraded, but rather continuously recycled. The enzymes responsible for Chl de- and re-esterification are unknown. It remains to be shown whether a mechanism for Chl degradation beyond the level of Chlide is active in plants at steady state. The involvement of the PAO pathway is rather unlikely. For example, catabolites like FCCs or NCCs have never been observed before the initiation of senescence (Pružinská et al. 2005, 2007), and most of the Chl catabolic enzymes known to date are expressed in a senescence-related fashion (Pružinská et al. 2003; Kusaba et al. 2007; Park et al. 2007; Schelbert et al. 2009). In addition, turnover of D1 under normal illumination is not affected in a Festuca mutant that is deficient in the PAO pathway of Chl breakdown, but D1 exhibits an unusual stability during senescence in this mutant (Hilditch et al. 1986).

III. Chlorophyll Breakdown During Leaf Senescence

Until some 20 years ago, the fate of Chl during leaf senescence was enigmatic and the pigment seemed to disappear without leaving a trace (Hendry et al. 1987; Brown et al. 1991). Within the last two decades this

situation has changed dramatically, starting with the pioneering identification of the first structure of a NCC from barley, *Hv*-NCC-1, (Kräutler et al. 1991). To date more than two dozen structures of different catabolites of Chl occurring transiently ('intermediary' catabolites) or permanently ('final' catabolites) during leaf senescence have been discovered. The identification of these catabolites was a key step for the elucidation of the sequence of reactions in the PAO pathway. Structures of final catabolites of Chl, i.e. FCCs and NCCs, will be presented in a separate section, while intermediary catabolites will be introduced along with the enzymes catalyzing their synthesis and further metabolism.

A. 'Final' Chlorophyll Catabolites

1. Nonfluorescent Chlorophyll Catabolites

When senescent leaves are extracted with methanolic buffers and the extracts subsequently analyzed by reversed-phase HPLC, NCCs can usually be identified by their typical UV absorption spectrum, exhibiting a characteristic peak maximum at around 315 nm. Depending on the plant species analyzed, patterns of NCCs can be complex, i.e. containing several different NCCs, such as in *Arabidopsis thaliana* (Arabidopsis), or simple with one major NCC, such as in the case of senescent leaves of the deciduous tree *Cercidiphyllum japonicum*. Furthermore, NCCs from different species are often unique and not found in other species. The reason for this complexity arises from the fact that although the open tetrapyrrole backbone of all NCCs is identical, they vary with regard to one or more of three side positions depicted as R^1 to R^3 in Fig. 16.1. In addition, the chiral center of C1 provides further variability in NCCs, because, depending on the plant species analyzed, the pyrrole ring A is present in the *R* or the *S* configuration. This results from the stereospecific action of red Chl catabolite reductases (RCCRs) from different plant species (Hörtensteiner et al. 2000; Pružinská et al. 2007), which are responsible for the introduction of the C1-stereocenter during the formation of *p*FCC from red Chl catabolite (RCC) (see below). Thus, two possible stereoisomers, *p*FCC and *epi-p*FCC, can occur and this ultimately results in C1-isomeric NCCs. With the exception of *At*-NCC-3 from Arabidopsis, all NCCs identified so far from plants have a methyl group at C7 and are therefore derived from Chl *a* (Kräutler 2003; Kräutler and Hörtensteiner 2006). It has been shown (see below) that conversion of Chl *b* to Chl *a* is a prerequisite for Chl breakdown through the PAO pathway, which explains this specificity. By contrast, RCC-like catabolites derived from both Chl *a* and Chl *b* have been identified in *Auxenochlorella protothecoides* when the green alga is forced to de-green under heterotrophic and N-limiting conditions in the dark (Iturraspe et al. 1994; Engel et al. 1996). *At*-NCC-3 has a hydroxymethyl group at C7, indicating that it derives from a slightly divergent, as yet unknown, PAO pathway (Müller et al. 2006). All major NCCs found so far carry a hydroxyl group at $C8^2$, indicating that this is an important and common modification of NCCs.

To date NCCs have been structurally characterized from senescent leaves of the following species (Table 16.1): Arabidopsis (5) (Pružinská et al. 2005; Müller et al. 2006), canola (*Brassica napus*; 4) (Mühlecker et al. 1993; Mühlecker and Kräutler 1996; Pružinská et al. 2005), maize (*Zea mays*; 2) (Berghold et al. 2006), *C. japonicum* (2) (Curty and Engel 1996; Oberhuber et al. 2003), barley (1) (Kräutler et al. 1991), Aztec tobacco (*Nicotiana rustica*; 2) (Berghold et al. 2004), *Liquidambar orientalis* (1) (Iturraspe et al. 1995), *Liquidambar styraciflua* (1) (Iturraspe et al. 1995), spinach (*Spinacia oleacea*; 5) (Berghold et al. 2002), peace lily (*Spathiphyllum wallisii*; 1) (Kräutler et al. 2010), pear (*Pyrus communis*; 2) (Müller et al. 2007). Despite the complexity of modifications occurring in NCCs, identical compounds were identified in several cases (Table 16.1). In particular *Cj*-NCC-1, which is derived from *epi-p*FCC and is hydroxylated at $C8^2$, is identical to

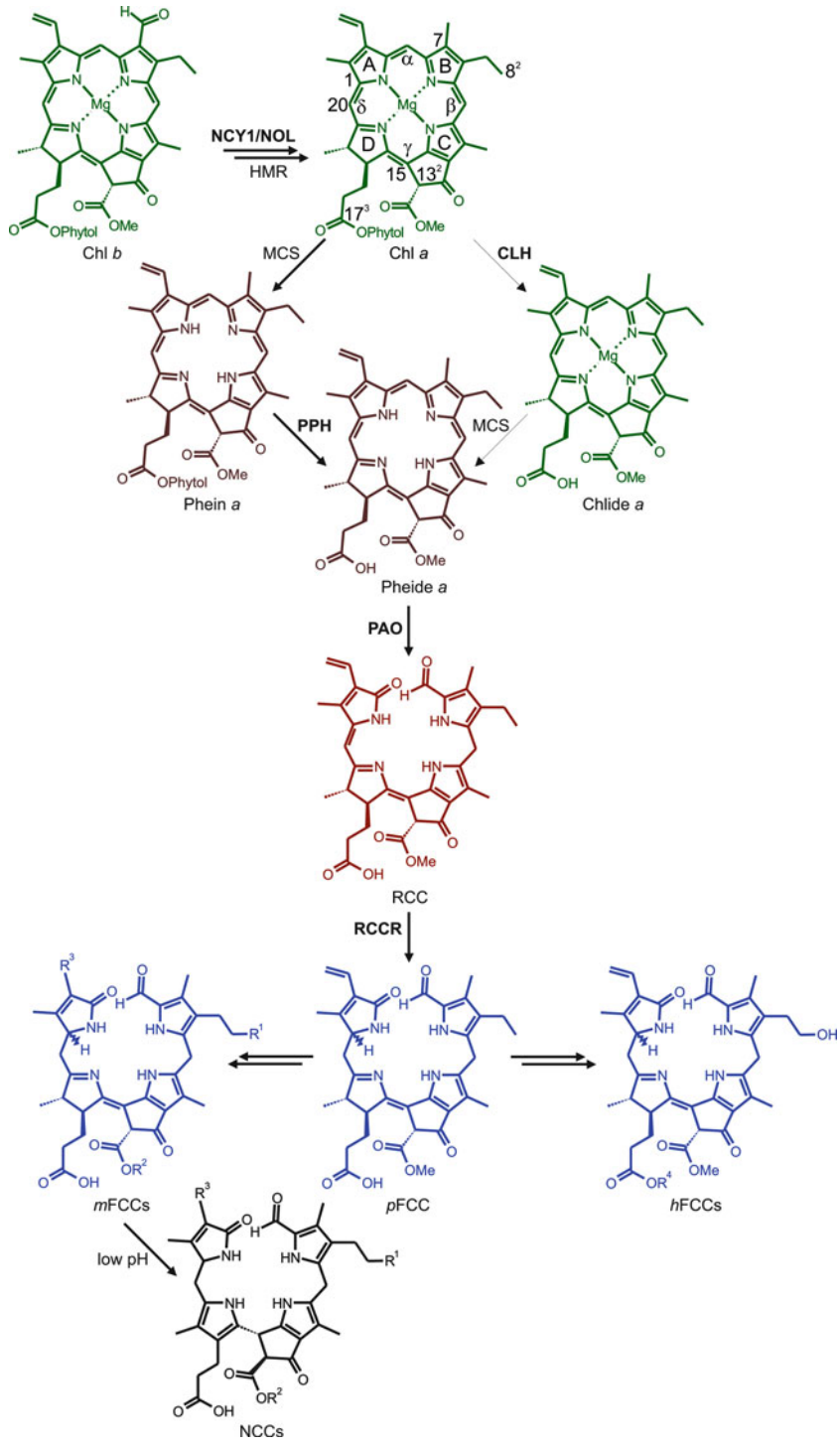

Fig. 16.1. The PAO pathway of chlorophyll breakdown active in senescing leaves. Chemical structures of Chl and of Chl catabolites are shown along with the enzymes involved in the pathway. Enzymes, for which genes and mutants have been characterized in the past, are in *bold*. Note that dephytylation and Mg-dechelation were recently shown to proceed through Phein and that the alternative path involving CLH plays, if at all, a minor role. Note also that the PAO pathway is split at the later reactions, resulting in the production of either *h*FCCs or (ultimately) NCCs. Pyrrole rings (A-D), methane bridges (α-δ) and relevant carbon atoms are labeled in Chl *a*. R^1-R^4 in FCCs and NCCs indicate modifications as outlined in Table 16.1. For abbreviations see the text.

Table 16.1. List of NCCs and of modified and hypermodified FCCs structurally identified from higher plants

Name	R^{1} [a]	R^{2} [a]	R^{3} [a]	R^{4} [a]	C1-epimer [b]	Source	Reference
At-FCC-1 [c]	OH	H	Vinyl	H	1	L	Pružinská et al. (2005)
At-FCC-2 [c]	H	H	Vinyl	H	1	L	Pružinská et al. (2005)
Mc-FCC-49 [d]	*O*-glucosyl	CH_3	Vinyl	Daucic acid	*epi*	F	Moser et al. (2009)
Mc-FCC-56 [d]	OH	CH_3	Vinyl	Daucic acid	*epi*	F	Moser et al. (2008a)
Ma-FCC-61 [d]	OH	CH_3	Vinyl	Digalactosyl-glyceryl	*epi*	L	Banala et al. (2010)
Sw-FCC-62 [d]	OH	CH_3	Vinyl	Dihydroxypheny-lethylglucosyl	1	L	Kräutler et al. (2010)
At-NCC-1 [c]	*O*-glucosyl	H	Vinyl	H	1	L	Pružinská et al. (2005)
At-NCC-2 [c]	OH	H	Vinyl	H	1	L	Pružinská et al. (2005)
At-NCC-3 [c]	OH [e]	H	Vinyl	H	1	L	Pružinská et al. (2005)
At-NCC-4 [c]	*O*-glucosyl	CH_3	Vinyl	H	1	L	Pružinská et al. (2005)
At-NCC-5 [c]	H	H	Vinyl	H	1	L	Pružinská et al. (2005)
Bn-NCC-1 [c]	*O*-malonyl	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-2 [c]	*O*-glucosyl	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-3 [c]	OH	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-4 [c]	H	H	Vinyl	H	1	L	Pružinská et al. (2005)
Cj-NCC-1 [c]	OH	CH_3	Vinyl	H	*epi*	L	Curty and Engel (1996)
Cj-NCC-2 [c]	H	CH_3	Vinyl	H	*epi*	L	Oberhuber et al. (2003)
Hv-NCC-1 [c]	OH	CH_3	Dihydro-xyethyl	H	1	L	Kräutler et al. (1991)
Lo-NCC-1 [c]	OH	CH_3	Vinyl	H	nd	L	Iturraspe et al. (1995)
Ls-NCC-1 [c]	OH	CH_3	Vinyl	H	nd	L	Iturraspe et al. (1995)
Ms-NCC-2 [c]	OH	CH_3	Vinyl	H	*epi*	F	Müller et al. (2007)
Nr-NCC-1 [c]	*O*-glucosyl-malonyl	CH_3	Vinyl	H	*epi*	L	Berghold et al. (2004)
Nr-NCC-2 [c]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	L	Berghold et al. (2004)
Pc-NCC-1 [c]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	F	Müller et al. (2007)
Pc-NCC-2 [c]	OH	CH_3	Vinyl	H	*epi*	F	Müller et al. (2007)
So-NCC-1 [c]	OH	H	Dihydro-xyethyl	H	*epi*	L	Berghold et al. (2002)
So-NCC-2 [c]	OH	CH_3	Dihydro-xyethyl	H	*epi*	L	Oberhuber et al. (2001)
So-NCC-3 [c]	OH	H	Vinyl	H	*epi*	L	Berghold et al. (2002)
So-NCC-4 [c]	OH	CH_3	Vinyl	H	*epi*	L	Berghold et al. (2002)
So-NCC-5 [c]	H	CH_3	Vinyl	H	*epi*	L	Berghold et al. (2002)
Sw-NCC-58 [d]	OH	CH_3	Vinyl	H	1	L	Kräutler et al. (2010)
Zm-NCC-1 [c]	*O*-glucosyl	CH_3	Dihydro-xyethyl	H	*epi*	L	Berghold et al. (2006)
Zm-NCC-2 [c]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	L	Berghold et al. (2006)

[a] R^{1}-R^{4} indicate residues at C3, $C8^{2}$, $C13^{2}$ and $C17^{3}$ side positions, respectively, of FCCs and NCCs as shown in Fig. 16.1

[b] C1 stereochemistry refers to the type of *p*FCC, i.e. *p*FCC (1) or *epi*-*p*FCC (*epi*), formed in the respective species, *nd* not determined

[c] A nomenclature for NCCs (and FCCs) has been defined (Ginsburg and Matile 1993) in which a prefix indicates the plant species and a suffix number indicates decreasing polarity in reversed-phase HPLC

[d] These catabolites are indexed according to their retention time in HPLC analysis. *At Arabidopsis thaliana, Bn Brassica napus, Cj Cercidiphyllum japonicum, Hv Hordeum vulgare, Lo Liquidambar orientalis, Ls Liquidambar styraciflua, Ma Muca acuminate, Mc Musa cavendish*, *Ms Malus sylvestris, Nr Nicotiana rustica, So Spinacia oleracea, Zm Zea mays*

[e] In *At*-NCC-3, the site of hydroxylation is indicated to be $C7^{1}$ (rather than $C8^{2}$)

Source: leaf (L) or fruit (F)

NCCs isolated from three other plant species (Berghold et al. 2002; Müller et al. 2007).

The identification of NCCs as the major degradation end products of Chl brings into question the relevance of different types of (green) Chl catabolites that have been discussed as being genuine Chl breakdown intermediates. These include $C13^2$-hydroxylated Chls (Maunders et al. 1983; Schoch et al. 1984) and $C13^2$-decarboxymethylated (so-called 'pyro'-) forms of pheophorbide or pheophytin (Schoch et al. 1981; Ziegler et al. 1988; Shimokawa et al. 1990). These intermediates might arise from unspecific oxidative reactions or be the result of artifacts of tissue extraction. For instance, the release of Mg^{2+} or the formation of pyro-forms readily takes place under acidic conditions (Amir-Shapira et al. 1987; Engel et al. 1996). In addition, the identification of pyropheophorbide as the 'end' product of in vitro Chl breakdown using enzyme preparations isolated from senescent leaves (Shioi et al. 1991) is possibly due to impropriate assay conditions that did not allow degradation beyond the level of pheophorbide (Pheide).

Recently, analysis of NCC structures has been extended to fruit ripening (Kräutler 2008). Different NCCs were isolated from the peels of ripening apples and pears and their constitution resolved (Müller et al. 2007). In addition, further, so far structurally uncharacterized, NCCs were identified in the peels of yellow bananas (*Musa cavendish*) (Moser et al. 2008a). The structures of the NCCs from fruits contain peripheral substituents like those found in NCCs isolated from leaves, and in the case of pear the same two NCCs were found in fruit peel and leaves (Müller et al. 2007). This indicates that the pathway of Chl breakdown is identical during leaf senescence and fruit ripening.

In most plant species analyzed so far, NCCs are the predominating final catabolites and FCCs are generally low in abundance. NCCs were shown to accumulate inside the vacuoles of senescing cells (Matile et al. 1988; Hinder et al. 1996) and for a long time their synthesis (from FCCs) was considered to occur before vacuolar import (Matile et al. 1996). However, experiments on Chl catabolite transport into isolated barley vacuoles (see below), indicated that FCCs rather than NCCs are imported into the vacuole (Hinder et al. 1996). This was corroborated by in vitro isomerization under acidic conditions of *epi-p*FCC to its corresponding NCC, which is identical in structure to *Cj*-NCC-2 (Oberhuber et al. 2003). Thus, after import into the vacuole, FCCs seem to be rapidly converted to NCCs by an acid-catalyzed isomerization. According to a mechanism proposed by Oberhuber et al. (2003), a free propionic acid side chain at C17 is required for this isomerization. This view is supported by the identification of persistent FCCs that are conjugated at $C17^3$ (see below). Furthermore, the FCC-to-NCC isomerization forms NCCs with a defined stereochemistry at C15, in which the sterically demanding functions at $C13^2$ and C15 are in *trans* configuration.

2. Fluorescent Chlorophyll Catabolites

As mentioned above, FCCs are generally low in abundance. However, several FCCs have been identified in senescent leaf extracts from different species (Ginsburg and Matile 1993; Bachmann et al. 1994; Pružinská et al. 2005) and the constitution of *p*FCC and some *m*FCCs have been resolved (Table 16.1). The fact that the modifications found in FCCs are identical to the ones of NCCs (Pružinská et al. 2005) supports the view that modification of these side positions occurs at the level of FCC rather than NCC. FCCs exhibit a characteristic UV absorption spectrum with maxima at 320 and 360 nm. In addition, they are blue fluorescing with a broad emission maximum around 430 nm. The fluorescence is due to the Schiff's base configuration of the unsaturated γ-methine bridge linking pyrrole rings C and D, which is lost upon FCC-to-NCC isomerization.

In contrast to the low abundance of FCCs in senescent leaves of many species, it was

recently shown that ripening bananas accumulate FCCs to rather high concentrations. These FCCs seem therefore not to occur transiently, but to persist to late ripening stages (Moser et al. 2008a, 2009). Subsequently, persistent FCCs have also been identified from senescent banana (Banala et al. 2010) and peace lily leaves (Kräutler et al. 2010). This new and unexpected finding is explained by the structures of these FCCs, which has been elucidated in some cases; in these FCCs, the C17 propionic acid group is conjugated with different moieties, including unusual moieties such as daucic acid and different C6-linked substituted pyranose units (Table 16.1). As a consequence of C17-modification, the normal acid-induced isomerization to NCCs does not occur and these FCCs have been termed 'hypermodified' (*h*FCC) (Moser et al. 2009). The identification of *h*FCCs indicates the existence of a second variant fate of Chl catabolites within the PAO pathway, i.e. C17-modified catabolites persist as FCCs, whereas unmodified ones are converted to NCCs. Interestingly, both variants, i.e. *h*FCCs and NCCs, occur simultaneously in banana and peace lily, pointing to the possibility that *h*FCCs might have some physiological role (Moser et al. 2009), e.g. in producing an optical effect protecting, for instance, against herbivores. Yet so far, *h*FCCs have been identified in only a few species, and large scale screening of senescent tissue for the presence of *h*FCC is needed in order to show how wide-spread hypermodification of FCCs is within the plant kingdom. This will be a prerequisite for investigating the possible physiological function of *h*FCCs.

3. Degradation Beyond FCCs/NCC?

In most plant species analyzed so far, abundance of NCCs increases with progression of senescence, in agreement with the idea that they are the final products of Chl breakdown. This is supported by calculations for *C. japonicum* (Curty and Engel 1996) and canola (Ginsburg and Matile 1993) that show that the amounts of NCCs account for almost all metabolized Chl. However, several further breakdown products have been identified, indicating a possible degradation beyond the NCC level. An urobilinogenoidic derivative of *Hv*-NCC-1 (Losey and Engel 2001) and monopyrrolic Chl catabolites were identified in barley (Suzuki and Shioi 1999). Furthermore, senescent leaves of *C. japonicum* contained small amounts of a yellow-colored Chl catabolite (YCC), an oxidation product of *Cj*-NCC-1 (Moser et al. 2008b). It remains to be shown whether any of these compounds are synthesized by enzymes as part of a defined pathway within intact senescing cells, or whether they are formed by unspecific oxidation events after tissue disintegration at late stages of senescence.

B. Biochemistry of the PAO Pathway of Chlorophyll Breakdown

FCCs and NCCs exhibit a common tetrapyrrolic backbone structure, which is the result of the activity of PAO. This points to the existence of a basic common pathway, which we nowadays call the PAO pathway. Analysis of mutants affected in different steps of Chl breakdown as well as inhibition and cell fractionation studies allowed the identification of further intermediates of Chl breakdown, in particular Pheide *a*. Furthermore, the establishment of in vitro enzyme assays and crucial efforts in chemical synthesis of potential intermediates (Kräutler et al. 1997; Oberhuber et al. 2008) provided the basis for the elucidation of most of the individual steps of the pathway and allowed the recent cloning of Chl catabolic genes. Mutants are available for all known enzymes involved in the pathway. The following sections summarize our current knowledge of the PAO pathway.

1. Chlorophyll b to Chlorophyll a Reduction

Apart from *At*-NCC-3 (Müller et al. 2006), all FCCs and NCCs identified so far from higher plants are derived from Chl *a*. In addition, barley leaf senescence in the presence of D_2O partially labeled *Hv*-

NCC-1, indicating it to derive in part from Chl *b* (Folley and Engel 1999). Furthermore, PAO was shown to accept Pheide *a*, but not Pheide *b*, as substrate (Hörtensteiner et al. 1995), and PAO mutants (see below) specifically accumulated Pheide *a* (Pružinská et al. 2003; Tanaka et al. 2003). Together these data demonstrated that conversion of Chl *b* to Chl *a* occurs before further degradation via PAO and upstream of Pheide formation. Biochemically, Chl *b*-to-Chl *a* conversion constitutes the reductive half of the so-called Chl cycle, which allows interconversion of Chl(ide) *a* and Chl(ide) *b* via C7-hydroxymethyl Chl(ide) (Rüdiger 2002). Substrate preferences of the enzymes involved indicate that the oxidative part is predominantly active towards Chlide (Oster et al. 2000), while the reductive part mainly uses phytylated pigments as substrate (Scheumann et al. 1999). The cycle is important to balance the ratio of *a*- and *b*-type pigments for adaptation to particular physiological conditions, for example changing light intensities.

Both oxygenation steps from Chlide *a* to Chlide *b* are catalyzed by Chlide *a* oxygenase, a Rieske-monooxygenase (Tanaka et al. 1998; Oster et al. 2000), which was recently shown to be regulated by a feedback mechanism (Sakuraba et al. 2007). Chl *b* modulates the stability of Chlide *a* oxygenase through the activity of the chloroplast Clp protease system (Sakuraba et al. 2009).

Chl *b* reduction was shown to require two different enzymes, Chl *b* reductase and hydroxymethyl Chl reductase (Ito et al. 1996; Scheumann et al. 1998). Whereas the latter is a stroma-localized, ferredoxin (Fd)-dependent enzyme whose molecular nature is not yet known, Chl *b* reductase has been identified in a forward genetic screen for stay-green mutants in rice (Kusaba et al. 2007). The protein identified, NON-YELLOW COLORING1 (NYC1), is a member of the family of short-chain dehydrogenases/reductases with three predicted transmembrane spanning domains. These features fit with the biochemically identified dependence on NADPH as electron source and the predicted localization of Chl *b* reductase activity at the thylakoid membrane (Scheumann et al. 1999). Furthermore, *NYC1* gene expression correlated with leaf senescence (Kusaba et al. 2007), confirming the increase of Chl *b* reductase activity which was demonstrated in senescing barley leaves (Scheumann et al. 1999). The analysis of the rice *nyc1* mutant, which showed particular retention of LHCII subunits and Chl *b*, favored the assumption that *NYC1* encodes Chl *b* reductase (Kusaba et al. 2007). This was largely confirmed when analyzing Arabidopsis mutants defective in the ortholog of NYC1 (At4g13250) (Horie et al. 2009). However, in vitro activity could not be demonstrated for either rice or Arabidopsis NYC1 (Kusaba et al. 2007; Horie et al. 2009), possibly because of the hydrophobic nature of these proteins. In both rice and Arabidopsis a close homolog of NYC1, NYC1 LIKE (NOL), is present and both proteins were shown to exhibit Chl *b* reductase activity when expressed in *E. coli* (Horie et al. 2009; Sato et al. 2009). In addition, rice NYC1 and NOL physically interact, and in line with this, NOL, although predicted to be a soluble protein, co-purifies with the thylakoid membrane (Sato et al. 2009). Chl *b* reductase is an early (or the initial) step of Chl breakdown and NOL of Arabidopsis was shown to reduce Chl *b* to hydroxymethyl Chl when isolated trimeric LHCII complexes were used as substrate. In addition, this reaction was sufficient to release Chl from the complexes, indicating that Chl *b* reductase activity is an initial reaction required for both Chl breakdown and degradation of LHCII proteins (Horie et al. 2009).

2. Mg-Dechelation and Dephytylation

In Bf 993, a stay-green mutant of *F. pratensis*, Pheide *a* was shown to accumulate upon senescence induction (Vicentini et al. 1995a). Likewise, inhibition of Chl breakdown through addition of iron chelators, such as 2,2' dipyridyl, caused Pheide *a* accumulation, indicating

that Pheide *a* is a genuine intermediate of Chl breakdown (Langmeier et al. 1993; Vicentini et al. 1995a). The establishment of an in vitro assay that involves PAO and RCCR and converts Pheide *a* to *p*FCC (Hörtensteiner et al. 1995) corroborated this assumption. It demonstrated that removal of phytol and the central magnesium atom of Chl precedes the chlorine ring opening reaction through PAO. There was still a puzzling question concerning the order of these two reactions. Until recently, phytol removal was considered to precede Mg-chelation (Hörtensteiner 2006; Tanaka and Tanaka 2006), although in some instances, pheophytin (Phein) had been identified as an intermediate indicating the possibility of the reactions occurring in inverse order (Amir-Shapira et al. 1987; Heaton and Marangoni 1996).

It has been suggested that phytol, after hydrolysis from Chl, is re-utilized for the synthesis of α-tocopherol because its abundance increases during senescence (Peisker et al. 1989; Rise et al. 1989). This requires the activation of phytol to phytyl pyrophosphate, the co-substrate of condensation with homogentisic acid, and nucleotide-dependent phosphorylation of phytol was demonstrated in spinach chloroplast extracts (Soll et al. 1980). A phytol salvage pathway for tocopherol biosynthesis was recently established (Ischebeck et al. 2006). It involves two sequential phosphorylation steps, the first of which is catalyzed by VTE5 of Arabidopsis (Valentin et al. 2006) in a CTP-dependent reaction (Ischebeck et al. 2006). The second enzyme, phytylphosphate kinase, has not yet been cloned.

In addition to feeding Chl-derived phytol into α-tocopherol biosynthesis, substantial quantities of phytol are found during senescence or nitrogen deprivation in esterified form, mainly with various fatty acids (Ischebeck et al. 2006; Gaude et al. 2007). These fatty acid phytyl esters accumulate predominantly within plastoglobules, lipoprotein vesicles of plastids, whose abundance and size increases during senescence and which are considered to have an important role in lipid metabolism (Bréhélin et al. 2007).

Chlorophyllase hydrolyzing phytol from Chl was biochemically identified 100 years ago (Willstätter and Stoll 1913) and was believed to be active in Chl breakdown during senescence. The biochemical properties of chlorophyllase are intriguing because the enzyme was shown to be highly active at temperatures above 45°C and at high concentrations of acetone. In addition, it hydrolyzes a wide variety of hydrophobic substrates, such as Chl, Phein and fatty acid esters (McFeeters 1975; Arkus et al. 2005), and also catalyzes transesterification reactions (Fiedor et al. 1992). Chlorophyllase activity was shown to be present constitutively, but a relationship between chlorophyllase and senescence-related Chl breakdown was inferred from the latency of the enzyme and its proposed localization at the chloroplast envelope (Matile et al. 1997). Thus, in plant tissue extracts, chlorophyllase activity can only be measured after solubilization with solvents or detergents (Trebitsh et al. 1993; Matile et al. 1997). In addition, it was considered that chlorophyllase was able to act on Chl only after its release from Chl-binding proteins and shuttle to the chloroplast envelope, involving an unknown Chl carrier (Matile et al. 1999).

In 1999, two groups independently succeeded in cloning chlorophyllase (*CLH*) genes from orange (*Citrus sinensis*) and white goosefoot (*Chenopodium album*) (Jakob-Wilk et al. 1999; Tsuchiya et al. 1999) based on amino acid sequence information obtained from purified protein fractions exhibiting in vitro chlorophyllase activity (Trebitsh et al. 1993; Tsuchiya et al. 1997). Since then, *CLH* genes have been described from a few other plant species, including Arabidopsis, broccoli (*Brassica oleracea*), *Ginkgo biloba* and wheat (Tsuchiya et al. 1999; Tang et al. 2004; Arkus et al. 2005; Chen et al. 2008). Surprisingly, prediction of subcellular localization revealed that some of the cloned CLHs might localize outside plastids, i.e. in the cytosol or the vacuole. CLHs were therefore considered to localize to different compart-

ments, implying the existence of multiple pathways for Chl breakdown (Takamiya et al. 2000). Experimental analysis of subcellular CLH localization using different methods resulted in inconsistent conclusions. The two Arabidopsis CLHs, AtCLH1 and AtCLH2, localized to the cytosol when tagged with GFP (Schenk et al. 2007), while the N-terminus of *G. biloba* CLH targeted GFP to the chloroplast (Okazawa et al. 2006). Lemon (*Citrus limon*) CLH was shown by in situ immunofluorescence to reside inside the chloroplast in lemon flavedo tissue (Azoulay Shemer et al. 2008) and to co-purify with chloroplast membranes after heterologous expression in tobacco mesophyll protoplasts (Harpaz-Saad et al. 2007). Experiments aiming to investigate the involvement of CLHs in Chl breakdown produced similarly contradictory results: absence of one or both of AtCLH1 and AtCLH2 in the respective mutants (Schenk et al. 2007) as well as RNAi-based gene silencing of AtCLH1 (Kariola et al. 2005) or AtCLH2 (Liao et al. 2007) had little or no effect on Chl breakdown during leaf senescence. In addition, expression patterns of *AtCLHs* do not correlate with Chl breakdown (Zimmermann et al. 2004; Liao et al. 2007). This indicated that in Arabidopsis CLHs are not required for leaf senescence-related Chl breakdown. In contrast, investigations in some other species support an involvement of CLH in Chl breakdown. For example in broccoli, antisense-suppression of CLH delayed rates of postharvest head yellowing (Chen et al. 2008). Yet in the same tissue, expression of CLH genes does not correlate with progression of Chl breakdown (Büchert et al. 2011). During fruit ripening in *Citrus* species, CLH was convincingly shown to participate in Chl breakdown and also to promote Chl breakdown when expressed in squash (*Cucurbita pepo*) leaves or tobacco protoplasts (Harpaz-Saad et al. 2007; Azoulay Shemer et al. 2008).

The proposed non-involvement of CLHs in Arabidopsis leaf senescence prompted a search for alternative phytol-cleaving esterases. A candidate esterase was identified in three independent approaches, i.e. a functional genomics screen of the Arabidopsis proteome (Schelbert et al. 2009), a screen for genes co-expressed with *NYE1/SGR* in Arabidopsis (see below) (Ren et al. 2010) and a screen for stay-green mutants in rice (Morita et al. 2009). Yet only in one of these investigations, a functional analysis of the enzyme as a possible phytol hydrolase was performed (Schelbert et al. 2009). After heterologous expression, the Arabidopsis protein (At5g13800) exhibited esterase activity with Phein *a* or Phein *b* as substrate yielding the respective Pheide pigment. Surprisingly, the enzyme did not dephytylate Chl, pointing to an intriguing specificity towards metal-free pigments. The enzyme was therefore termed pheophytinase (PPH). This substrate specificity was consistent with accumulation of Phein *a* in senescent leaves of Arabidopsis *pph* mutants. *pph* exhibits a stay-green phenotype similar to the one observed in *nyc1* mutants with high retention during senescence of thylakoid membrane structures, LHCII subunits and Chl (Morita et al. 2009; Schelbert et al. 2009; Ren et al. 2010). As expected for a Chl-dephytylating enzyme, PPH and its rice ortholog, NCY3, were shown to localize to the chloroplast. In addition, *PPH*/*NYC3* mRNA increases with senescence, thus exhibiting high level of co-expression with other Chl catabolic genes such as *PAO* and *NYE1*/*SGR* (Ren et al. 2010). Similarly, PPH expression correlated with yellowing of broccoli heads, indicating that also during postharvest PPH but not CLH is active (Büchert et al. 2011).

In summary, there is increasing evidence that dephytylation occurs only after removal of Mg from Chl, and PPH/NYC3 is likely to be responsible for this reaction. This is certainly true for Arabidopsis and rice leaf senescence. However, for other systems, in particular fruit ripening, CLH could have a role (in addition?) and further investigations are required to solve this riddle. From a metabolic perspective it is understandable that Chl breakdown should proceed via PPH and not CLH, because then Chlide, the last precursor of Chl biosynthesis, is not simultane-

ously an intermediate of Chl breakdown. Hence, biosynthetic and catabolic reactions are entirely separated allowing better metabolic control of overall Chl metabolism.

The mechanism of Mg-dechelation has not been clear until now. Two types of activities have been described in the literature. In two cases, white goosefoot (Shioi et al. 1996a; Suzuki and Shioi 2002) and strawberry (*Fragaria* x *ananassa*) (Costa et al. 2002), heat-stable low-molecular weight compounds have been described that catalyze Mg-dechelation. These compounds were termed metal-chelating substance (MCS) and they have molecular weights of <400 Da (goosefoot) and 2,180 Da (strawberry). However, their molecular nature has so far not been determined. Inhibition studies indicated that MCS compounds may contain active SH-groups, pointing to a possible proteic nature (Shioi et al. 1996a; Costa et al. 2002). The speculation that MCS compounds merely represent prosthetic groups of Mg-dechelating proteins (Matile et al. 1996) has been refuted (Suzuki et al. 2005). The second type of Mg-dechelating activity was attributed to heat-labile proteins, termed Mg-releasing proteins (MRP) (Vicentini et al. 1995b; Suzuki and Shioi 2002). MRP activity has so far only been demonstrated using the artificial substrate chlorophyllin, i.e. alkali-hydrolyzed Chl, not with Chlide as substrate. This was interpreted as MCS being active in vivo (Kunieda et al. 2005). Considering the fact that, at least in some systems, Mg-dechelation occurs before dephytylation (Schelbert et al. 2009), it is possible that Chlide is not the natural substrate for Mg-dechelation. Hence, there is a need to re-examine MCS and MRP-like activities with Chl as substrate.

3. Macrocycle Ring Opening

The establishment of an in vitro assay that catalyzes conversion of Pheide *a* to *p*FCC (Ginsburg et al. 1994; Hörtensteiner et al. 1995) and the chemical synthesis of RCC (Kräutler et al. 1997), which is an intermediate of this reaction, were crucial for elucidating the mechanism of the two-step macrocycle ring opening reaction. A still puzzling aspect of this reaction is the fact that both individual steps, i.e. Pheide *a*-to-RCC conversion catalyzed by PAO and RCC-to-*p*FCC conversion catalyzed by RCCR, are inefficient on their own, and large amounts of *p*FCC can be produced only in the coupled reaction (Rodoni et al. 1997a). This biochemical property and the demonstration of physical interaction between PAO and RCCR using a bacterial two-hybrid system (Pružinská et al. 2007) strongly indicates that metabolic channeling takes place in the PAO/RCCR reaction.

PAO is an iron-dependent oxygenase, whose activity was shown to be inhibited by chelating substances such as 2,2′-dipyridyl or *o*-phenanthroline (Ginsburg et al. 1994). In PAO, two iron centers, a Rieske iron-sulfur center and a mononuclear iron center, are present (Pružinská et al. 2003), the latter of which is responsible for the activation of molecular oxygen (Schmidt and Shaw 2001). *p*FCC-labeling experiments using $^{18}O_2$ showed that PAO is a monooxygenase that specifically incorporates an oxygen atom derived from molecular oxygen at the formyl group attached to pyrrole ring B (Hörtensteiner et al. 1998). As for other Rieske type oxygenases, electrons required to drive the iron-redox cycle of PAO are supplied through reduced Fd (Ginsburg et al. 1994; Pružinská et al. 2003). Based on the distribution of its activity, PAO was believed to reside in the chloroplast envelope (Matile and Schellenberg 1996). PAO proteins do contain two C-terminally located transmembrane helices, and recent proteomics data indicate that PAO could also localize to thylakoid membranes (Joyard et al. 2009). A further C-terminal motif containing conserved cysteine residues was identified as a target of thioredoxin regulation, indicating redox-regulation of PAO function (Bartsch et al. 2008). As mentioned above, PAO exhibits an intriguing specificity for Pheide *a*, with Pheide *b* inhibiting the activity in a competitive manner. This specificity provides an explanation for the almost unique occurrence of Chl *a*-derived FCCs and NCCs.

Based on the biochemical properties of PAO, a functional genomics screen was performed in Arabidopsis to identify PAO at the molecular level (Pružinská et al. 2003). PAO turned out to be identical to *A*CCELERATED *C*ELL *D*EATH (ACD) 1 (At3g44880) (Greenberg and Ausubel 1993), which after heterologous expression in *E. coli* exhibited PAO activity with properties similar to those of native PAO (Pružinská et al. 2003). In an independent approach using antisense silencing of candidate genes, *ACD1* was also identified as likely to be *PAO* (Tanaka et al. 2003). PAO/ACD1 is the ortholog of LETHAL LEAF SPOT 1 in maize (Gray et al. 1997), and absence of these proteins in corresponding mutants or antisense lines from Arabidopsis, rice or tomato results in premature cell death phenotypes (Greenberg and Ausubel 1993; Gray et al. 2002; Spassieva and Hille 2002; Pružinská et al. 2003, 2005; Tanaka et al. 2003). In all cases investigated, Pheide *a* was shown to accumulate to high concentrations. The phototoxicity of Pheide was considered to trigger the observed cell death phenotype in a light-dependent manner. However, cell death in Arabidopsis PAO-antisense lines was recently shown to be light-independent and a cell death signaling mechanism involving Pheide *a* was proposed (Hirashima et al. 2009). Components of such a pathway have not yet been identified and it remains to be confirmed to what extent direct phototoxicity of Pheide *a* contributes to cell death.

RCCR is a soluble protein of about 30 kDa that catalyzes the C1/C20 reduction of RCC to *p*FCC. As in the case of PAO, electrons are supplied from reduced Fd, but the protein does not contain any known domain that would indicate the mechanism of the reaction. However, RCCR is distantly related to a family of Fd-dependent bilin reductases (Frankenberg et al. 2001). These include bilin reductases from algae and cyanobacteria required for phycobilin biosynthesis, such as phycoerythrobilin synthase (PebS) and phycocyanobilin:ferredoxin oxidoreductase (PcyA), as well as phytochromobilin synthase (HY2) from higher plants that catalyzes the final step in phytochrome chromophore biosynthesis (Kohchi et al. 2001). For PcyA and HY2, a reaction mechanism has been proposed that involves a radical intermediate resulting from direct transfer of an electron from Fd to a critical (conserved) glutamate residue in the enzymes (Tu et al. 2004, 2008). The recent elucidation of the crystal structures of Arabidopsis RCCR in the absence or presence of RCC (Sugishima et al. 2009, 2010) demonstrated a high degree of structural similarity to the 3D structures of PebS and PcyA (Hagiwara et al. 2006; Dammeyer et al. 2008), supporting the idea that in RCCR, a radical mechanism involving a glutamate residue (glutamate$_{154}$ of Arabidopsis RCCR) is also active.

As mentioned above, formation of *p*FCC by RCCR is highly stereospecific, i.e. two possible C1-stereoisomers, *p*FCC or *epi-p*FCC, are formed. The constitution of both C1-epimers was verified by one- and two-dimensional NMR methods (Mühlecker et al. 1997, 2000). The source of RCCR defines this specificity, as shown by the analysis of more than 50 plant species (Hörtensteiner et al. 2000; Pružinská et al. 2007). In order to analyze this biochemically, chimeric proteins were produced in *E. coli*, in which parts of Arabidopsis RCCR (At4g37000) producing *p*FCC were replaced by the corresponding parts of tomato RCCR (specifically forming *epi-p*FCC). This attempt identified phenylalanine$_{218}$, which when replaced by valine (present in tomato RCCR) switched Arabidopsis RCCR from *p*FCC to *epi-p*FCC production (Pružinská et al. 2007). Interestingly, this residue is located within the RCC binding pocket in the crystal structure (Sugishima et al. 2009), but 3D structure analysis of the phenylalanine$_{218}$-to-valine variant did not convincingly explain the altered stereospecificity (Sugishima et al. 2010).

RCCR has been cloned from barley based on amino acid sequence information obtained from the purified protein (Rodoni et al. 1997b; Wüthrich et al. 2000). The partial cDNA sequence obtained exhibited high homology to *ACD2*, which had been identified

in a genetic screen for accelerated cell death in Arabidopsis and which had been considered to be a component of a cell death signaling pathway (Greenberg et al. 1994). Chloroplast-import experiments confirmed the proposed (Ginsburg et al. 1994; Rodoni et al. 1997a) chloroplast localization of RCCR (Wüthrich et al. 2000). However, ACD2/RCCR also partially localizes to mitochondria (Mach et al. 2001), in particular in response to stress conditions, such as pathogen infection or protoporphyrin IX treatment (Yao and Greenberg 2006). Mitochondrial localization of ACD2 was believed to have a possible function in preventing the cellular death that is observed in *acd2* mutants. Cell death in *acd2* is preceded by an early mitochondrial oxidative burst (Yao et al. 2004) and it was proposed that ACD2 could protect mitochondria from such an oxidative burst (Yao and Greenberg 2006), but the mechanism of this protection has not been resolved yet. This, together with the observation that cell death in *acd2* also occurred in Chl-free root protoplasts, called into question the role of RCCR/ACD2 as a Chl catabolic enzyme (Yao and Greenberg 2006). However recently, the in vivo participation of RCCR in Chl breakdown during senescence has been demonstrated (see below) (Pružinská et al. 2007).

When analyzing *acd2* with respect to Chl breakdown, it turned out that progression of cell death in the mutant was highly correlated with the accumulation of RCC and RCC-like pigments (Pružinská et al. 2007). Cell death was light-dependent and coincided with the production of singlet oxygen, indicating a phototoxic effect of RCCs. Whether RCCs are directly phototoxic, or whether cell death is the result of a death signaling pathway involving ROS, remains to be demonstrated. In the case of the Arabidopsis *flu* mutant, which accumulates high levels of protochlorophyllide (Meskauskiene et al. 2001), cell death does not occur because of direct phototoxicity of this Chl biosynthetic intermediate. Instead, it was shown that protochlorophyllide-dependent production of singlet oxygen (op den Camp et al. 2003) triggers a cell death pathway that involves EXECUTER 1 and EXECUTER 2, two novel chloroplast-localized proteins with so far unknown function (Wagner et al. 2004; Lee et al. 2007).

Surprisingly, *acd2* mutants are not entirely blocked at the level of RCCR, because despite the accumulation of RCCs they still produce FCCs and NCCs (Pružinská et al. 2007). Interestingly, for several of these catabolites, including *p*FCC, the presence of both C1-epimers could be shown. This implied a loss of stereospecificity during RCC reduction, indicating that this reduction occurs through a stereo-unselective mechanism that is unknown so far. In electrochemical reduction experiments on RCC, *p*FCC and *epi*-*p*FCC are obtained in equal quantities (Oberhuber et al. 2008). This demonstrates that under appropriate conditions, which are possibly also present in chloroplasts, (stereo-unselective) RCC-to-*p*FCC reduction could occur without the involvement of an enzyme. Complementation of *acd2* with versions of RCCR that exhibited different C1-specificities in vitro resulted in stereospecifically uniform patterns of catabolites, which corresponded to the specificity of the complementing enzyme (Pružinská et al. 2007). These experiments allowed it to be unambiguously conclude that RCCR is active in Chl breakdown.

4. Modifications of the Primary Fluorescent Chlorophyll Catabolite

The diversity of known FCC- and NCC-type catabolites indicates that additional reactions occur after the common formation of *p*FCC (or *epi*-*p*FCC). Overall, *p*FCC side group modification has so far been shown to be possible at four different positions. While hydroxylation at $C8^2$ appears to be a common reaction, other modifications occur in a species-specific manner. For example, although several identical NCCs are found in canola and Arabidopsis, *Bn*-NCC-1, carrying a malonyl group at $C8^2$, is absent from Arabidopsis. Enzymes catalyzing

these modifications have so far not been identified at the molecular level and only for some reactions have biochemical activities been demonstrated.

$C13^2$-demethylated catabolites have been identified in Brassicaceae, but not in many other species such as barley (Table 16.1). This pattern of occurrence of demethylated forms of catabolites fits well with the presence in these species of an enzyme termed pheophorbidase (Suzuki et al. 2002). Pheophorbidase (PPD) is capable of hydrolyzing the $C13^2$-methylester of Pheide, but it does not accept metal-containing chlorins or *Cj*-NCC-1 as substrate, indicating a high degree of substrate specificity (Suzuki et al. 2006). The product of the reaction, $C13^2$-carboxyl pyropheophorbide, was shown to decarboxylate spontaneously to pyropheophorbide (Shioi et al. 1996b), a proposed product of Chl breakdown found mainly in algae and during post-harvest senescence (Ziegler et al. 1988; Aiamla-or et al. 2010). PPD was recently cloned from radish (*Raphanus sativus*) and was predicted to localize to the cytosol (Suzuki et al. 2006). It is arguable whether Pheide is the true substrate for $C13^2$-demethylation, because Pheide *a* formation and further metabolism occurs in plastids (Hörtensteiner 2006; Kräutler and Hörtensteiner 2006), hence PPD action on Pheide would require an unlikely export from, and re-import into, senescing chloroplasts. In addition, Arabidopsis *pao1* does not accumulate $C13^2$-carboxyl pyropheophorbide or pyropheophorbide (Pružinská et al. 2005). PPD is a serine-type esterase and is highly homologous to the Arabidopsis methyl esterase (MES) protein family. The closest homolog of PPD, MES16, was shown to hydrolyze methyl esters of jasmonic acid and indole acetic acid (Yang et al. 2008).

An NCC that carries a malonyl group attached to $C8^2$ has been found in canola (Mühlecker et al. 1993). In this case, $C8^2$-hydroxylation by a (so far unknown) mechanism precedes malonylation (Hörtensteiner 1998). Likewise, *Nr*-NCC-2, a $C8^2$-glucosylated NCC from tobacco, was shown to have a malonylated counterpart, *Nr*-NCC-1 (Berghold et al. 2004). In both cases malonyltransferase reactions have been demonstrated that transfer the malonyl moiety from malonyl-coenzyme A to the NCC substrate. The activity isolated from canola was shown to be specific for Chl catabolites, but because of the presumed cytosolic localization of the transferase (Hörtensteiner 1998), FCCs rather than NCCs are the likely in vivo substrates.

C. Subcellular Localization of the Pathway and Catabolite Transport

Chl is localized in chloroplasts and the early catabolic reactions (at least up to *p*FCC) also localize to this organelle. This view is corroborated by the finding that isolated senescent chloroplasts are capable of *p*FCC formation and release (Matile et al. 1992). The site of *p*FCC-modifying activities is most probably located in the cytosol. This is deduced from the biochemical properties of the modifying enzymes characterized so far (Hörtensteiner 1998; Suzuki et al. 2006). A possible exception might be $C8^2$ hydroxylation. In addition to *p*FCC, isolated chloroplasts produce a second more polar FCC (Schellenberg et al. 1990; Ginsburg et al. 1994), which can serve as the substrate for malonylation, indicating it to be hydroxylated (own unpublished results).

Despite the undoubted plastidial localization of the Chl catabolic enzymes involved in the above-described PAO pathway (Wüthrich et al. 2000; Kusaba et al. 2007; Ren et al. 2007; Schelbert et al. 2009), several reports hint at extra-plastidial pathways for Chl catabolism (Takamiya et al. 2000). These include the identification of different types of chloroplast-derived vesicles, which participate in the breakdown of chloroplast constituents (Guiamét et al. 1999; Otegui et al. 2005; Martínez et al. 2008; see also Costa et al., Chap. 18), and a possible role for autophagic processes playing a role in chloroplast degradation during senescence (Ishida et al. 2008; Wada et al. 2009; see also

Wada and Ishida, Chap. 19). However, to date none of these processes have demonstrated to involve Chl catabolism. The stay-green and cell death phenotypes of Chl catabolic mutants suggest that the majority of Chl is degraded via the PAO pathway inside senescing chloroplasts and that extra-plastidial pathways, may make – if at all – only a minor contribution.

Different experimental approaches, such as fluorescent protein fusion analysis, proteomics and chloroplast-subfractionation studies, have been used to address the sub-chloroplast localization of the PAO pathway enzymes. The results are partially conflicting. Thus, NYC1 protein and Chl *b* reductase activity reside in the thylakoid membrane (Scheumann et al. 1999; Sato et al. 2009), while NOL, which was shown to interact with NYC1, localized to the envelope in a proteomics study (Joyard et al. 2009). Likewise, RCCR is a soluble protein localizing to the stroma (Rodoni et al. 1997b; Joyard et al. 2009), yet RCCR physically interacts with PAO (Pružinská et al. 2007), which was shown to localize to the envelope (Matile and Schellenberg 1996; Joyard et al. 2009). Finally, PPH was also localized to the stroma (Schelbert et al. 2009), but since from its activity towards Phein it has been inferred that it is likely to be attached to LHCs, localization in the thylakoid membrane would be expected. In summary, the data obtained so far imply that Chl catabolism may occur at both thylakoid and envelope membranes (Fig. 16.2). As a consequence a mechanism for shuttling Chl pigments from the thylakoid to the envelope has been postulated (Matile et al. 1999), but the nature of the Chl shuttle is unknown. Alternatively, the formation of contact between thylakoid and envelope membranes at sites of active Chl catabolism could overcome the spatial separation of enzymes of the PAO pathway.

Chl catabolites are deposited in the vacuoles of senescing cells (Matile et al. 1988; Hinder et al. 1996), indicating that two membranes, the chloroplast envelope and the tonoplast, need to be crossed (Fig. 16.2). In both cases, active transport processes are involved, but only for vacuolar import has the transport been shown to be primary active (Matile et al. 1992; Hinder et al. 1996). This implied the participation of members of the ATP binding cassette (ABC) transporter family, and for two of these transporters, ABCC2 and ABCC3, transport activity for *Bn*-NCC-1 has been demonstrated (Lu et al. 1998; Tommasini et al. 1998). However, the nature of the in vivo transporter(s) remains uncertain and an Arabidopsis ABCC2 mutant was only marginally affected in senescence (Frelet-Barrand et al. 2008). This might be due to functional redundancy of different transporters. Export from senescing chloroplasts required the (extra-plastidial) presence of a hydrolysable nucleotide, but ATP could be replaced by UTP (Matile et al. 1992). The nature of the transporter at the chloroplast envelope is unknown.

IV. Chlorophyll Breakdown and Its Relation to Stress Response

In addition to leaf senescence and fruit ripening, many biotic and abiotic stresses cause loss of Chl. However, the mechanism of Chl disappearance is not well understood and in only a few cases has a direct relation to the PAO pathway been demonstrated (Yang et al. 2004; Mur et al. 2010). Analysis of a large number of microarray studies investigating stress-related gene expression (Zimmermann et al. 2004) indicates that many Chl catabolic genes are regulated in response to different abiotic stress conditions, in particular drought and osmotic stress, as well as in response to challenge by several pathogens, including *Pseudomonas syringae*. For most abiotic stress conditions that result in Chl degradation, it remains to be established to what extent the PAO pathway contributes. In situations where stress results in cell death, Chl disappearance could be due to unspecific peroxidative or photooxidative pathways that become active after tissue death.

In contrast, a large body of evidence exists that relates Chl breakdown to pathogen infection as an active response process of plants. Thus, the Chl catabolic mutants of

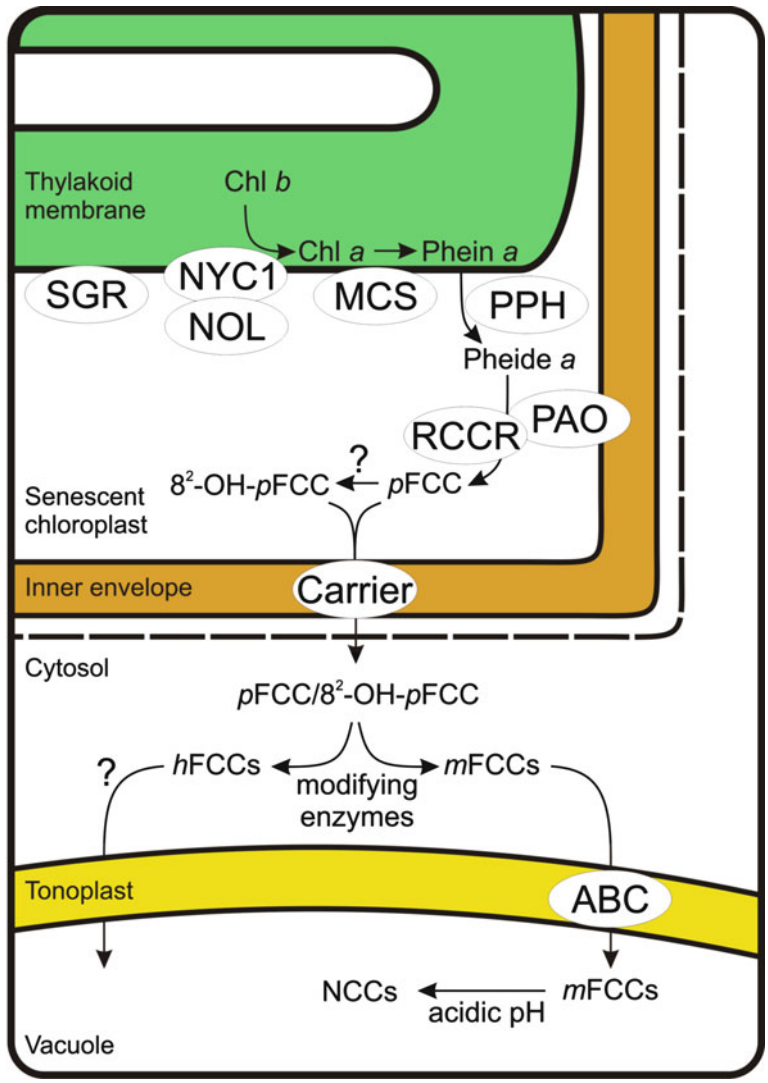

Fig. 16.2. Topographical model of the PAO pathway of Chl breakdown. The model updates the current state of knowledge about the pathway and incorporates the (presumed) subcellular localization of Chl catabolites and Chl catabolic enzymes. Putative steps are labeled with *question marks*. For abbreviations see the text.

Arabidopsis, *acd1* and *acd2*, were originally identified because of accelerated cell death in response to infection with *P. syringae* (Greenberg and Ausubel 1993; Greenberg et al. 1994). Furthermore, execution of the hypersensitive response (HR) in the Arabidopsis-*P. syringae* pathogenesis system is linked to light, i.e. a plant's defense potential following an incompatible interaction that results in localized cell death is increased in the light compared to the dark (Zeier et al. 2004; Griebel and Zeier 2008). Among other mechanisms, imbalances in Chl metabolism were considered to trigger pathogen defense through ROS signaling. As shown for several lesion mimic mutants (Ishikawa et al. 2001; Mach et al. 2001; Meskauskiene et al. 2001; Pružinská et al. 2003) and transgenic plants de-regulated in Chl metabolism (Kruse et al. 1995; Tanaka et al. 2003), ROS production is linked to the photodynamic properties of many Chl metabolic intermediates.

Recently, a stay-green mutant of Arabidopsis was shown to suppress HR-related cell death, and Pheide *a* played a crucial role in ROS-mediated establishment of the HR (Mur et al. 2010). This indicates that Chl breakdown could have an impact on cell death during the HR, explaining at least in part the known light-dependency of HR in Arabidopsis as elicited by some strains of *P. syringae*, but possibly also causing HR-like phenotypes in other instances of plant-pathogen and plant-herbivore interactions (Roberts and Paul 2006). However, it remains to be shown whether Chl catabolites are directly involved in the execution of cell death after pathogen attack.

V. Regulation of Chlorophyll Breakdown

A. The Stay-Green Protein

In many species, stay-green mutants are known that upon induction of senescence show retention of greenness (as compared to the respective wild types). These have been categorized into two principal groups: functional and non-functional (also called cosmetic) stay-green mutants (Thomas and Howarth 2000). The difference between these categories is whether retention of greenness is coupled to loss (cosmetic stay-greens) or retention (functional stay-greens) of photosynthetic capacity. Thus, cosmetic stay-green mutants [categorized as type C (Thomas and Howarth 2000)] show normal senescence behavior, but retain green color, indicating them to be defective in Chl breakdown. As outlined above, mutations affecting some Chl catabolic enzymes such as PPH or NYC1 have been shown to fall into the type C category. However, most cosmetic stay-green mutants that have been identified from naturally occurring varieties of different species or during screening programs for stay-green phenotypes are defective in a different gene, termed *STAY-GREEN* (*SGR*). Plant varieties in which SGR deficiency has been proven include fruit ripening mutants of bell pepper (*Capsicum annuum*; *chlorophyll retainer*) (Efrati et al. 2005; Barry et al. 2008) and tomato (*green flesh*) (Barry et al. 2008) as well as Gregor Mendel's famous *I* locus mutant of pea (*Pisum sativum*) (Armstead et al. 2007; Sato et al. 2007), Arabidopsis *nonyellowing1* (*nye1*) (Ren et al. 2007), different rice mutants (Jiang et al. 2007; Park et al. 2007; Sato et al. 2007) and Bf 993 of *F. pratensis* (Armstead et al. 2006, 2007). It is to be expected that molecular defects in SGR are also present in other phenotypic type C mutants, such as soybean (*Glycine max*) d_1d_2 (Guiamét et al. 1991) and Arabidopsis *ore10* (Oh et al. 2003).

SGR proteins of different species share a high degree of sequence similarity (Hörtensteiner 2009), contain a C-terminally located cysteine-rich consensus sequence of unknown function (Aubry et al. 2008) and are targeted to the chloroplast (Park et al. 2007; Ren et al. 2007; Sato et al. 2007). However, a clear (biochemical) function for SGR remains elusive. For the Mendel's *I* locus mutant and Bf 993 a link to PAO function was postulated, because the mutants had diminished PAO activity and accumulated Chlide and Pheide *a* (Vicentini et al. 1995a; Thomas et al. 1996). However, a recent detailed re-analysis of this hypothesis showed that SGR acts independently and upstream of PAO (Aubry et al. 2008). Interestingly, rice SGR was shown to interact with LHCII, but not LHCI, subunits, and this interaction was not compromised in a valine$_{99}$ point mutation of rice SGR, which causes the stay-green phenotype (Park et al. 2007). Thus, it has been speculated that SGR could be important for destabilization of Chl-protein complexes as a prerequisite for subsequent Chl and apoprotein degradation, but the (point-) mutations shown to be present in several *SGR* mutants (Hörtensteiner 2009) could (in addition) affect an so far unknown enzyme activity or may affect binding of other factors required for Chl-apoprotein degradation (Park et al. 2007). This view is corroborated by the fact that during senescence mutants defective in SGR specifically retain LHCII subunits. In contrast, LHCI peptides are less affected and a corresponding activity that may

control Chl-apoprotein degradation in PSI awaits detection. Interestingly, most plant species analyzed so far contain at least two *SGR* genes, but whether the respective paralogs could be involved in LHCI degradation remains to be shown. In the case of Arabidopsis, only SGR1 (At4g22920) deficiency causes the stay-green phenotype described; absence of SGR2 (At4g11910) does not affect Chl breakdown (Aubry et al. 2008).

In summary, SGR seems to function in destabilization of Chl-binding proteins. According to this view it is not itself a Chl catabolic enzyme, but may be required to allow the catabolic enzymes to access their substrates. Thus, SGR can be considered a regulator of both Chl and apoprotein degradation, which acts at the level of Chl-apoprotein complex stability. It is interesting to note that during senescence, Bf 993 accumulates N-terminally truncated fragments of LHCII subunits, indicating that absence of SGR prevents the membrane-embedded core part of the complexes from being proteolytically degraded (Thomas and Howarth 2000). SGR might work in addition to or in concert with NYC1/NOL. Stability of Chl-apoprotein complexes was shown to require a defined ratio of Chl *a* and Chl *b* (Horn and Paulsen 2004) and as a consequence Chl *b*-less mutants are pale and LHCII apoproteins are unstable in these mutants (Harrison et al. 1993).

B. Chlorophyll Breakdown and Its Relation to Nitrogen Metabolism

Remobilization of nutrients from leaves to storage organs or seeds is an intrinsic feature of leaf senescence. Nitrogen in particular is efficiently remobilized and the photosynthetic apparatus, containing some 20% of total cellular nitrogen (Peoples and Dalling 1988), contributes a major source. In contrast, Chl only accounts for about 2% of nitrogen in a leaf cell, a fraction which is lost when FCC/NCC-containing senescent leaves shed from the plant. Thus, Chl degradation is not aimed at recycling pigment-bound nitrogen, but Chl breakdown can be seen as an important detoxification process that is a prerequisite for the remobilization of nitrogen bound in Chl-apoproteins. Cosmetic stay-green mutants defective in SGR, NYC1 or PPH retain large fractions of these Chl-binding proteins, but so far the impact of this loss, e.g. for seed filling, has not been thoroughly investigated. It can be hypothesized that annual plants may be less affected than perennial species, such as for example deciduous trees. In addition, severe effects might only be seen under nitrogen-limiting growth conditions.

Except for D1 of the PSII reaction center, which is turned over by the joint activity of at least two types of proteases, DegP and FtsH (Adam et al. 2006; Sakamoto 2006), proteases responsible for the degradation of Chl-binding proteins are largely unknown, although members of the families of Lon, Clp and FtsH proteases have been implicated (Sakamoto 2006; Liu et al. 2008).

C. Transcriptional Control of the PAO Pathway

Chl breakdown is the visible symptom of senescence and the status of yellowing has been proposed as a biomarker of leaf senescence (Ougham et al. 2008). Thus, the phenotypic progression of senescence is mostly correlated with Chl breakdown. Nevertheless, the process of yellowing is integrated into fundamental metabolic changes that occur during leaf senescence and that are termed the ‘senescence syndrome’ (Smart 1994). These include structural changes, such as the chloroplast-to-gerontoplast transition, as well as biochemical processes largely aiming at the recycling of nutrients. Initiation and progression of the syndrome depend on a regulatory network, which is only partially understood so far (Lim et al. 2006). Regulation of Chl breakdown is integral to the regulation of leaf senescence. This is evident from the fact that, although specific sets of genes are up-regulated when different types of leaf senescence are compared, Chl catabolic genes belong to those genes that are up-regulated under all conditions tested (Buchanan-Wollaston et al. 2005; Van der Graaff et al. 2006). Yet in Arabidopsis, several

Chl catabolic genes are, for example, also expressed in petals, which do not contain Chl (Zimmermann et al. 2004). This indicates that regulation of the PAO pathway could go beyond leaf senescence. In line with this is the observation that the genes of the PAO pathway that are known to be transcriptionally regulated, i.e. *SGR*, *NYC1*, *PPH* and *PAO*, are highly co-regulated (Ren et al. 2010) and consequently cluster closely together when performing gene network analyses (Fig. 16.3) (Obayashi et al. 2009). This suggests the possible involvement of specific transcription factors regulating the PAO pathway. Although several hundreds of transcription factors have been shown to be differentially regulated during leaf senescence (Balazadeh et al. 2008), factors that specifically target Chl catabolic genes are unknown. Interestingly, Arabidopsis *RCCR* is not co-regulated with the other known PAO pathway genes (Fig. 16.3), supporting the idea that RCCR may have additional functions (Yao et al. 2004; Yao and Greenberg 2006). Similarly, expression of Arabidopsis *NOL* clusters with *RCCR* rather than with the other genes. This is different from the situation in rice, where *NOL* and *NYC1* both show senescence-related enhancement of expression (Sato et al. 2009).

D. Metabolic Control of the PAO Pathway?

Chl catabolic mutants such as *pph-1* and *pao1* retain large quantities of Chl and accumulate only comparatively small quantities of the respective intermediates, Phein *a* and Pheide *a* (Pružinská et al. 2005; Schelbert et al. 2009). This indicates the existence of feedback control mechanisms that prevent further degradation of Chl if the pathway is blocked. In the case of PAO mutants, retention of greenness was shown to correlate with reduced abundance of SGR transcripts (Park et al. 2007). Thus, a retrograde signaling pathway seems to exist that regulates Chl catabolic gene expression in response to disturbances of the chloroplast-localized PAO pathway. The mechanism of signaling remains to be elucidated.

VI. Conclusions and Outlook

Since the first elucidation of an NCC structure 20 years ago (Kräutler et al. 1991), the 'biological enigma' of Chl breakdown has largely been solved. Most of the Chl catabolic enzymes of the PAO pathway are known along with their respective catabolic intermediates and end products. Despite this major progress, Chl breakdown remains mysterious in several aspects. The surprising recent identification of persistent *h*FCCs implies that the pathway is even more complex than the proposed linear conversion of Chl down to NCCs (Hörtensteiner and Kräutler 2011). It also raises the intriguing possibility that Chl catabolites might not only be by-products of Chl detoxification, but that they might have some biological role. For example, NCCs have a high antioxidative potential (Müller et al. 2007), and *h*FCCs and a *C. japonicum* YCC have been shown to contribute, respectively, to the optical appearance of fruits and to the fall colors of deciduous trees (Moser et al. 2008b; Kräutler et al. 2010). Regarding the biochemistry of the PAO pathway, none of the enzymes that convert *p*FCC to *m*FCCs or *h*FCCs has been identified at the molecular level. Cloning the genes encoding these enzymes will not be an easy task, in particular because several side group modifying reactions occur in a species-specific manner. Furthermore, the molecular mechanism of Mg-dechelation is unclear, and the steps involved in Chl turnover at the steady state also remain enigmatic.

Chl breakdown occurs massively during fruit ripening as well as during leaf senescence. Several lines of evidence indicate that the PAO pathway is active as well. FCCs and NCCs have been identified from fruit sources (Moser et al. 2008a, 2009), and PAO and RCCR have been isolated from bell pepper chromoplast membranes (Moser and Matile 1997). Uncertainty remains concerning dephytylation, i.e. there is a need to show whether PPH and/or CLH is active during fruit ripening.

On the basis of sequence homology of the Chl catabolic enzymes, Chl breakdown

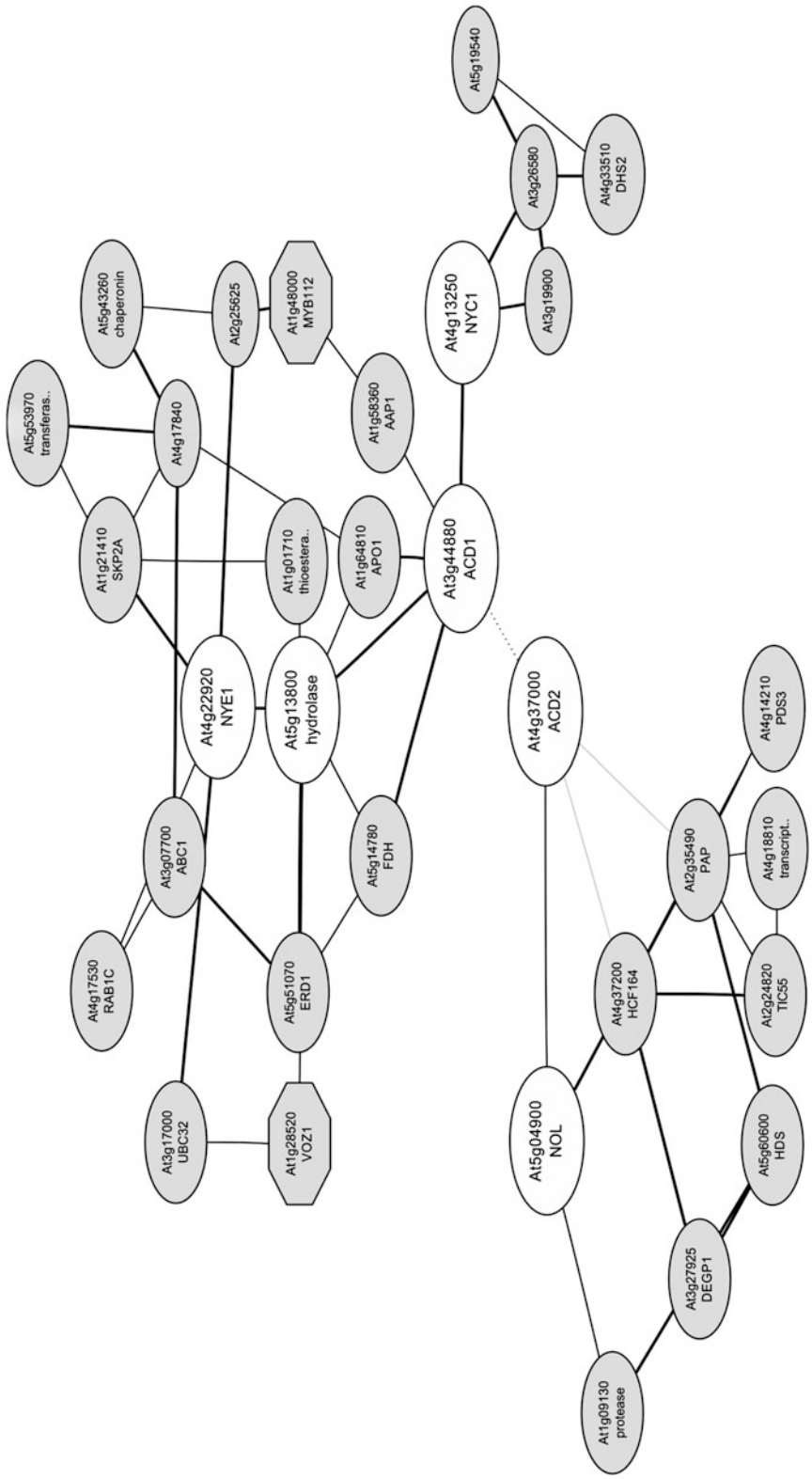

Fig. 16.3. Gene network of Chl catabolic genes in *Arabidopsis thaliana*. Coexpression analysis was performed with the NetworkDrawer tool of the ATTED2 platform (http://atted.jp/top_draw.shtml#NetworkDrawer) using as input the Chl catabolic genes shown in light gray shading (SGR1, NYE1, At4g22920; PPH, hydrolase, At5g13800; PAO, ACD1, At3g44880; NYC1, At4g13250; RCCR, ACD2, At4g37000; NOL, At5g04900). Thickness of lines connecting the different genes indicates relative extent of coexpression with the thickest lines denoting the highest levels of coexpression. Note that the *dotted line* between PAO (ACD1) and RCCR (ACD2) indicates that the two proteins were shown to physically interact, but there is no coexpression between the two respective genes. Transcription factor genes are shown with *octagon-shaped* symbols, whereas all other genes have *circular* symbols.

through the PAO pathway is a common occurrence in higher plants, but comparison of the proteins to the available genomic sequences of lower plants indicates the presence of RCCR-, PPH- and/or PAO-like proteins in algae and even in cyanobacteria (Gray et al. 2004; Pružinská et al. 2007; Schelbert et al. 2009; Thomas et al. 2009). It remains to be demonstrated whether any of these homologs does indeed encode catalytically active Chl breakdown enzymes.

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Chapter 17

Regulation of Leaf Senescence: Role of Reactive Oxygen Species

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Summary

Leaf senescence causes a genetically programmed decline in various cellular processes including photosynthesis and involves hydrolysis of macromolecules including proteins, lipids and nucleic acids. Environmental stresses and reproductive structures influence the rate of

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senescence. The process of senescence and abiotic stress response are associated with the overproduction of reactive oxygen species (ROS) which are highly reactive and toxic compounds, and ultimately result in oxidative stress. ROS contribute to the progression of leaf senescence, as the antioxidant capacity of the leaf declines. Arabidopsis mutants and transgenic plants, in which antioxidant enzymes were manipulated, substantiate direct involvement of the ROS in leaf senescence. Infact, there is an intrinsic link between oxidative damage and leaf senescence and the free radical theory of aging seems to apply to plant senescence. Chloroplasts may play a regulatory role during leaf senescence similar to that of mitochondria during animal programmed cell death. Peroxisomes have a ROS mediated cellular function in leaf senescence and stress response. Reproductive sinks act as a stress leading to higher oxidative damage to proteins, drive the mobilization of nitrogen to the developing seeds and hence regulate the rate of senescence. The photosynthetic organelles are the main targets of ROS linked damage in plants experiencing various environmental stresses and natural senescence with decline in ROS detoxification mechanisms. At the same time, ROS play an important signaling role in plants controlling the processes such as growth, development, senescence, responses to environmental stimuli and programmed cell death. Plants adapt to environmental stresses through the process of acclimation, which involves less ROS production coupled with an efficient antioxidant defence. Among the different ROS, H_2O_2 appears to be the key regulatory molecule involved both in senescence and stress acclimation. In addition to redox control of chloroplast, a considerable cross-talk is observed in the regulatory networks involving hormones, ROS and transcription factors both in natural and stress induced senescence and abiotic stress responses. In this chapter an attempt has been made to review and analyse the role of ROS in senescence and abiotic stress responses, since both involve oxidative stress.

I. Introduction

Senescence is the final stage in a plant's life ultimately leading to death. However, this process is not chaotic, but progresses gradually in a genetically programmed manner. Hence, it is a form of programmed cell death (PCD). It is highly co-ordinated at the molecular, cellular, biochemical and physiological levels and is accompanied by degradation of chlorophyll, proteins, lipids and nucleic acids and a decline in the rate of photosynthesis. During senescence there is also an increase in proteolytic activity accompanied by nutrient remobilization in plants.

Leaf senescence is subject to regulation by endogenous and environmental factors (Gan and Amasino 1997; Munné-Bosch and Alegre 2004). Endogenous factors include reproductive development, hormones, ROS, and environmental factors include biotic and abiotic stresses. In monocarpic plants, the developing reproductive sink (grain/pod) often governs the senescence of the whole plant, and the rate of senescence is a determinant of crop productivity.

Both, reproductive sinks and abiotic stresses hasten the process of senescence. Senescence, as in the case of other PCD processes, is also associated with an increased production of ROS such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and more toxic derivative hydroxyl ion ($HO^{\cdot}$) (Breusegem and Dat 2006). These toxic ROS oxidize proteins, lipids and DNA

Abbreviations: ABA – Abscisic acid; APX – Ascorbate peroxidase; AsA – Ascorbic acid (reduced); CAT – Catalase; DHA – Ascorbic acid (oxidized); DHAR – Dehydroascorbate reductase; GPX – Glutathione peroxidase; GR – Glutathione reductase; GSH – Glutathione (reduced); GSSG – Glutathione (oxidized); MAPK – Mitogen activated protein kinase; MDAR – Monodehydroascorbate reductase; PCD – Programmed cell death; ROS – Reactive oxygen species; SA – Salicylic acid; SAG – Senescence associated gene; SOD – Superoxide dismutase

resulting in lipid peroxidation, cellular damage and ultimately cell death.

Abiotic stresses such as drought, salinity, extreme temperature, excess light, ultra violet radiation, ozone and hypoxia disrupt the cellular homeostasis and the delicate balance between multiple pathways that reside in different cellular compartments of the plant cell (Noctor et al. 2002; Møller et al. 2007). When different pathways which involve electron transfer, such as photorespiration, photosynthesis, mitochondrial respiration, fatty acid β-oxidation, etc. are not coupled with their respective redox element; the electrons are subsequently transferred to molecular oxygen thereby generating ROS (Mittler 2002). ROS are mainly produced at a low level as a byproduct in organelles such as chloroplasts, mitochondria and peroxisomes. However, during stress, their rate of production is dramatically elevated leading to cellular toxicity and damage.

In this chapter, we deal with ROS metabolism during senescence and abiotic stress responses, as oxidative stress is involved in both these processes. The antioxidant defense in leaves during senescence, stress responses and its regulation by ROS is highlighted. ROS signaling which involves communication between chloroplast and nucleus and plays a major role in developmental senescence, stress induced senescence and stress acclimation is also discussed.

II. Reactive Oxygen Species (ROS) Generation and Detoxification Mechanisms

A. ROS Generation

In chloroplasts, Photosystem I (PSI) and photosystem II (PSII) in thylakoids are the major sites of ROS generation. Under drought conditions, reduced CO_2 availability due to stomatal closure and exposure of plants to continuous excessive light, direct electron transfer towards molecular oxygen, generating $O_2^{\cdot -}$ at PSI by the Mehler reaction. Production of $^1O_2$ occurs at PSII by excited triplet-state chlorophyll at the P680 reaction center and in the light-harvesting complex when the electron transport chain is over-reduced (Asada 2006). Singlet oxygen can activate a genetic program leading to growth inhibition and lethality through the EXECUTER1 and EXECUTER2 pathways (K.P. Lee et al. 2007).

Cytotoxicity of $^1O_2$ has been shown to cause lipid peroxidation and extensive tissue damage in leaves under photo-oxidative condition that could directly lead to cellular death (Møller et al. 2007).

Water deficit and stomatal closure decrease the ratio of CO_2 and O_2 in mesophyll cells and increase photorespiration leading to production of glycolate in chloroplasts. Glycolate oxidation by glycolate-oxidase in peroxisomes accounts for the majority of H_2O_2 production during photorespiration (Noctor et al. 2002; Karpinski et al. 2003). Additional sources of H_2O_2 production in peroxisomes include fatty acid β-oxidation, operation of the flavin oxidase pathway and the dismutation of superoxide radicals (Palma et al. 2009).

Mitochondria generate smaller amounts of ROS compared to chloroplasts and peroxisomes (Foyer and Noctor 2005; Rhoads et al. 2006). In mitochondria, complex I and complex III in the electron transport chain (mtETC) are the major sites of ROS production (Møller et al. 2007). An intermediate, ubi-semiquinone formed at complex I and III, donates electrons to oxygen and generates $O_2^{\cdot -}$, and further $O_2^{\cdot -}$ is reduced to H_2O_2 (Rhoads et al. 2006).

It has been reported that, the apoplast is an important site for H_2O_2 production in response to abscisic acid (ABA) and adverse environmental conditions such as drought and salinity (X. Hu et al. 2006; Jubany-Marí et al. 2009). In Arabidopsis *AtRbohD* and *AtRbohF* encode two major NADPH oxidases expressed in guard and mesophyll cells responsible for apoplastic ROS generation that is required for ABA-induced stomatal closure (Kwak et al. 2003; Torres and Dangl 2005). Other apoplastic ROS-forming enzymes are cell wall-associated oxidases, peroxidases and polyamine oxidases (Moschou et al. 2008).

Increased concentration of H_2O_2 in the apoplast is thought to be involved in acclimation response of plants to drought and salt stress (Jubany-Marí et al. 2009).

B. ROS Detoxification Mechanisms

Abiotic stresses and senescence are associated with enhanced ROS production in plants. Plants protect themselves against ROS by employing antioxidant defense systems in cells where these toxic oxygen intermediates are produced. These include both enzymatic and non-enzymatic ROS scavenging mechanisms. Enzymatic ROS scavenging mechanisms involve superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), and nonenzymatic ROS scavenging mechanisms include antioxidants such as glutathione (GSH), ascorbic acid (AsA), carotenoids, tocopherols and flavonoids.

1. Enzymatic ROS Scavenging Mechanisms

In these mechanisms, SODs act as the first line of defense against ROS, converting superoxide to H_2O_2. CAT subsequently detoxifies H_2O_2. In contrast to CAT, APX requires an ascorbate and GSH regeneration system, the ascorbate-glutathione cycle. Detoxifying H_2O_2 to H_2O by APX occurs by oxidation of ascorbate to monodehydroascorbate (MDA), which can be regenerated by MDA reductase (MDAR) using NAD(P)H as reducing equivalent. MDA can spontaneously be converted into dehydroascorbate (DHA). Ascorbate regeneration is mediated by DHAR driven by the oxidation of GSH to GSSG. Finally, through glutathione peroxidase cycle, GR can regenerate GSH from GSSG using NAD(P)H as a reducing agent. Like APX, glutathione peroxidase (GPX) also detoxifies H_2O_2 to H_2O, but uses GSH directly as a reducing agent. The GPX cycle is closed by regeneration of GSH from GSSG by GR.

2. Non-enzymatic ROS Scavenging Mechanisms

Nonenzymatic antioxidants are equally important for scavenging ROS; these antioxidants include the major cellular redox buffers AsA, GSH, tocopherol, flavonoids, alkaloids and carotenoids. Mutants with decreased AsA levels (Conklin et al. 1996) or altered GSH content (Creissen et al. 1999) are hypersensitive to stress. Whereas GSH is oxidized by ROS forming GSSG, ascorbate is oxidized to MDA and DHA. Through the ascorbate-glutathione cycle, GSSG, MDA, and DHA can be reduced reforming GSH and ascorbate. α-tocopherol (vitamin E) on the other hand, is a lipid antioxidant that can scavenge ROS and protect lipids from oxidation (Li et al. 2008). Flavonoids neutralize ROS radicals while carotenoids scavenge $^1O_2$ efficiently.

III. Alleviation of ROS Generation Under Various Abiotic Stresses

A. Enzymatic Antioxidants

SOD is a metalloenzyme that catalyzes the dismutation of $O_2\cdot^-$ to oxygen and H_2O_2. Based on the metal cofactors present in their active sites, SODs are classified into three known types: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD), which are localized in different cellular compartments ubiquitous in all aerobic organisms (Mittler 2002). SOD plays an important role in preventing radical mediated chain oxidation of GSH, thereby enabling GSH to act physiologically as an active antioxidant and to prevent cells from oxidative stress (Asada 1999). Many stresses are reported to cause an increase in the foliar SOD activity (Pastori et al. 2000).

Ascorbate peroxidase (APX) is one of the most important antioxidant enzymes that plays an important role in detoxifying H_2O_2 using ascorbate as a reducing power in cell. It is ubiquitous in nature and is present in algae, Euglena and higher plants. Various

isoforms of APX are active in chloroplasts, the cytosol and in microsomes. APX along with CAT represent the major enzymes for H_2O_2 quenching (Vanacker et al. 1998). Overexpression of APX in the chloroplasts of *Nicotiana tabacum* increased salt and water deficit tolerance in the plant (Badawi et al. 2004). Transgenic Arabidopsis plants over-expressing *OsAPXa* or *OsAPXb* exhibited increased tolerance to salt stress (S.H. Lee et al. 2007).

Catalase (CAT) is a tetrameric heme containing enzyme that catalyzes the dismutation of metabolic oxidizing agent H_2O_2 into H_2O and O_2. Different CAT isozymes have been extensively studied in higher plants (Polidoros and Scandalios 1999). Proliferation of peroxisomes was observed during environmental stresses, which might be helping in scavenging of H_2O_2, which can diffuse from the cytosol (Lopez-Huertas et al. 2000). Transgenic rice plants overexpressing a type 1 metallothionein *OsMT1a* demonstrated enhanced CAT activity and thus enhanced drought tolerance (Yang et al. 2009).

B. Non-enzymatic Antioxidants

AsA (vitamin C) is the most abundant, powerful and water soluble antioxidant and has been detected in majority of plant cell types, in the organelles as well as in the apoplast (Giovannoni 2007). It is involved in detoxification of many types of free radicals affecting activities of several enzymes, and it is also required for regeneration of α-tocopherol. The most important reducing substrate for H_2O_2 removal in plant cells is AsA, which also acts as a co-factor of violaxanthin deepoxidase and favours dissipation of excess excitation energy (Smirnoff 2000). AsA detoxifies superoxide radicals and singlet oxygen and dismutates H_2O_2 by the action of APX. AsA is regenerated through the conversion of MDA using MDAR in a NADPH-dependent reaction or conversion of DHA through DHAR in a GSH-dependent reaction (Smirnoff 2000; Hamada 2000). AsA can also directly scavenge $O_2^{\cdot -}$, $^1O_2$ and $^{\cdot}OH$ and regenerate tocopherol from tocopheroxy radical, contributing to membrane stability (Laing et al. 2007).

GSH which is a major source of non-protein thiols in most plant cells is a tripeptide (α-glutamyl cysteinylglycine) and is localized in all cell compartments like cytosol, chloroplasts, endoplasmic reticulum, vacuoles and mitochondria (Jimènez et al. 1997). Glutathione occurs abundantly in reduced form (GSH), and its concentration is highest in the chloroplasts (May et al. 1998). It is an important redox component in plant cells. Therefore, changes in intracellular GSH status may drastically affect cell differentiation, cell death, senescence and enzymatic regulation (Creissen et al. 1999). GSH has a thiol group which makes it water soluble, and which enables the formation of mercaptide bonds with metals and protects plants against oxidative stress and heavy metal stress (Noctor et al. 2002). Accumulation of GSH has been observed in heat stressed tomato seedlings (Rivero et al. 2007). In fact, heat stress also increased GSH levels in the flag leaf of two wheat genotypes with contrasting behaviour in heat tolerance during progressive senescence (Srivalli and Khanna-Chopra 2008).

α-tocopherols are lipophilic antioxidants synthesized by all plants. The antioxidants interact with the polyunsaturated acyl groups of lipids, stabilize membranes and scavenge various ROS and lipid soluble byproducts of oxidative stress response (Wang and Quinn 2000). Because of their chromanol ring structure, tocopherols are capable of donating a single electron to form the resonance stabilized tocopheroxyl radical (Yamaguchi et al. 2001). Lipid peroxy radicals are scavenged by α-tocopherols which generate a tocopheroxyl radical that can be recycled back to the corresponding α-tocopherol by reacting with AsA or other antioxidants. α-tocopherol is synthesized in chloroplasts and proplastids and is located in membranes of the cells, especially in the thylakoid membranes of the chloroplasts and protects membrane from oxidative damage by preventing the propagation of lipid peroxidation (McDermott 2000). Studies conducted with

different plants including soybean, rosemary and mediterranean shrubs showed that drought stress resulted in an increase in α-tocopherol levels (Shao et al. 2008a; Munné-Bosch et al. 2009). It is also reported that, over-expression of the Arabidopsis tocopherol cyclase *(VTE1)* gene, encoding an enzyme required for vitamin E synthesis, in tobacco enhanced both the vitamin E level and the tolerance to drought stress (Liu et al. 2008).

Flavonoids are a family of plant secondary metabolites of >9,000 individual molecules present in all tissues and organs and can be classified into flavonols, flavones, isoflavones, and anthocyanins based primarily on their structural features. Flavonoids are synthesized mainly in the cytosol, in multi-enzymatic complexes linked to the endoplasmic reticulum membrane, from where they are transported to their subcellular destinations. Glycosylated flavonoids are present in chloroplasts and have also been detected in plant nuclei (Feucht et al. 2004). In vitro antioxidant tests show that the antioxidant capacities of flavonoids are several fold higher than those of AsA or α-tocopherol (Rice-Evans et al. 1997). Flavonoids serve as ROS scavengers by locating and neutralizing radicals before they damage the cell, and thus they are important for plants under adverse environmental conditions (Løvdal et al. 2010). Flavonoids and other phenolic compounds absorb and screen UV light, and plants capable to synthesize these compounds are found more tolerant to high UV irradiation than mutants impaired in the flavonoid biosynthetic pathway (Clé et al. 2008). Mutant plants deficient in chalcone synthase and chalcone isomerase were unable to accumulate flavonoids and were found to be more sensitive to UV light (Filkowski et al. 2004).

Carotenoids comprise one of the largest classes of pigments in nature, and are produced in plants from isoprenoids in the chloroplasts of photosynthetic tissues and chromoplasts of fruits and flowers (Galpaz et al. 2006). These pigments are lipid soluble antioxidants playing a multitude of functions in plant metabolism including oxidative stress tolerance. They serve two major functions, as accessory pigments for light harvesting and as protective agents for scavenging the ROS produced in the photosynthetic apparatus (Tao et al. 2007). In some plants, heavy metal stress, however, is reported to reduce the level of carotenoids. Singh et al. (2008) reported decreased carotenoid and chlorophyll contents in *Vigna mungo* plants with increasing cadmium (Cd) concentration. The carotenoid content of *Hordeum vulgare* seedlings decreased under Cd-stress (Demirevska-Kepova et al. 2006). Over-expression of carotenoid biosynthesis genes enhances tolerance to stress including high light, UV, herbicides and salt in Arabidopsis and tobacco (Davison et al. 2002; Han et al. 2008).

IV. Genetic Engineering of Plants for Improving Abiotic Stress Tolerance by Enhancing ROS Defence

Since many environmental stresses are accompanied by oxidative stress, several researchers in recent years have taken the approach of attempting to improve stress tolerance in plants by modifying their ability to scavenge ROS that are generated during stress response (Table 17.1). Over-expression of one or more ROS-scavenging enzymes in various cellular compartments of plants can potentially increase their tolerance to various abiotic stresses. For example, ROS scavenging enzymes such as APXs and SODs, that function in the chloroplasts, were shown to improve photosynthesis under hyperosmotic conditions (S.H. Lee et al. 2007; Tseng et al. 2007).

Overexpressing ROS-responsive regulatory genes encoding transcription factors such as Zat10, Zat12 or JERF3 enhanced the expression of ROS-scavenging genes and tolerance to salt, drought or osmotic stress (Sakamoto et al. 2004; Devletova et al. 2005; Wu et al. 2008). In addition, overexpression of the mitogen-activated kinase 1 (*MKK1*) gene in Arabidopsis enhanced the activity of MAPK cascade, which reduced the stress-associated ROS levels and increased tolerance to abiotic stresses (Xing et al. 2008).

Table 17.1. Transgenic plants overexpressing ROS scavenging antioxidants showing abiotic stress tolerance

Gene	Transgenic plant	Gene source	Tolerance to abiotic stresses	Reference
Superoxide dismutase (SOD)				
Cu/Zn SOD	*Oryza sativa*	*Avicennia marina*	Transgenic plants were more tolerant to methyl viologen (MV) mediated oxidative stress measured as electrolyte leakage, showed more growth and yield under salt and drought stress	Prashanth et al. 2008
Cu/Zn SOD	*Nicotiana tabacum*	*Oryza sativa* L.	Enhanced tolerance to salt, drought stress and enhancement in chloroplast antioxidant system	Badawi et al. 2004
Mn SOD	*Populus davidiana* × *P. bolleana*	*Tamarix androssowii*	Salt tolerance, increase in relative weight gains of the transgenic plants and increase in SOD activity	Wang et al. 2010
Mn SOD	*Triticum aestivum*	*Nicotiana plumbaginifolia*	Photo-oxidative stress tolerance, lower oxidative damage, higher H_2O_2 and significant increase in GR and SOD activities	Melchiorre et al. 2009
Mn SOD + CAT	*Brassica campestris* L.	*Escherichia coli*	Increase in the activities of SOD, CAT, APX and GR	Tseng et al. 2007
Cu/Zn SOD + APX	*Festuca arundinacea* Schreb.	*Nicotiana tabacum*	Oxidative stress and heavy metals (Cu, Cd and As) tolerance, Low ion leakage and chlorophyll degradation	S.H. Lee et al. 2007
Catalase (CAT)				
CAT3	*Nicotiana tabacum*	*Brassica juncea*	Cadmium stress tolerance, better seedling growth and longer roots	Gichner et al. 2004
katE	*Nicotiana tabacum*	*Escherichia coli*	katE expression increases the resistance of the chloroplast translational machinery to salt stress by scavenging hydrogen peroxide	Al-Taweel et al. 2007

(continued)

Table 17.1. (continued)

Gene	Transgenic plant	Gene source	Tolerance to abiotic stresses	Reference
Ascorbate peroxidase (APX)				
cAPX	*Lycopersicon esculentum*	*Pisum sativum*	Enhanced tolerance to UV-B, heat, drought and chilling stresses, increase in APX and SOD activity	Wang et al. 2006
APX1	*Arabidopsis thaliana*	*Hordeum vulgare* L.	Salt tolerance due to higher APX, SOD, GR and CAT and low H_2O_2 content	Xu et al. 2008
swpa4	*Nicotiana tabacum*	*Ipomoea batatas*	Resistance to various stresses like MV, H_2O_2, NaCl and mannitol	Kim et al. 2008
Monodehydroascorbate reductase (MDAR)				
MDAR1	*Nicotiana tabacum*	*Arabidopsis thaliana*	Salt, ozone and PEG stress tolerance due to higher MDAR activity	Eltayeb et al. 2007
Dehydroascorbate reductase (DHAR)				
DHAR	*Arabidopsis thaliana*	*Oryza sativa*	Salt tolerance due to slight increase in DHAR activity and total ascorbate	Chen and Gallie 2005
DHAR	*Nicotiana tabacum*	*Arabidopsis thaliana*	Drought and ozone tolerance with higher DHAR activity	Ushimaru et al. 2006
Glutathione reductase (GR)				
GR	*Triticum aestivum*	*Escherichia coli*	Higher GSH content and GSH/GSH+GSSG ratio than control	Melchiorre et al. 2009
GR	*Gossypium hirsutum*	*Arabidopsis thaliana*	Photoprotection and cold tolerance	Kornyeyev et al. 2003
Glutathione peroxidase (GPX)				
GPX	*Nicotiana tabacum*	Chlamydomonas	Tolerant to oxidative, chilling and salt stress due to low MDA and high photosynthesis and antioxidative system	Yoshimura et al. 2004
GPX-2	*Arabidopsis thaliana*	Synechocystis PCC 6803	Tolerance to H_2O_2, Fe ions, MV, chilling, high salinity or drought stresses	Gaber et al. 2006

Similarly, overexpression of genes leading to enhanced levels of non-enzymatic antioxidants plays a significant role in combating different abiotic stresses. A 95% decrease in α-tocopherol in *γ-TMT (VTE4)*-RNAi tobacco lines although resulted in hypersensitivity to salt but dramatically enhanced tolerance to osmotic and oxidative stress in plants grown on high sorbitol concentrations. The reduction in α-tocopherol was compensated by γ-tocopherol accumulation in plants, revealing that γ-tocopherol is more potent than α-tocopherol in protecting against desiccation (Abbasi et al. 2007).

No systematic effort has been made to study stress induced senescence in transgenic plants with enhanced antioxidant defence, although chlorophyll degradation and lipid peroxidation are often used as markers for abiotic stress tolerance. In some studies, enhanced photosynthesis and/or PSII activity have also been shown in transgenics overexpressing SOD (Badawi et al. 2004), APX (Kim et al. 2008), MDAR (Eltayeb et al. 2007), DHAR (Chen and Gallie 2005) and GR (Kornyeyev et al. 2003). However the effect of reduced oxidative stress on cysteine protease activity or *SAG12* gene expression, a marker of senescence, has never been examined. It can be speculated that enhancing antioxidant defence may slow down stress induced senescence, but this needs to be confirmed experimentally.

V. Antioxidant Defence in Leaves During Senescence

Senescence, as in the case of other PCD processes, is associated with an increased production of ROS which oxidize proteins, lipids and DNA resulting in lipid peroxidation, cellular damage and cell death, as the antioxidant status of the leaf is reduced (Kukavica and Veljovic-Jovanovic 2004). A wealth of data exists on the association of leaf senescence with oxidative damage. The increase in oxidative stress occurs with increase in plant age especially in chloroplasts (Munné-Bosch and Alegre 2002). The biosynthesis and content of AsA decrease during senescence (Queval and Noctor 2007; Srivalli and Khanna-Chopra 2009). The process is also accompanied by a decrease in the activities of antioxidant enzymes such as SOD, CAT, APX and pyridine nucleotides (Jiménez et al. 1998; Orendi et al. 2001; Srivalli et al. 2001; Queval and Noctor 2007; Srivalli and Khanna-Chopra 2009).

Stress-induced senescence is also accompanied with an increase in ROS and a decrease in the activity of antioxidant enzymes (Hodges and Forney 2000; Sandalio et al. 2001; Santos et al. 2001). Leaf senescence and the expression of various *SAG*s were promoted in old leaves upon exposure to ozone and other treatments with increased level of ROS (Miller et al. 1999; Navabpour et al. 2003). The reduction in the antioxidant capacity might be important in the activation of proteases such as Cys proteases, which tend to be inhibited by reductants such as GSH (Groten et al. 2006). These results provide circumstantial evidence that ROS contribute to the progression of leaf senescence.

Mutant analysis and studies on transgenic plants provide a more straightforward support for the role of ROS in senescence. Kurepa et al. (1998) reported that the Arabidopsis later-flowering mutant *gigantea* was more tolerant to paraquat demonstrating a direct link between oxidative tolerance and longevity. Novel roles of NAD in signaling and regulation of cell longevity are being elucidated in plants (Noctor et al. 2006; Ying 2008). The Arabidopsis mutant *old 5*, being disrupted in NAD synthesis, showed early leaf senescence (Jing et al. 2005). The timing of senescence is also altered in the AsA-deficient Arabidopsis mutant *vtc1* (Barth et al. 2004), but this may be due to the enhanced susceptibility of individual AsA-deficient cells to PCD (Pavet et al. 2005). The delayed leaf senescence mutants of *Arabidopsis thaliana*, *ore*1, *ore*3 and *ore*9 were found to be more tolerant towards oxidative stress. Since the activities of antioxidant enzymes were not enhanced, the data provided genetic evidence, that oxidative stress tolerance is linked to control of leaf longevity in plants (Woo et al. 2004). On the other hand, transgenic plants, in which the

antioxidant enzymes were manipulated, exhibited altered leaf senescence (Orvar and Ellis 1997; Willekens et al. 1997). Thus these molecular analyses substantiate the direct involvement of ROS in leaf senescence.

ROS have a tight relationship with membranes and lipid dynamics since the membrane associated NAD(P)H oxidases can sense both endogenous and exogenous stresses and are one of the major generators of ROS (Mittler 2002). The involvement of lipid metabolism in leaf senescence was demonstrated by studying phospholipid catabolism. In Arabidopsis, antisense suppression of phopholipase Dα delayed ABA- or ethylene-induced leaf senescence (Fan et al. 1997) and the Arabidopsis *SAG101* gene encoding an acyl hydrolase was also shown to be involved in leaf senescence (He and Gan 2002). Lipids are produced by fatty acid biosynthesis pathways, and mutations in these pathways were also shown to modulate leaf senescence (Mou et al. 2002). Thus, ROS-induced membrane lipid metabolism is not a passive wear and tear process but actively involved in leaf senescence.

A. Role of Different Cell Compartments

1. Chloroplast

Chloroplast antioxidant defense plays a crucial role during oxidative stress induced senescence. Triple null mutants of Arabidopsis in stromal APX (sAPX), thylakoidal APX (tAPX) and low cellular AsA levels, showed bleaching and necrosis accompanied by accumulation of H_2O_2 during light stress (Giacomelli et al. 2007). It seems that chloroplasts may play a regulatory role during leaf senescence, similar to that of the mitochondria during animal PCD, where-in they integrate signals of apoptotic proteins regulating the release of cytochrome *c* and the production of ROS that direct subsequent apoptotic processes (Dufur and Larsson 2004). In chloroplasts, the Ndh complex regulates the redox level of cyclic electron transporters by providing electrons that are removed by the Mehler reaction and the coordinated action of SOD and POX when transporters become over-reduced. The level and activity of the Ndh complex increase during leaf senescence (Zapata et al. 2005; see also Sabater and Martín, Chap. 23). Transgenic tobacco with a knockout of the plastid *ndhF* gene shows low levels of the plastid Ndh complex, reduced ROS levels and a considerable delay in leaf senescence with respect to wild-type tobacco plants (Zapata et al. 2005). On the other hand, cytochrome *c* release from mitochondria and the decrease of Calvin cycle activity in chloroplasts both lead to an increased generation of ROS in the respective organelle. In addition, the decrease of SOD activity (Jiménez et al. 1998) in both organelles would amplify the levels of ROS most likely triggering further senescence processes. Chloroplast control of leaf senescence provides an unexpected role of the plastid *ndh* genes that are present in higher plants and opens up the question, whether plastids are targets for factors similar to apoptotic proteins affecting mitochondria in animal PCD.

2. Peroxisomes

Peroxisomes have a ROS-mediated cellular function in leaf senescence and in stress responses induced by xenobiotics and heavy metals. Peroxisomes could also have a role in plant cells as a source of signal molecules like NO, $O_2^{\cdot -}$, H_2O_2 (del Rio et al. 2002). Whereas the activities of $O_2^{\cdot -}$ and H_2O_2 generating enzymes like xanthine oxidase, urate oxidase or Mn-SOD and the NADPH-dependent generation of activities of $O_2^{\cdot -}$ on the membranes of peroxisomes increased during senescence, catalase activity almost completely decreased (del Rio et al. 1998; Tiwari et al. 2009). The enzymes of the ascorbate–glutathione cycle in the peroxisomes were also notably affected by progression of senescence, and the GSH and GSSG pools were considerably increased in peroxisomes (Jiménez et al. 1998). Moreover, it is very likely that the peroxisomal NADH-dependent production of $O_2^{\cdot -}$ radicals is intensified by the reverse transition of leaf

peroxisomes to glyoxysomes during senescence, since more NADH would be available as a result of the induction of fatty acid β-oxidation and the glyoxylate cycle.

3. Mitochondria

Mitochondria remain functional till the last phase of senescence but do show deterioration in membrane structure. It is therefore unlikely that plant mitochondria trigger plant senescence in the same way they do in animal PCD. However, the antioxidant defence declines as revealed by the decrease of APX and MDHAR activities in mitochondrial membranes contributing to higher H_2O_2 production (Jiménez et al. 1998). In parallel, the alternative respiration pathway is activated during senescence (Maxwell et al. 2002). Alternate oxidase minimizes the formation of ROS in mitochondria by preventing the overreduction of the electron transport chain and hence is regarded as a mechanism to protect the plant from oxidative stress. However, minimizing H_2O_2 production in the mitochondria by long-term antimycin A treatment in Arabidopsis by inhibiting cytochrome c dependent electron transport and stimulating alternative respiration pathway did not delay senescence. (Zentgraf et al. 2012). There is need for more studies to understand the precise role of alternate oxidase during senescence.

B. Regulation by Reproductive Sinks

Reproductive sinks modulate the rate of senescence in plants and are associated with mobilization of nutrients (Srivalli and Khanna-Chopra 2004, 2009). Wheat plants devoid of sinks exhibited delayed senescence and maintained lower ROS coupled with increased activity of antioxidant defense enzymes SOD, APX, CAT, POX and enzymes of ascorbate–glutathione cycle (Fig. 17.1) and higher GSH/GSSG ratio than plants with reproductive sink. These differences were apparent 21 days after anthesis (DAA). These sink-less wheat plants maintained higher oxidatively damaged proteins and nitrogen levels as compared to plants with reproductive sinks during monocarpic senescence. Mitochondria had higher levels of damaged proteins than chloroplasts at 7 DAA, and this was related to lower SOD and APX activity in mitochondria as compared to chloroplasts (Fig. 17.2). Removal of sinks enhanced SOD activity both in stroma and thylakoids compared to plants with sink, but SOD activity declined with progressive senescence. It appears that oxidative damage to the proteins, when not followed by high proteolytic activities, led to a slower nitrogen mobilization in wheat plants lacking a reproductive sink. Thus, the reproductive sink is able to drive forward the mobilization process through high ROS levels which mediate damage to the proteins and also influence proteolytic activities. Higher SOD and CAT activities were also observed in a stay green maize cultivar compared to an early senescent cultivar (He et al. 2005). DHAR activity is important for establishing the cellular AsA redox state and affects leaf growth. Suppression of DHAR activity accelerated chlorophyll degradation and enhanced leaf aging (Chen and Gallie 2006). Protein oxidation is widespread and often used as a diagnostic marker for oxidative stress (Møller et al. 2007). Mitochondria have been shown to contain the highest concentrations of oxidatively modified proteins as compared to chloroplasts and peroxisomes under normal, non-stressful conditions (Bartoli et al. 2004) and during progression of senescence (Jiménez et al. 1998; Rosenwasser et al. 2011).

VI. Regulation of Senescence by ROS

Dark-induced senescence in the leaves of Pelargonium was manifested in chlorophyll breakdown and an increase in ROS levels, followed by induction of two SAG transcripts: senescence-related transcription factor *WRKY6-1* and cysteine protease homolog *SAG12-1* (Rosenwasser et al. 2006). ROS-generated lipid peroxidation is an inherent feature of senescing cells and a

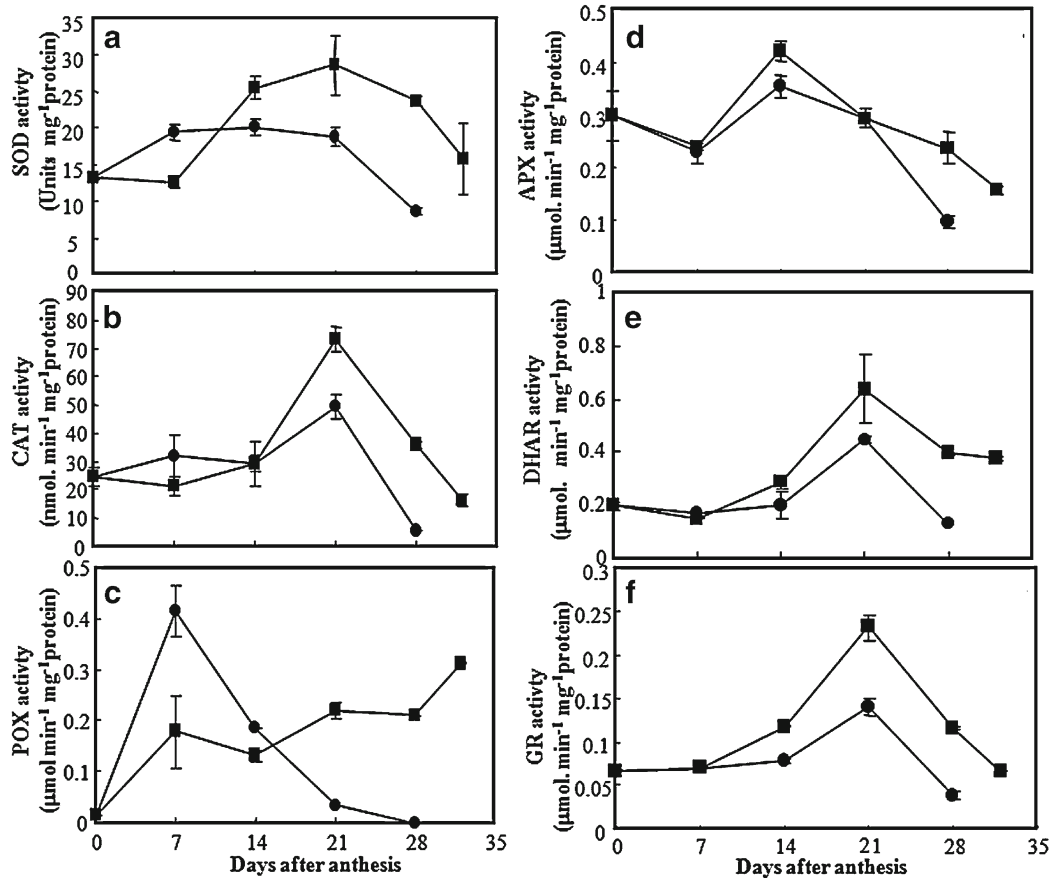

Fig. 17.1. Effect of spikelet removal on antioxidant enzyme activity in the flag leaf of *T. aestivum* cv. Kalyansona. Plants without spikelet maintained higher activity of SOD, APX, CAT, POX and of the enzymes of ascorbate-glutathione cycle than plants with sinks during progression of senescence starting from 21 DAA. SOD activity (**a**), CAT activity (**b**), POX activity (**c**), APX activity (**d**), DHAR activity (**e**) and GR activity (**f**). Control plants with intact spikes (●); plants with spikelets removed (■) (Srivalli and Khanna-Chopra 2009).

source of ROS, especially alkoxy, peroxy radicals and $^1O_2$ which are highly toxic. The increase in the leaf H_2O_2 level occurs in parallel with increases in lipid peroxidation and protein oxidation in senescent leaves (Vanacker et al. 2006). In Arabidopsis, the onset of senescence is triggered by the H_2O_2 peak during bolting time which is due to the downregulation of *CAT2* by the bZIP transcription factor GBF1. H_2O_2 inhibits APX activity and activates the systemic expression of the senescence transcription factors, i.e. WRKY53 (Smykowski et al. 2011)

Our current understanding of the relationship between environmental responses and leaf senescence mostly comes from the study of senescence responses to phytohormones such as abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid (SA) that are extensively involved in response to various abiotic and biotic stresses (Lim et al. 2007). To cite an example, ABA controls both the cellular protection activities and some of the events associated with senescence, and the balance between these two seems to be important in controlling the

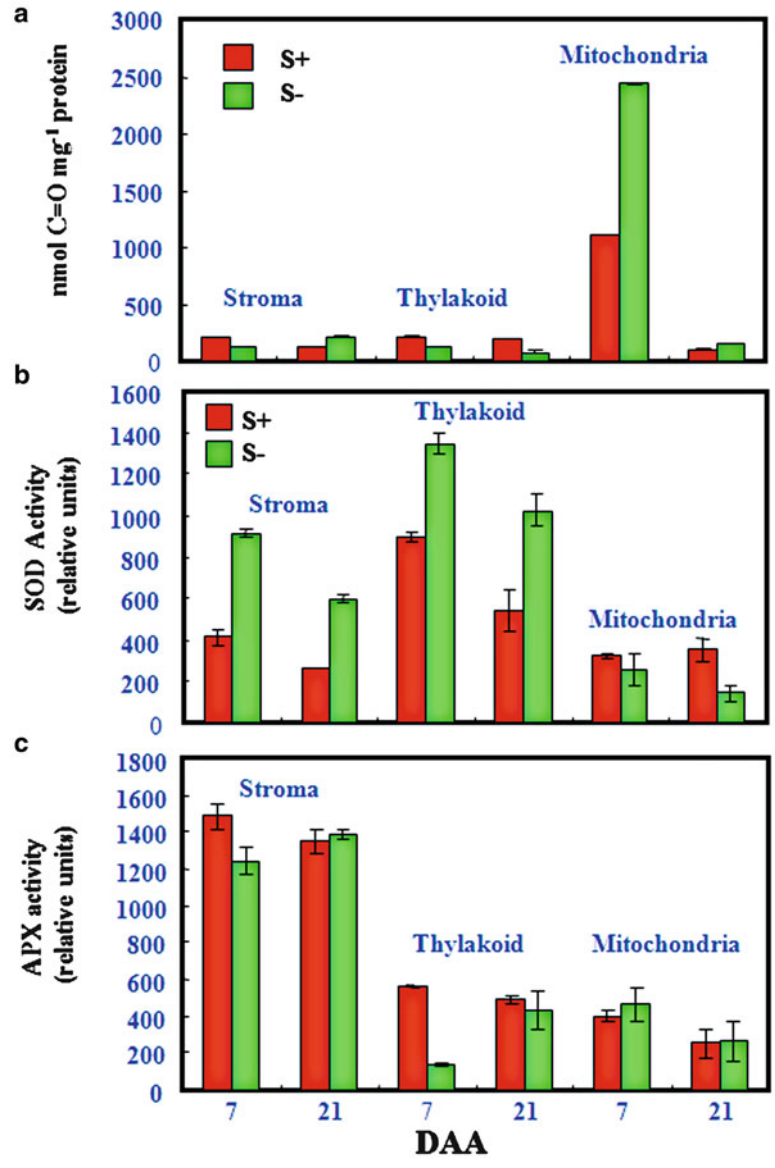

Fig. 17.2. Oxidative damage to proteins and antioxidant defense in stroma, thylakoids and mitochondria from the flag leaf of *T. aestivum* cv Kalyansona at 7 and 21 days after anthesis from control plants with intact spikes (S+) and plants with spikelets removed (S−). Wheat plants maintained higher oxidatively damaged proteins in mitochondria compared to chloroplasts at 7 DAA in both sink (+) and sink (−) plants. Mitochondria had lower SOD and APX activity than chloroplasts during monocarpic senescence. Protein carbonylation (**a**), SOD activity (**b**) and APX activity (**c**) (Srivalli and Khanna-Chopra 2009). DAA-Days after anthesis.

progression of leaf senescence (Hung and Kao 2003, 2004). It is known that ABA plays an important role in improving cold stress tolerance and triggering leaf senescence. ABA-induced expression of genes of the antioxidant system may also contribute to the enhancement of cold tolerance by managing cold stress induced ROS production (Xue-Xuan et al. 2010). The hormone plays a key role in modulating signal transduction

during variation in environmental conditions of plants (Mariya et al. 2010). The ABA signaling pathway participates in the regulatory network of cold and leaf senescence pathways involving other signaling molecules such as sugars, ethylene and ROS.

Similarly, cytokinins may be involved both in plant adaptation to drought stress and may influence the rate of senescence. Enhancing cytokinin synthesis in transgenics by overexpressing the *IPT* gene under a senescence-activated promoter (*SAG12-IPT*) induced drought tolerance and delayed drought induced leaf senescence in tobacco and bent grass (Rivero et al. 2007; Merewitz et al. 2011). Transgenics also exhibited less ROS and lipid peroxidation coupled with enhanced antioxidant defence in terms of enzyme activities and AsA and GSH content compared to the normal plants. Reduced oxidative stress protected the photosynthetic system leading to higher water use efficiency under drought stress in the transgenics plants.

VII. ROS Signaling During Senescence and Abiotic Stresses

A characteristic response is provoked depending on the type of ROS or its subcellular production site. The specificity of ROS-driven transcript expression has been assessed in a recent study comparing transcriptome data generated from ROS-related microarray experiments (Gadjev et al. 2006). On the other hand, five transcripts were identified as general oxidative stress response markers based on their responsiveness to several of the ROS-generating stresses. These include pathogenesis related proteins and antioxidant defense enzymes such as glutathione-S-transferase etc. This study provided a framework for future studies on ROS signals and oxidative stress response in plants.

Over the last few years, it has become evident that ROS signaling plays an important role in various physiological responses, including pathogen defense and stomatal opening/closure. On the other hand, ROS overproduction is detrimental for proper plant growth and development, indicating that the regulation of an appropriate redox balance is essential for plants. Plants can sense, transduce, and translate the ROS signals into appropriate cellular responses. Therefore, cells require the presence of redox-sensitive proteins that can undergo reversible oxidation/reduction and may turn on and turn off depending upon the cellular redox state (Foyer and Noctor 2003). ROS can oxidize the redox-sensitive proteins directly or indirectly via the redox-sensitive molecules, such as GSH or thioredoxins, which control the cellular redox state in higher plants (Nakashihma and Yamaguchi-Shinozaki 2006). Redox-sensitive metabolic enzymes may directly modulate corresponding cellular metabolism, whereas redox-sensitive signaling proteins execute their function via downstream signaling components, such as kinases, phosphatases, and transcription factors (Foyer and Noctor 2005). Mainly, two molecular mechanisms of redox-sensitive regulation of protein function prevail in living organisms (Shao et al. 2008b). Signaling mediated by ROS involves hetero-trimeric G-proteins (Pfannschmidt et al. 2003) and protein phosphorylation regulated by specific MAP kinases and protein Tyr phosphatases (Foyer and Noctor 2005).

ROS homeostasis in plants involves the mitogen-activated protein kinase (MAPK) pathway. A sequential phosphorylation-activation relay starts that transmits and amplifies the signal from the MAPKKK to the target, which can be a transcription factor (TF) whose activity, localization or half-life is affected by the phosphorylation. The signaling through MAPKKKs and MAPKKs could also proceed through other mechanisms than phosphorylation of their direct downstream target expanding their function outside the neatly hierarchical MAPK cascade. This is the case with the Arabidopsis MAPKKK, mitogen-activated protein kinase kinase kinase 1 (MEKK1), which might phosphorylate the WRKY53 TF directly and in addition bind to its promoter functioning

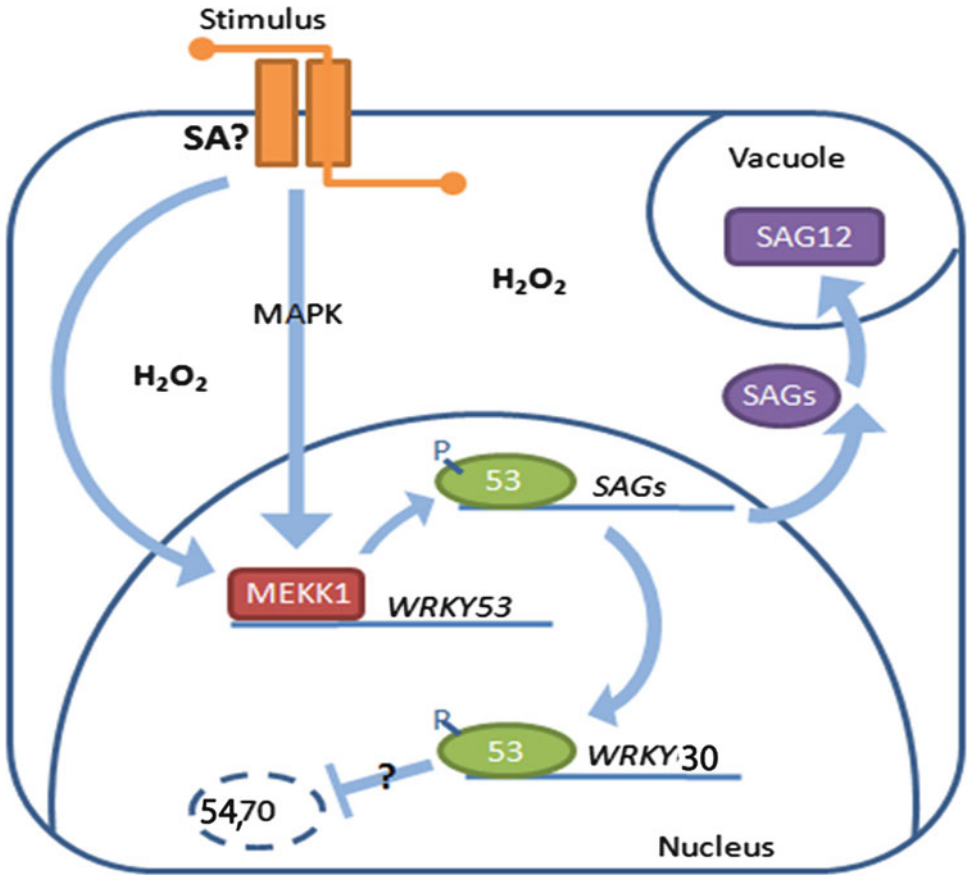

Fig. 17.3. Transcriptional regulation of senescence e.g. WRKY53. Several transcription factors are involved in the regulation of senescence including members of the WRKY family. Many members of the family are active during oxidative stress. *WRKY 53* is directly regulated by MEKK1 which binds to the *WRKY53* promoter and directly phosphorylates it. WRKY53 is responsible for the induction of many *SAGs* including vacuolar protease SAG12. WRKY53 and WRKY30 are positive regulators of senescence. WRKY54, WRKY70 and WRKY53 interact independently with WRKY30 which is expressed during developmental leaf senescence and hence could be part of senescence regulatory network.

as a transcriptional activator (Fig. 17.3, Miao et al. 2007). WRKY53 was shown to induce the expression of stress and defence-related as well as senescence-associated genes such as *SAG12* (Miao et al. 2004). The cold and salt responsive MEKK1-MKK1/2-MPK4/6 signaling cascade, seems to have a bi-directional interaction with ROS. The MEKK1 protein was found to be activated and stabilized by H_2O_2. On the other hand, the MAPK components like MPK4 and MPK6 were modulated by ROS and different abiotic stresses (Teige et al. 2004).

A major role of the MEKK1-MKK1/2-MPK4 pathway in ROS signaling was highlighted by Pitzke et al. (2009) using mutants of the pathway. Arabidopsis mutants lacking MEKK1, MKK1/2 or MPK4 exhibited formation of spontaneous lesions as well as accumulation of ROS and SA. Gene expression analysis showed that out of 32 transcription factors reported to be highly responsive to multiple ROS-inducing conditions, 20 are regulated by the MEKK1, predominantly via the MEKK1–MKK1/2–MPK4 pathway. The mutants have altered transcript levels of

genes encoding ROS scavenging enzymes indicating major imbalances in redox homeostasis. The mutant lines have reduced transcript levels of the gene encoding tAPX leading to enhanced H_2O_2 levels. The expression of *CAT2* encoding the major H_2O_2 scavenging enzyme (Vandenabeele et al. 2004) is reduced in *mekk1* and *mpk4*, but unaffected in *mkk1/2*. CAT2 down-regulation precedes the onset of senescence in Arabidopsis (Zimmermann et al. 2006). Hence *CAT2* expression is controlled by a MEKK1–MPK4 pathway by passing MKK1/MKK2.

The close relationship between cellular redox balance and senescence was highlighted by mutants of the *CPR5/OLD1* gene in Arabidopsis. A study by Jing et al. (2008) suggested that the early onset of senescence observed in *cpr5/old1* (constitutive expression of pathogenesis related genes 5) mutants of Arabidopsis is caused by a change of cellular redox balance. The mutants exhibited uncontrolled ROS generation and altered ROS scavenging as the earliest altered cellular events. Hence, *CPR5* was proposed as a senescence regulatory gene with a major function of regulating ROS generation and cellular redox status. It would be interesting to pursue the downstream components of *cpr5* involved in redox regulation.

Transcription factors regulate both senescence and abiotic stress responses (Nakashima et al. 2009). In fact, considerable crosstalk exists between the gene expression patterns of the transcription factor genes that are induced in both the processes. For example, among the 100 transcription factor genes that are induced during senescence, 28 genes are also induced during various stresses (Lim et al. 2007; Balazadeh et al. 2008). The transcription factors WRKY30, WRKY 53 and NAM, ATAF, CUC (NAC) act as positive regulators while WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence (Fig. 17.3, Besseau et al. 2012) WRKY30 and WRKY53 also respond to H_2O_2 while all four *WRKY* genes are dependent on SA for maximal expression during senescence. WRKY54, WRKY70 and WRKY53 interact independently with WRKY30 which is expressed during developmental leaf senescence and hence could be part of senescence regulatory network. The NAC regulon also plays an important role in abiotic stress responsive gene expression. Over-expression of the stress responsive *NAC1 (SNAC1)* and stress responsive *NAC2 (SNAC2)* genes enhanced drought and salt tolerance in rice without growth retardation and yield penalty (H. Hu et al. 2006). WRKY transcription factors play pivotal roles in regulating many stress responses in plants. However, unravelling their precise roles in abiotic stress responses needs more study. Over-expression studies have shown that WRKY transcription factors enhance salt, heat, cold and drought tolerance and hence form part of the signaling processes associated with transcriptional reprogramming when plants encounter abiotic stresses (Rushton et al. 2010).

As earlier discussed, H_2O_2 acts as a signaling molecule, a second messenger, mediating the acquisition of tolerance to both biotic and abiotic stresses (Desikan et al. 2003). It is involved in various responses including plant senescence. It can modulate the activities of many components in signaling, such as protein phosphatases, protein kinases and TFs. It also communicates with other signal molecules and the pathway forming part of signaling network that controls response downstream (Quan et al. 2008).

VIII. Acclimation and Communication Between Chloroplasts and Nucleus

The oxidation-reduction cascades of the photosynthetic and respiratory electron transport chains not only provide the driving forces for metabolism but also generate various redox signals. PQ, thioredoxin and ROS have all been shown to have signaling functions. Moreover, the intrinsic involvement of molecular oxygen in electron transport processes with the inherent generation of $^1O_2$, $O_2^{\cdot -}$ and H_2O_2 provides a repertoire of additional extremely powerful signals. Accumulating evidence implicates as major redox buffers of plant cells, AsA and GSH, in redox signal transduction. The network of redox signals from energy-generating

organelles orchestrates metabolism to adjust energy production to utilization, interfacing with hormone signaling to respond to environmental change at every stage of plant development (Foyer and Noctor 2003).

The abiotic stress factors produce a cellular oxidative environment which activates GSH synthesis. The redox state of the cytoplasmic GSH system affects gene expression through various redox-sensitive regulatory proteins, such as nonexpressor of pathogenenis-related protein 1 (NPR1) which is reduced, transported to the nucleus, interacts with TGACG motif binding transcription factors (TGA) and then induces *PR* gene expression. Chloroplasts provide the precursor of GSH and hence play an important role in regulating the GSH pool in cytoplasm. This model links stress induced changes in plastid -specific redox signal with nuclear gene expression which may contribute towards acclimation in plants (Foyer and Noctor 2005; see also Pfannschmidt and Munné-Bosch, Chap. 22).

Cellular redox homeostasis is significantly affected by stress-induced production of ROS. ROS, as discussed earlier, generate signals for the synthesis of defence enzymes and other antioxidant systems against stress. Foyer and Noctor (2005) have proposed a model suggesting interaction of ROS and antioxidants as an interface between metabolic and stress signals. Redox homeostasis occurs through ROS–antioxidant interaction and is a metabolic interface between stress perception and physiological and molecular adaptive responses of plant cells. This could be either induction of acclimatory processes or PCD. Although some basic information on ROS-induced expression of appropriate genes and their downstream regulation is available, the mechanisms of ROS signaling and their role in cellular redox homeostasis is not yet clearly understood (Brosche et al. 2010).

Current data suggest that GSH is a key arbiter of the intracellular redox potential, and AsA is particularly influential in setting thresholds for apoplastic and cytoplasmic redox signaling (Foyer and Noctor 2005). Differential antioxidant concentrations between compartments permit antioxidant-driven vectorial signaling through processes such as ascorbate-driven electron transport. GSH status may be perceived by the cell through several mechanisms, including protein glutathionylation which may be driven by an increase in GSSG or ROS or may be enzyme catalysed i.e. glutaredoxins (Noctor et al. 2012). This protects proteins from an irreversible damage to cysteinyl residues. Many stresses cause an initial increase in GSSG, which in turn stimulates GSH synthesis to maintain cellular homeostasis, often leading to an increase in glutathione (Srivalli and Khanna-Chopra 2008). Deviation from a high GSH/GSSG ratio during stress can trigger PCD.

Redox stimuli generated during abiotic stresses cause adjustment of plastid and leaf cell metabolism leading to acclimation (Dietz and Pfannschmidt 2011). A major target of photosynthetic redox signals is the plastid-encoded RNA polymerase (PEP). The reduction of the PQ pool acts as a signal that controls the phosphorylation of the light harvesting complexes of PSII via a thylakoid-associated kinase STN7 (Pesaresi et al. 2010). The same signals also trigger a phosphorylation cascade towards the PEP enzyme that results in changes of photosynthetic reaction center genes *psbA* and *psaA/B*.

The chloroplast is an excellent model for understanding redox-regulated plant gene expression (Pfannschmidt et al. 1999). The light-driven chemistry of photosynthesis consists of a series of redox steps involving structural components or functionally coupled pools of redox active compounds, such as thioredoxin (TRX), AsA and GSH. Changes in the redox state of these components regulate the expression of both plastome and nuclear-encoded chloroplast proteins. This redox information co-ordinates expression in both compartments (Allen and Pfannschmidt 2000). Redox signals have been shown to control post-translational modification of proteins via phosphorylation, redox modulation of assimilatory reactions and control of gene transcription and translation. Photosynthetic control of gene expression can be described for the genes located in chloroplasts themselves, both

at transcriptional and post-transcriptional levels (Pfannschmidt et al. 1999; Dietz and Pfannschmidt 2011).

However even today, the identity of the intracellular signaling molecule(s) postulated to emanate from plastids to modify nuclear gene expression remains elusive (see Pfannschmidt and Munné-Bosch, Chap. 22). Pertinent signals are thought to derive from various sources, including the tetrapyrrole pathway, protein synthesis, ROS, or the redox state of the organelle (Pfannschmidtt 2010). Recent studies have cast doubt on the most studied signaling molecule, the tetrapyrrole pathway intermediate Mg-protoporphyrin IX, indicating that chloroplast activity might control nuclear gene expression indirectly by affecting cytosolic metabolite levels or redox states (metabolic signaling). So far no true signaling molecule leaving the plastid, however, has been identified. Most ROS (maybe with the exception of H_2O_2) are very short-lived and therefore, dissociate before they can cross the chloroplast envelope and serve as a direct signal. Furthermore, ROS are rather unspecific signaling molecules because many other stress-related processes such as pathogen defence or wounding responses also involve the action of ROS. Therefore, plastid-generated ROS may initiate signaling cascades within the organelle, which then pass the envelope by unknown means (Apel and Hirt 2004). Photosynthetic redox signals from the PQ pool seem to be converted into a phosphorylation cascade but no substrates have so far been identified. Finally, many metabolites have been suggested to pass the plastidial envelope via several more or less specialized transporters. However, the metabolites leaving the plastids play an essential and intrinsic part in the cell´s metabolism, and it is difficult to assign a specific signaling role to a particular molecule (Pfannschmidt 2010). Hence there is need for more research on plastid signaling under physiologically relevant conditions and in relation to other organelles such as mitochondria with which the photosynthetic organelles are biochemically connected.

IX. Conclusions and Future Research

Senescence is a complex process and involves several regulatory molecules. Considerable crosstalk is observed in the regulatory networks involving hormones, ROS and transcription factors both in natural and stress induced senescence and abiotic stress responses. ROS mediated signaling is controlled by a very delicate balance between their production and scavenging at cellular and organelle level and plays an important role in modulation of developmental phenomena including senescence and stress responses. There is need to understand the initiation of ROS signaling, the sensing and response mechanisms and its relationship with the known signal transduction pathways involved in senescence, growth and stress responses.

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Chapter 18

Chloroplast Protein Degradation: Involvement of Senescence-Associated Vacuoles

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Summary

Senescence, the last developmental phase in the life of a leaf, is characterized by massive degradation of chloroplast proteins and redistribution of the released amino acids and peptides to other parts of the plant. Chloroplast protein degradation plays an important role in the nitrogen economy of plants. Loss of chloroplast proteins is associated with cessation of protein synthesis and an increase in rates of protein degradation. For some photosynthesis-associated proteins, there is clear evidence for degradation within the plastid itself. For example, chloroplastic FtsH metalloproteases and DegP serine-proteases are involved in the breakdown of the D1 protein upon photoinhibition of photosystem II, and these same proteases might degrade D1 during senescence. The involvement of chloroplast proteases in the degradation of Rubisco, the most abundant leaf protein, is less clear. Senescence-associated vacuoles (SAVs) are a class of small, acidic, lytic vacuoles that occur in senescing leaf cells. They develop in chloroplast-containing cells (i.e., mesophyll and guard cells) and are characterized by high peptidase activity, particularly of cysteine-type proteases. SAVs seem

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to be different from "Rubisco Containing Bodies", and development of SAVs does not depend on the operation of the autophagic pathway. A role for SAVs in chloroplast protein degradation can be implied from the fact that stromal proteins of the chloroplast and PSI are re-located to SAVs during senescence. In vitro, cysteine-type proteases within SAVs degrade the chloroplast proteins contained in these vacuoles. SAVs may be part of an extra-plastidial degradation pathway for chloroplast stroma and PSI proteins.

I. Introduction

Chloroplasts are the main source of remobilizable N in leaves of most species, particularly in C_3 plants, having amounts of proteins, nucleic acids and other nitrogenous compounds which may represent up to 70% of leaf N (Mae 2004; Krupinska 2007). Chloroplast proteins are extensively degraded during senescence of leaves, and the peptides and amino acids released are exported to other parts of the plant (Mae 2004). Therefore, senescent leaves of deciduous trees and shrubs, or of annual plants, are usually shed with no more than traces of photosynthetic proteins (Guiamet et al. 2002; Mae 2004; Krupinska 2007). Chloroplast protein degradation during senescence can be viewed as a mechanism to recycle this vast resource of nutrients, which endows plants with a remarkable plasticity in terms of internal nutrient redistribution. Depending on environmental conditions and agronomic practices, in monocarpic crop plants N remobilization associated with senescence of leaves may contribute 20–80% of protein harvested in grains of, e.g., wheat, rice, maize and other crops (Mae 2004; Gregersen et al. 2008; Masclaux-Daubresse et al. 2008). In deciduous perennial trees, N resorption from senescing leaves is likewise important to store N in the bark over the winter to sustain early growth during the following season (May and Killingbeck 1992; Killingbeck 2004). Thus, degradation of chloroplast proteins during senescence plays an important role in the nutrient balance of plants, and in nutrient cycles in nature. However, this also causes a substantial decline in the photosynthetic capacity of leaves. At the same time crops are often in need for photosynthate to fill immature seeds/grains. Thus, in many crops it might be desirable to delay senescence-associated degradation of chloroplast proteins to extend the photosynthetic activity of the canopy (Thomas and Howarth 2000). Some evidence suggests that this has been already partially accomplished by plant breeding during the past decades (e.g., Ding et al. 2005; Giunta et al. 2008). In other cases, senescence acceleration might lead to increased nutrient redistribution and, thereby, increased mineral content of grains (Uauy et al. 2006). Whether the goal is to delay or to accelerate senescence, a better understanding of the mechanism(s) of chloroplast protein degradation might provide tools to manipulate senescence to increase crop yield or the nutritional quality of grains.

Early ultrastructural studies showed that chloroplasts are degraded ahead of other cellular compartments (Noodén 1988). In many cases chloroplast number per cell does not seem to decline during senescence, i.e., the components of each chloroplast are progressively broken down, whereas some studies have recorded a decrease in the number of plastids per cell (reviewed in Krupinska 2007). Mitochondria are lost at a later stage, and mitochondrial functions (e.g., respiration) are maintained until quite late during senescence (Keech et al. 2007). To a large extent, the early and mid stages of

Abbreviations: GFP – Green fluorescent protein; Lhcb – Apoproteins of the light-harvesting complex associated to photosystem II; PCD – Programed cell death; PSI – Photosystem I; PSII – Photosystem II; RCBs – Rubisco-containing bodies; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; *SAGs* – Senescence associated genes; SAVs – Senescence-associated vacuoles

senescence seem to represent mostly a chloroplast dismantling process, leading to the appearance of "gerontoplasts" (Krupinska 2007). The dismantling of the photosynthetic apparatus is a massive process involving the degradation of the most abundant proteins on Earth (e.g., Rubisco and the light-harvesting chlorophyll-protein complexes of photosystem II).

II. Degradation of Chloroplast Proteins During Senescence

Typically, degradation of the stromal proteins involved in the carbon reduction cycle starts early, and the loss of these proteins (e.g., Rubisco, glyceraldehyde 3-phosphate dehydrogenase and ribulose 5-phosphate kinase) proceeds roughly in parallel, at similar rates (Krupinska 2007). Also non-photosynthetic, stromal proteins, such as those involved in nitrogen and sulfur assimilation are degraded early during senescence (e.g., Schmutz et al. 1983; Kamachi et al. 1992). Thylakoid membrane proteins and chlorophylls may persist a bit longer after the onset of stromal protein degradation. Among the thylakoid complexes involved in photosynthetic electron transport and ATP synthesis, there is evidence that the cytochrome b_6/f complex is lost ahead of photosystems I and II, and of ATP synthase, therefore the connection between PSII and PSI becomes the limiting step in the photochemical reactions of photosynthesis (Holloway et al. 1983; Humbeck et al. 1996; Guiamet et al. 2002). Although in many cases PSII and PSI are broken down with a similar time-course, there are also cases where PSI is lost before PSII (e.g., Miersch et al. 2000; Tang et al. 2005). Regarding each complex, degradation of PSII core proteins may be a coordinated event, since many studies have found a relatively constant F_v/F_m ratio, i.e., the maximum quantum yield of PSII, in the early phases of senescence (Miersch et al. 2000; Lu et al. 2001; Guiamet et al. 2002). However, F_v/F_m decreases during senescence of Arabidopsis and barley (Miersch et al. 2000; Woo et al. 2001). In contrast, breakdown of PSII antenna proteins may be uncoupled from degradation of PSII reaction centers, particularly under conditions of limiting irradiance where light-harvesting protein degradation is delayed (Hidema et al. 1991, 1992). There is even evidence that changes in irradiance differentially affect the degradation of the inner (CP29, Lhcb4) versus the peripheral (LHCII) antenna proteins of PSII in senescing leaves of barley (Humbeck and Krupinska 2003).

The differential timing in the degradation of the various complexes that make up the photosynthetic apparatus suggests that different, relatively specific proteases, proteolytic mechanisms or specific processes tagging proteins for degradation, might operate to achieve chloroplast protein degradation. This might afford a significant degree of control and adjustment to environmental conditions (e.g., irradiance). Particularly, balancing thylakoid versus stromal protein degradation might help to prevent overreduction of the photosynthetic electron transport chain, which might otherwise lead to the excessive production of reactive oxygen species. For example, it may be crucial to maintain a sizable pool of oxidized Q_A (the primary electron acceptor of PSII) to lower the probabilities of free radical formation, and this may be achieved by balancing the rates of degradation of light harvesting complexes in relation to consumption of reducing power. In fact, photochemical quenching estimations suggest that the pool of reduced Q_A remains fairly constant during leaf senescence in some species (Lu et al. 2001; Guiamet et al. 2002). Chloroplast breakdown must be tightly regulated to prevent runaway oxidative stress and premature cell damage before nutrient redistribution is complete.

III. Senescence-Associated Proteases

Except possibly for the case of the D1 protein of PSII, which is actively translated even at late stages of senescence (Droillard et al. 1992), the loss of photosynthetic proteins is

preceded by a decrease in their transcript levels (Lim et al. 2007) and shutdown of their synthesis (Mae 2004). However, the decisive event leading to decreased steady-state levels of photosynthetic proteins is an increase in the rates of protein degradation (Lamattina et al. 1985; Mae 2004). This is supported by correlative evidence showing an increase in proteolytic activity during senescence (e.g., Peoples and Dalling 1988; Feller 2004) as well as by the observation that inhibition of protease activity by pharmacological treatments with protease inhibitors can reduce chloroplast protein degradation in vivo (Thoenen et al. 2007).

Studies in Arabidopsis, Populus, wheat and barley have identified cysteine-, serine-, aspartic- and metalloproteases whose transcript levels increase during senescence of leaves (Buchanan-Wollaston et al. 2003; Andersson et al. 2004; Gregersen and Holm 2007; Parrott et al. 2010). Among these, the cysteine proteases appear to be the group of proteases most frequently associated with senescence. Also components of the autophagic machinery are up-regulated during leaf senescence (Thompson et al. 2005; Wada et al. 2009). The list of senescence-associated proteases includes proteins with a clear-cut transit peptide for chloroplast targeting as well as proteases synthesized by the endoplasmatic reticulum and targeted to their destinations by the secretory pathway. Apart from correlative evidence linking the expression (mRNA) of these proteases to the progression of senescence, increases in protein levels in parallel with mRNA have been shown for only a few proteases (e.g., SAG2 and SAG12 of Arabidopsis, Grbic 2003). There are cases of non-parallel changes in mRNA and its corresponding protein, for example the mRNA levels of ERD1, a regulatory subunit of the Clp proteolytic complex, increase during senescence of Arabidopsis leaves (Nakashima et al. 1997), whereas the ERD1 protein content declines (Weaver et al. 1999). Direct evidence implicating specific proteases with degradation of chloroplast proteins, e.g., through the use of knock out or knock down lines, is lacking in most cases, except for a few proteases which have been studied in more detail (see below).

A. Evidence for the Involvement of Chloroplast Proteases

The involvement of plastid-localized proteases in chloroplast protein degradation was put forward a few decades ago (reviewed in Gepstein 1988). More recently, genomic approaches revealed a large number of proteases with plastid targeting peptides (Adam and Clarke 2002; Sakamoto 2006), and transcriptomic analysis have shown that a number of these chloroplast proteases, particularly proteases of the Clp, DegP and FtsH families, are upregulated during senescence in various species (Bhalerao et al. 2003; Buchanan-Wollaston et al. 2003; Andersson et al. 2004; Guo et al. 2004; Gregersen and Holm 2007).

While some members of the ClpP family (i.e., nclpP3 and nclpP5) are up-regulated during senescence (Nakabayashi et al. 1999), the expression of other Clp proteases is constitutive, with no changes during leaf ontogeny (e.g., Shanklin et al. 1995). Clp proteases may play such an important role early during chloroplast biogenesis that knock out lines for the ClpP subunit are not viable (Zheng et al. 2006), which complicates the use of reverse genetic approaches to understand their role during senescence.

Proteases of the FtsH and DegP families seem to play a role in the degradation of the D1 protein of photosystem II. Members of the FtsH family are thylakoid-bound, ATP-dependent metalloproteases (Adam and Clarke 2002), and some of them seem to perform an important function in chloroplast biogenesis, as implied from the variegated phenotype of knock out mutants for FtsH2 and FtsH5 (Zaltsman et al. 2005). Interestingly, these alterations of chloroplast biogenesis are exacerbated in FtsH2/FtsH8 and FtsH5/FtsH1 double mutants, suggesting that these proteases act redundantly (Zaltsman et al. 2005). FtsH2 and FtsH5 seem to be involved in the degradation of

the D1 protein during the PSII repair cycle following photoinhibition (Zaltsman et al. 2005; Kato et al. 2009). The DegP family encompasses a number of serine-type, plastid proteases (Adam and Clarke 2002). Chloroplastic Deg proteases may cooperate with FtsHs in D1 degradation during photoinhibition (Kapri-Pardes et al. 2007; Sun et al. 2007, 2010). Although senescence-associated degradation of D1 has not been studied in detail, the breakdown of D1 associated with the repair cycle of photoinhibition is active enough (Aro et al. 1993; Guiamet et al. 2002), that there does not seem to be a need for a separate, senescence-specific pathway.

In vitro studies with thylakoids isolated from knock-out lines for the thylakoid-associated FtsH6 metalloprotease linked this protease to the degradation of the Lhcb antenna complexes of photosystem II (Zelisko et al. 2005). Thylakoids isolated from senescing leaves degraded Lhcb3 in vitro, and this degradation was inhibited by phosphoramidon (an inhibitor of metalloproteases) and EDTA. Addition of Zn^{2+} to EDTA-treated thylakoids restored the proteolytic activity against Lhcb3. Degradation of Lhcb1 and Lhcb3 was not affected in knock-out lines for FtsH5 or FtsH11, but degradation of Lhcb3 was slowed down in thylakoids of an FtsH6 knock out line (Zelisko et al. 2005). This is consistent with previous observations showing that senescence-associated degradation of Lhcb3 in isolated thylakoids is carried out by a zinc-binding metalloprotease (Zelisko and Jackowski 2004). However, in vivo studies with an FtsH6 knock out line failed to confirm the involvement of this protease in Lhcb degradation during senescence or high-irradiance acclimation (Wagner et al. 2011).

There is no clear-cut evidence for senescence-associated breakdown of Rubisco within chloroplasts. In isolated chloroplasts, oxidative stress (e.g., high irradiance, high O_2 concentrations, addition of paraquat, etc.) leads to the formation of insoluble Rubisco aggregates, which are degraded (Desimone et al. 1996). Likewise, isolated chloroplasts can degrade Rubisco and other stromal proteins in the presence of light (Roulin and Feller 1998), even if irradiance is moderate to low, and, therefore, probably not relevant during oxidative stress. However, light, which accelerates Rubisco degradation in in vitro experiments, most often delays, rather than accelerates chloroplast protein degradation in vivo (Noodén and Schneider 2004). In darkness, isolated chloroplasts break down Rubisco at very low rates (Roulin and Feller 1998). In some cases, incubation of isolated chloroplasts yields Rubisco fragments, but degradation does not proceed beyond this initial cleavage (Kokubun et al. 2002). Autodigestion experiments with isolated chloroplasts do not provide clear and strong evidence for Rubisco breakdown within plastids of senescing leaves.

The involvement of the plastidic CND41 aspartic protease in Rubisco degradation was put forward based on phenotypic analysis of antisense lines (Kato et al. 2004, 2005). CND41 degrades partially denatured Rubisco in vitro (Kato et al. 2004), and the loss of Rubisco is retarded in antisense lines (Kato et al. 2005). However, CND41 is also a DNA-binding protein, and it might affect Rubisco degradation indirectly. For example, bioactive gibberellin levels are lower in CND41 antisense lines, therefore their growth is stunted (Nakano et al. 2003). Since leaf senescence during vegetative growth is controlled by correlative effects from the younger parts of the plant (i.e., the developing shoot apex, Noodén et al. 2004), delayed senescence and Rubisco degradation in CND41 antisense plants might be just an indirect effect of dwarfism in these plants (Martínez et al. 2008b). The direct involvement of CND41 in chloroplast protein degradation might be tested in plants where the dwarf phenotype is corrected through exogenous applications of gibberellin.

In brief, there is evidence for degradation of photosystem II proteins within the chloroplast itself, whereas the involvement of plastid proteases in the breakdown of Rubisco and other stromal proteins is not as conclusive. This is consistent with the observation that stay-green mutants of soybean, Festuca

and rice specifically fail to degrade Lhcb proteins while breakdown of other chloroplast proteins proceeds normally (Thomas and Howarth 2000; Guiamet et al. 2002; Kusaba et al. 2007), which points to the operation of specific pathways for degradation of PSII light-harvesting proteins and Rubisco, respectively.

B. Chloroplast Protein Degradation in the Central Vacuole

Proteolysis within the central vacuole seems a logical alternative to degradation within plastids. Most of the acidic protease activity of a higher plant cell resides in the central vacuole (Matile 1997; De 2000), which is also the location of several senescence-associated cysteine-type proteases, e.g., SENU3 and SENU4 of tomato (Drake et al. 1996) and AtALEU/SAG2 of Arabidopsis (Hensel et al. 1993). The activity of four cysteine proteases localized to the central vacuole increased during senescence induced by incubation in darkness, water deficit, or during normal monocarpic senescence associated with reproduction in wheat (Martínez et al. 2007). Moreover, vacuolar lysates can degrade Rubisco in vitro (Yoshida and Minimikawa 1996; Srivalli et al. 2001). However, the spatial separation between chloroplast proteins and vacuolar proteases is maintained during senescence (Evans et al. 2010), which at first glance would rule out an important role of the vacuole in photosynthetic protein degradation. Few papers have, however, provided evidence for engulfment of entire plastids within the central vacuole (e.g., Minimikawa et al. 2001; Niwa et al. 2004; see also Krupinska and Mulisch, Chap. 14).

Chloroplast-derived vesicular structures have been implicated in Rubisco export out of chloroplasts as part of an extra-plastidial Rubisco degradation pathway (Chiba et al. 2003; Ishida et al. 2008; Wada et al. 2009; see also Wada and Ishida, Chap. 19). These Rubisco Containing Bodies (RCBs) are spherical vesicles (0.4–1.2 μm in diameter) bound by a double membrane. They contain Rubisco and glutamine synthetase II, but no thylakoid proteins (Chiba et al. 2003). The number of RBCs per cell increased by about threefold concomitantly with the start of Rubisco loss in the course of natural senescence of the first leaf of wheat (Chiba et al. 2003), suggesting that they are implicated in the degradation of the stromal proteins of the chloroplast. In Arabidopsis leaves expressing GFP fused to a chloroplast targeting peptide, GFP-containing vesicles are apparent in leaves treated with concanamycin A, an inhibitor of H^+-ATPases that causes an increase in vacuolar pH and, thereby, decreases vacuolar proteolytic activity (Ishida et al. 2008). Some GFP-vesicles (RCBs) were clearly located in the central vacuole, which points to a transport system between the chloroplast and the central vacuole mediated by RCBs (Ishida et al. 2008). Development of RCBs and their internalization into the central vacuole seemed to involve the autophagic pathway, since the number of RCBs decreased markedly in the cytoplasm and the central vacuole of concanamycin A-treated cells of autophagy mutants, i.e., knock out lines for *atg5* or *atg4a-4b* (Ishida et al. 2008; Wada et al. 2009; see also Ishida, Chap. 19). These observations point to the operation of an autophagic pathway in which RCBs carrying stromal proteins are internalized into the central vacuole, where chloroplast proteins may be broken down by vacuolar proteases. This predicts that inactivation of the autophagic machinery should slow down or inhibit chloroplast protein degradation. However, the loss of chloroplast proteins, including Rubisco, is significantly accelerated in autophagy mutants (Doelling et al. 2002; Thompson et al. 2005), which implies that an alternative, redundant pathway may operate to bring about extraplastidic chloroplast protein degradation in an autophagy-independent way.

IV. Senescence-Associated Vacuoles (SAVs)

A. Characterization of SAVs

Typically, mature mesophyll cells contain a single, large central vacuole which occupies most of the cell volume and performs a number of important functions, including osmoregulation,

hydrolytic activities, sugar and amino acid storage, disposal of xenobiotics, etc. (De 2000). In special cases, mesophyll cells may have two types of vacuoles, as is the case for the salt-tolerant plant *Mesembryantemum crystallinum* where one vacuole sequesters NaCl as a mechanism to avoid Na^+ toxicity in the cytosol whereas a second vacuole accumulates malate at night during operation of CAM photosynthesis (Epimashko et al. 2004). Thus, there may be more than one type of vacuole in mesophyll cells.

During senescence of several dicots, e.g., Arabidopsis, soybeans and tobacco, "*S*enescence-*A*ssociated *V*acuoles" (SAVs) accumulate in chloroplast containing cells, i.e., mesophyll and guard cells (Otegui et al. 2005). SAVs are small (0.5–0.8 μm in diameter), and bound by a single membrane which contains at least one marker (i.e., vacuolar H^+ pyrophosphatase) in common with the central vacuole (Fig. 18.1). However, SAVs differ from the central vacuole in a number of features: SAVs are smaller and they lack

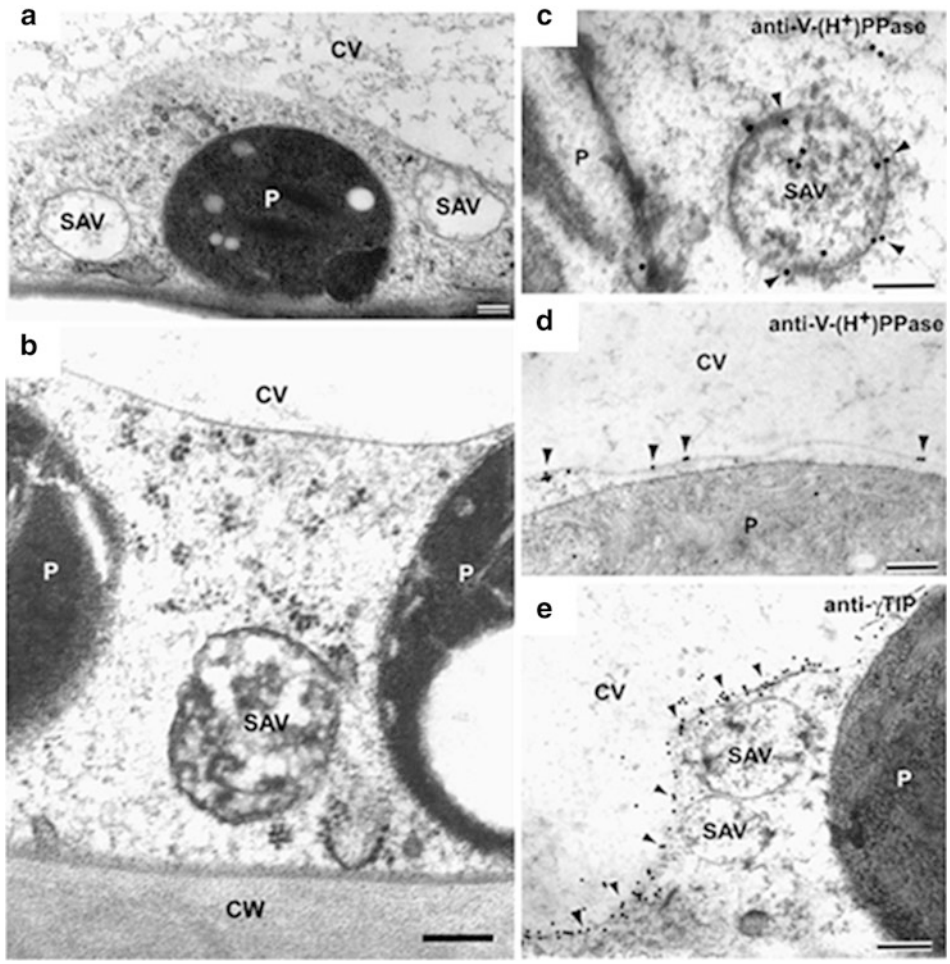

Fig. 18.1. Senescence-associated vacuoles (SAVs) of Arabidopsis and soybean observed by electron microscopy of high-pressure frozen/freeze-substituted samples. (**a**) SAVs develop in the peripheral cytoplasm surrounding the central vacuole of a senescing leaf cell of Arabidopsis. (**b**) SAV with dense contents in the peripheral cytoplasm of a senescing soybean cell. (**c**–**e**) Immunolocalization of vacuolar markers in senescing leaf cells of Arabidopsis. (**c**) and (**d**) Immunolocalization of the vacuolar H^+- pyrophosphatase, note labeling of the limiting membrane of SAVs (**c**) and the central vacuole tonoplast (**d**). (**e**) immunolabeling of γ-TIP, the tonoplast of the central vacuole is densely labeled, whereas the membrane of SAVs is not. *CV* central vacuole, *CW* cell wall, *P* plastid. *Scale bars* = 200 nm (**a** and **b**), 250 nm (**c**, **d**, and **e**). (Reprinted from Otegui et al. (2005).).

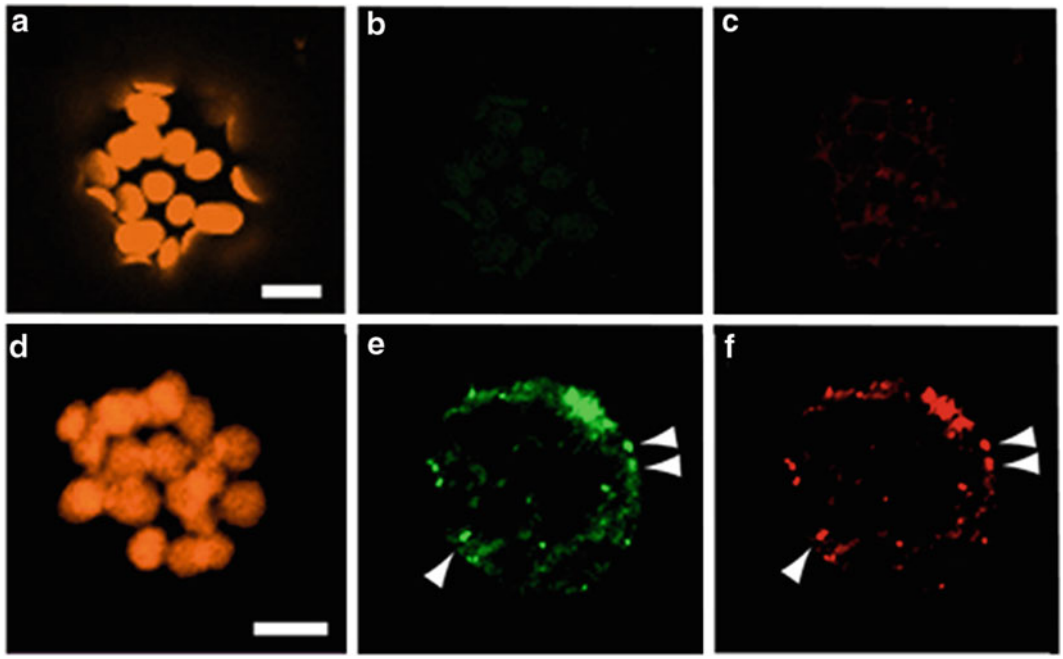

Fig. 18.2. Detection of cysteine-type protease activity in senescence-associated vacuoles. (**a** through **f**), protoplasts isolated from non-senescent (**a**, **b** and **c**) and senescent (**d**, **e** and **f**) leaves of Arabidopsis were incubated with Lysotracker Red (**c** and **f**) to label SAVs and the cysteine protease substrate R6502 (**b** and **e**). (**a** and **d**) show chlorophyll autofluorescence. Note the absence of Lysotracker-stained structures in non-senescent leaves, and the localization of R6502 within Lysotracker-stained SAVs in senescent leaves (*arrowheads* show a few representative SAVs). *Scale bar* = 10 μm. (Reprinted from Otegui et al. (2005).).

the vacuolar water channel γ-TIP (Otegui et al. 2005; Fig. 18.1). Besides, SAV are about 0.8 pH units more acidic than the central vacuole (Otegui et al. 2005), and this may be very significant in view of the fact that pH changes of this magnitude can exert a large influence on protein degradation within vacuoles (e.g., Hwang et al. 2003).

SAVs represent a novel class of lytic vacuoles, characterized by high peptidase activity. Confocal microscopy employing a fluorogenic peptidase substrate with affinity towards cysteine-type proteases showed that the highest peptidase activity of senescing mesophyll cells is located within SAVs (Otegui et al. 2005; Fig. 18.2). Although the interpretation of activity staining experiments in vivo may be complicated due to differences in surface-to-volume ratios of organelles with such contrasting sizes as SAVs and the central vacuole, subcellular fractionation studies confirmed these observations. Activity labeling with the biotinylated cysteine protease probe DCG-04 (Greenbaum et al. 2000; van der Hoorn et al. 2004) and detection in Western blots showed that labeled (and therefore active) cysteine proteases are mainly located in SAVs, compared to a crude leaf extract, or to chloroplasts isolated from senescing leaves (Fig. 18.3). Irrespective of the method employed for this analysis, it is quite clear that cysteine protease activity is enriched in SAVs, which is significant in view of the close association of cysteine proteases with the execution of the senescence program. SAG12, a strictly senescence-associated cysteine protease (Lohman et al. 1994), and a widely used marker for progression of senescence, also localized to SAVs (Otegui et al. 2005). Thus, we are tempted to conclude that SAVs represent the most active proteolytic compartment in senescing leaf cells.

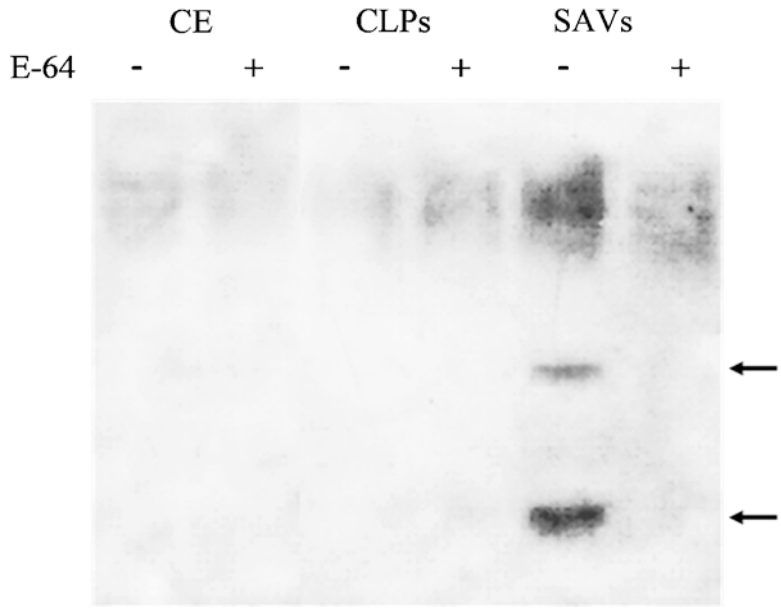

Fig. 18.3. Western blot analysis of cysteine-type proteases labeled with the biotinylated inhibitor DCG-04 and detected with a streptavidin-peroxidase conjugate. Equal amounts of protein (3 µg) from a crude extract (CE), purified chloroplasts (CLPs) and isolated SAVs were loaded in each lane. To control for non-specific binding of DCG-04 to proteins other than cysteine proteases, aliquots of the samples were incubated with the irreversible inhibitor E-64 prior to labeling with DCG-04 (Greenbaum et al. 2000; van der Hoorn et al. 2004). *Arrows* show two cysteine-type proteases of around 40 and 33 kDa apparent molecular mass which are detected only in SAVs; specific binding is confirmed by inhibition of labeling with E-64.

B. SAVs and Chloroplast Protein Degradation

The facts that SAVs develop in chloroplast containing cells during senescence, and that they have most of the cysteine protease activity of the cell suggest that SAVs might play a role in the breakdown of chloroplast proteins during senescence. The involvement of SAVs in breakdown of photosynthesis proteins would require re-location of chloroplast proteins to SAVs. To determine if a chloroplast protein may be re-located to SAVs during senescence, Martínez et al. (2008a) employed a transgenic tobacco line expressing GFP targeted to the plastid (Köhler et al. 1997). In mesophyll cells of mature, non-senescing leaves, GFP localized exclusively to chloroplasts (Martínez et al. 2008a). In contrast, in senescing leaves, a strong GFP signal was also detected in SAVs, indicating re-location of a chloroplast protein to SAVs (Fig. 18.4). Isolation and biochemical analysis of SAVs confirmed the presence of proteins from the chloroplast stroma (Rubisco and glutamine synthetase II, Martínez et al. 2008a), and photosystem I (Gomez et al. unpublished). Although chloroplasts can break down the large subunit of Rubisco (RbcL) to a 44 kDa fragment in vitro (Kokubun et al. 2002), normal sized RbcL was found in SAVs, which seems to rule out the possibility of an initial, partial proteolytic attack in plastids before transfer to SAVs (Martínez et al. 2008a). No PSII proteins were detected in SAVs, neither by immunological methods, modulated fluorescence measurements nor 77 K spectrofluorometry (Martínez et al. 2008a, Gomez et al. unpublished). These findings show that some chloroplast proteins are transported to SAVs, and hint at the operation of a novel sorting and trafficking pathway between chloroplasts and SAVs.

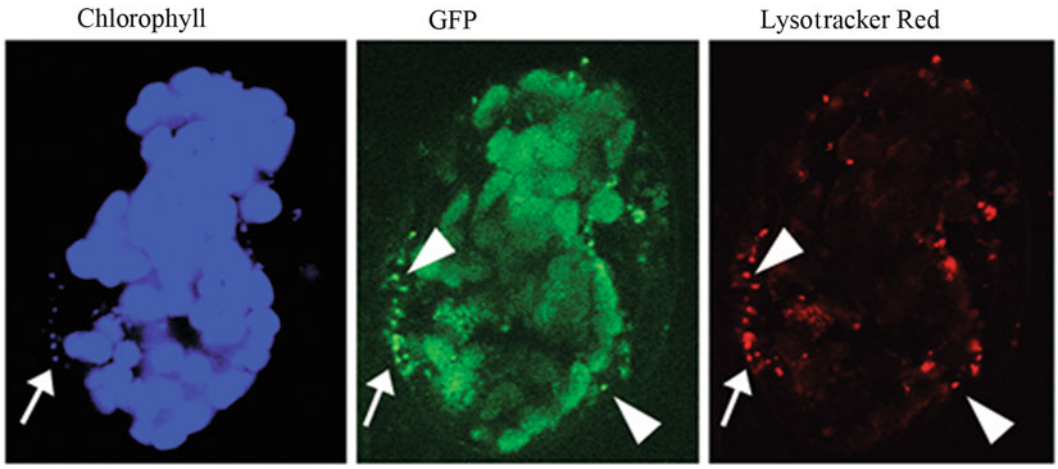

Fig. 18.4. Confocal section through a cell isolated from a senescing tobacco leaf expressing GFP targeted to the plastids and incubated with Lysotracker Red to detect SAVs. The punctuate pattern of GFP signal outside chloroplasts localizes to SAVs labeled with Lysotracker *Red* (*arrowheads* show a GFP-containing SAV). Less frequently, a fluorescence signal with the spectral properties of chlorophyll is also detected in SAVs (the *arrows* show SAVs with GFP and chlorophyll fluorescence). (Reprinted from Martínez et al. (2008a).).

Given their high peptidase activity, it is reasonable to presume that SAVs are not only a transport vesicle for chloroplast proteins. A role for SAVs in chloroplast protein breakdown seems likely, and this is supported by autodigestion experiments, where the chloroplast proteins contained in isolated SAVs were degraded by SAV-proteases (Martínez et al. 2008a; Gomez et al. unpublished). Though this supports the breakdown of chloroplast proteins in SAVs, *in planta* experiments (e.g., with knock out lines for SAV proteases) are required to confirm this issue.

C. Comparison with Other Lytic Compartments of Plant Cells

A number of small lytic compartments, different from the central vacuole, have been described in many tissues (for a review, see Müntz 2007). Of particular interest here are lytic compartments associated with senescence or cell death processes.

Aleurone cells of barley seeds develop small, acidic and proteolytically active secondary vacuoles after seed germination (Swanson et al. 1998). These secondary vacuoles contain cysteine protease activity, which might correspond, in part, to the papain-type protease aleurain. Gibberellic acid increases acidity of secondary vacuoles, whereas treatment with abscisic acid increases their lumenal pH (Swanson et al. 1998), which might affect the proteolytic activity of these vacuoles. Since gibberellins accelerate, while abscisic acid delays programmed cell death in aleurone cells, these observations seem to implicate secondary vacuoles in the execution of aleurone PCD.

Ricinosomes are small lytic vesicles in senescing endosperm cells of *Ricinus comunis* (Schmid et al. 1998, 1999; Gietl and Schmid 2001). Ricinosomes bud off the endoplasmic reticulum carrying the inactive 45 kDa precursor of the castor-bean cysteine endopeptidase Cys-EP, which carries a C-terminal KDEL motif for ER retention. The proCys-EP matures to the 35 kDa active form upon acidification of ricinosomes (Schmid et al. 2001). The number of ricinosomes per cell increases as nuclear DNA fragmentation (a marker for PCD) progresses in endosperm cells after germination (Schmid et al. 1999). At a late stage of PCD ricinosomes rupture releasing the active Cys-EP, which is then apparently involved in the final

clearing of the dead cell body. Similar organelles are found in senescing petals of *Hemerocallis* sp. (Schmid et al. 1999) and sporophytic tissues of tomato anthers (Senatore et al. 2009). KDEL-tailed cysteine proteases have been found in a number of species, but they do not seem to be expressed in senescing leaves (Gietl and Schmid 2001). SAV proteins already identified as transported through the endomembrane system (i.e., the cysteine-protease SAG12 and V-H^+ pyrophosphatase) lack an ER-retention motif, which is an important difference between SAVs and ricinosomes. Moreover, ricinosomes seem to act at a very late stage in programmed cell death, whereas SAVs appear early (Otegui et al. 2005), when leaf senescence is still a reversible process (Zavaleta-Mancera et al. 1999).

D. SAVs and Rubisco Containing Bodies

There are a number of similarities between SAVs and RCBs which might suggest that these are the same or closely related structures, with a similar function. Both, SAVs and RCBs develop during senescence of leaves, and both contain Rubisco, glutamine synthetase, and chloroplast-targeted GFP (Chiba et al. 2003; Otegui et al. 2005; Martínez et al. 2008a). However, RCBs do not carry chlorophyll, and are limited by a double membrane (Chiba et al. 2003), whereas SAVs have a single limiting membrane (Otegui et al. 2005) and carry chlorophyll (Martínez et al. 2008a). SAVs and RCBs accumulate in senescing leaves, therefore both might be present simultaneously in the same cells during senescence. However, in tobacco leaves senescing under continuous darkness, there are SAVs containing an acidic interior and chloroplast-targeted GFP, but apparently no RCBs (i.e., vesicles containing chloroplast-targeted GFP but not an acidic lumen) (Martínez, Costa, Guiamet, unpublished observations), which seems to rule out the simultaneous occurrence of both organelles in the same cells. A thorough examination of the presence of SAVs and RCBs in the same tissues under diverse developmental and environmental scenarios, or in response to specific hormones is needed, but this will require the discovery of specific molecular markers for RCBs in conjunction with the use of SAG12 as a marker for SAVs.

The development of SAVs does not seem to depend on the autophagic machinery, as SAV development is apparently normal in an *atg7* knock-out line of Arabidopsis (Otegui et al. 2005), where autophagy is completely disrupted (Doelling et al. 2002). In contrast, formation of RCBs seems to be impaired in an autophagy mutant (Wada et al. 2009; Wada and Ishida, Chap. 19). Equally important, active proteolysis takes place inside SAVs but so far no proteolytic activity has been described for RCBs. In this regard, SAVs are clearly lytic compartments whereas RCBs might represent a "shuttle" for chloroplast proteins on their way to the central vacuole. Thus, the present evidence suggests that RCBs and SAVs are part of two different mechanisms for chloroplast protein degradation during senescence. The observation that chloroplast dismantling and breakdown of their components is accelerated, rather than delayed, in autophagy mutants (e.g., Doelling et al. 2002; Hanaoka et al. 2002; Thompson et al. 2005; Xiong et al. 2005; Wada et al. 2009), where formation of RCBs is impaired (Wada et al. 2009), reinforces the idea that an active chloroplast protein degradation machinery, probably involving SAVs, operates independently of autophagy and RCBs.

V. Conclusions and Prospects

The evidence collected so far suggests that SAVs are a lytic compartment with high peptidase activity, where stromal proteins and PSI proteins of the chloroplast are re-located during senescence, setting the stage for their degradation (Fig. 18.5). A number of important questions remain to be addressed, including the origin of SAVs, how stromal and PSI proteins are sorted out from other chloroplast components for transport to SAVs, the transport mechanism itself, and the final fate of SAVs. The origin of SAVs may involve

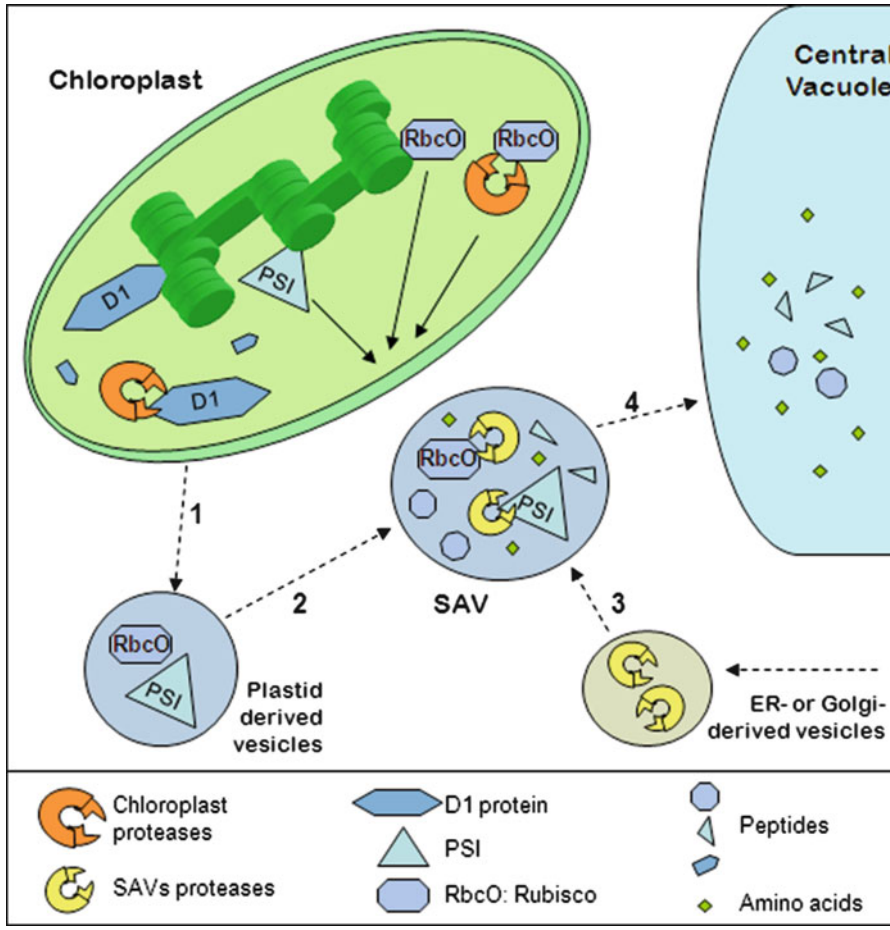

Fig. 18.5. A schematic view of the hypothetical involvement of senescence-associated vacuoles in the degradation of the most abundant chloroplast proteins during senescence of leaves. In this model, D1 (and possibly PSII and other chl-binding proteins) is broken down within the plastid by chloroplastic proteases (shown in *orange*). Rubisco (RbCO) and other stromal proteins are re-located from the plastid to SAVs (*1, 2*) and broken down there by non-chloroplastic proteases (shown in *yellow*). Probably whole PSI complexes are also re-located to SAVs for degradation. Fusion of SAVs with the central vacuole (4) might deliver amino acids and peptides (or even protein fragments which might be degraded to completion), and amino acids may be stored in the central vacuole until they are redistributed to other parts of the plant. The origin of SAVs possibly involving the fusion of plastid-derived (2) and ER- or Golgi-derived (3) vesicles, the mechanism sorting and transporting chloroplast proteins into SAVs and their final destination are areas that need further research. Modified from Martínez et al. (2008b).

events taking place in the chloroplast and in the secretory pathway, since some components of SAVs (e.g., SAG12) have a signal peptide for translocation into the endoplasmic reticulum (Grbic 2003), whereas other components of SAVs have a clear plastidic origin (e.g., chloroplast proteins). Plastids are fairly dynamic entities, with stromal protrusions (stromules) constantly extending out of, and retracting on the main plastid body (Gunning 2005; Hanson and Satterzadeh 2011). Stromule tips often enlarge, and cases where stromule tips were shed off have been recorded (Gunning 2005). Plastid protuberances, much shorter than stromules, are also common (Hanson and Satterzadeh 2011). Mass export of chloroplast components in the form of vesicles formed through scission

of chloroplast protuberances or shedding of stromule tips might supply the chloroplast molecules found in SAVs. However, stromules are found more frequently in non-green plastids than in chloroplasts (Hanson and Satterzadeh 2011; see also Gray, Chap. 9), which seems to rule out the involvement of stromules in the biogenesis of SAVs. In any event, these observations highlight the dynamic nature of the chloroplast and its envelope, suggesting that similar changes might result in the formation of vesicles containing chloroplast components. Chloroplast-derived vesicles might later fuse with vesicles coming from the ER and Golgi apparatus carrying, e.g., proteases, hydrolytic enzymes, etc. (Fig. 18.5). We surmise that SAVs may eventually fuse with the tonoplast and discharge their contents in the central vacuole, which might be a site for transient storage of amino acids released from protein degradation. In this regard, it is interesting that the vacuolar concentration of amino acids increases during senescence (Matile 1997), possibly placing the central vacuole as the destination of SAV contents. A thorough analysis of the protein composition of SAVs employing mass spectrometry approaches combined with reverse genetic approaches may help to unravel some of these questions in the future.

Different mechanisms may be involved in chloroplast protein degradation (Hörtensteiner and Feller 2002), and these include (1) degradation by chloroplast-targeted, nuclear-encoded hydrolases, (2) export of chloroplast components to lytic vacuoles or (3) re-location of vacuolar hydrolases to the chloroplast. There is evidence for the operation of the first two mechanisms, whereas, as far as we know, there is not much evidence for re-direction of vacuolar proteases to chloroplasts. Collectively, the evidence may be accommodated into a scheme where PSII components are degraded within the plastid, while stromal and PSI proteins are degraded in SAVs, at least partially, and the resulting peptides and/or amino acids transported to the central vacuole (Fig. 18.5). This does not rule out other mechanisms, e.g., autophagy-mediated degradation of RCBs, which might operate independently of SAVs. Separate, independently regulated plastidic and extra-plastidic pathways for the degradation of different chloroplast proteins might endow senescing cells with a high degree of flexibility, for example to adjust light harvesting and carboxylation capacity to the environmental conditions (e.g., senescence under shade vs. sunlight), which may be quite important during the initial stages of senescence when residual photosynthesis may supply much of the energy consumed by the degradation and export machinery.

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Chapter 19

Autophagy of Chloroplasts During Leaf Senescence

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Summary

During leaf senescence, chloroplasts undergo the programmed breakdown of both stromal and thylakoid components of the photosynthetic apparatus. This strategy has evolved to remobilize nutrients from old leaves into newly developing tissues and sustain maximal growth rates. After the remobilization of chloroplast components, some shrunken chloroplasts called gerontoplasts, which are plastid structures formed by the loss of the thylakoid membrane network, remain in the cytoplasm. Concomitantly, the population of chloroplasts

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is decreased in mesophyll cells. The morphological traits of senescing cells, including the capture of whole chloroplasts in the vacuole has been observed by electron microscopy since the early 1980s. Chloroplast degradation in the vacuole has been observed. Recent genome-wide analysis has shed light on autophagy, a bulk protein degradation system well-conserved in eukaryotes ranging from yeast to mammals, in plants. The improvement of techniques for imaging living cells has enabled researchers to describe the characteristic substrate trafficking across membranes from the cytoplasm into the vacuole. By applying our understanding of the degradation mechanism of autophagy characterized in yeasts to plants, chloroplasts were shown to be degraded by autophagy during leaf senescence. Chloroplast autophagy occurs by two different pathways. The first is partial degradation via vesicle trafficking. Chloroplasts produce vesicles, named Rubisco-containing bodies (RCBs), which contain only the stromal fraction. RCB formation is affected by the carbon status of the cell, and is specifically linked to photosynthesis inside chloroplasts. RCBs are finally transported and degraded in the vacuole by autophagy. The second pathway is the autophagy of whole chloroplasts, and their degradation inside the vacuole. These pathways of chloroplast autophagy exist as one of the degradation mechanisms of chloroplast components during leaf senescence, causing a decrease in chloroplast size and number.

I. Introduction

A. The Mechanism of Rubisco Protein Degradation and Chloroplast Autophagy

During leaf senescence, chloroplast proteins are rapidly degraded and remobilized into newly developing tissues as major nitrogen resources. In C_3 plant species, approximately 80% of leaf nitrogen is invested in chloroplasts mainly as photosynthetic proteins (Makino and Osmond 1991). The largest single fraction of this is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme of photosynthesis which is responsible for carbon fixation, comprising 20–30% of total leaf nitrogen. Remodeling the nutrient distribution in the whole plant and decreasing the photosynthetic capacity in individual leaves, the patterns and effects of Rubisco protein degradation during leaf senescence have been intensively studied for the past quarter-century (for review, see Feller et al. 2008); however, the molecular mechanism of Rubisco degradation is still largely unclear.

One of the controversial points is the pathway which connects Rubisco to the specific proteases, considering the subcellular compartmentation of both. Rubisco is localized in the stroma of chloroplasts, while most hydrolytic activity in mesophyll cells is in the vacuole. In fact, about 95% of hydrolytic activity directed against Rubisco has been detected in the vacuole in barley leaves (Thayer and Huffaker 1984). Three mechanisms of Rubisco degradation are postulated. The first is degradation within the chloroplast by chloroplast intrinsic proteases, while the second is degradation outside chloroplasts (presumably in the vacuole) via protein export from chloroplasts. The third mechanism is the degradation of whole chloroplasts in the vacuole, with the mechanism presumed to be the same as that for proteins exported from the chloroplast. In the first pathway, a number of proteases, including ATP-dependent prokaryotic AAA^+ proteases, are localized in the stroma and thylakoid membranes of chloroplasts (for review, see Sakamoto 2006). In particular, CND41, a 41 kD *c*hloroplast *n*ucleoid *D*NA-binding protease, is the only confirmed chloroplast

Abbreviations: *ATG* genes; – Autophagy related genes; GFP – Green fluorescent protein; IDL – Individually darkened leaves; RCBs – Rubisco-containing bodies

protease which has hydrolytic activity against Rubisco (Kato et al. 2004). However, the actual contribution of CND41 to nitrogen recycling during leaf senescence is still unclear.

Chloroplast derived vesicles exporting chloroplast components to the vacuole have been observed in Chlamydomonas (Park et al. 1999) and soybean (Guiamét et al. 1999). Based on ultrastructural observation by immuno-electron microscopy, Chiba et al. (2003) reported on the existence of Rubisco containing vesicular bodies localized in the cytosol and vacuole of senescing wheat leaves; these vesicular bodies were being named Rubisco-containing bodies (RCBs). Similar vesicles derived from chloroplasts have also been reported in other higher plants; soybean, Arabidopsis (Otegui et al. 2005: senescence associated vacuoles) and tobacco (Prins et al. 2008: Rubisco vesicular bodies). Krupinska (2007) reviewed the fate of the chloroplast and its constituents during leaf senescence in a past volume of this series. Our chapter describes the current understanding of the fate of chloroplasts during leaf senescence and also summarizes the possible involvement of several autophagic pathways in chloroplast degradation. During leaf senescence, chloroplasts transform into gerontoplasts, losing the thylakoid membrane network and accumulating many plastoglobuli. As shown by morphological analysis of senescing leaf tissues, chloroplasts shrink and decrease in number (Kura-Hotta et al. 1990; Inada et al. 1998). Specifically, ultrastructural analyses have shown chloroplasts translocated into the central vacuole, indicating the degradation of whole chloroplasts in the vacuole (Wittenbach et al. 1982; Minamikawa et al. 2001).

Since the completion of the Arabidopsis genome-project in 2000 (Kaul et al. 2000), "genome-wide" analysis has progressed rapidly in higher plants. As a part of the results, a number of genes homologous to the yeast autophagy related (*ATG*) genes have been identified in Arabidopsis. Autophagy is a major protein degradation system evolutionarily conserved in eukaryotes, initially characterized using budding yeast as a model system. It is found as a cellular response to the starvation stress in yeast, degrading a bulk protein fraction including entire organelles for the remodeling and redistribution of cellular nutrients (Ohsumi 2001). Reverse genetic analyses on *ATG* genes have revealed the existence of an active autophagy system in Arabidopsis. Inferred from the increased mortality of these *atg* mutants under nutrient starvation conditions, autophagy seems to play an important role in nutrient recycling in plants as well as yeasts (Thompson and Vierstra 2005).

The investigation of plant autophagy has only recently begun, and our understanding of the molecular details of plant autophagy is still developing. In this chapter, the yeast autophagy pathway, as the model system of autophagy, and its counterparts of Arabidopsis autophagy are first described. This is followed by an introduction to the autophagy of chloroplasts, which has recently been demonstrated as one of the chloroplast component degradation pathways for nutrient recycling (Ishida et al. 2008; Wada et al. 2009; Izumi et al. 2010).

II. Autophagy in Plants

A. Fundamental Mechanism of Autophagy: Learning from Yeast

Autophagy is a bulk protein degradation system in eukaryotes, which targets a portion of the cytoplasmic fraction, including entire organelles, for recycling in the vacuole/lysosome. It was defined in mammalian cells more than 50 years ago and is understood to be the process by which living cells achieve the degradation of their own constituents in the lysosome (Deduve and Wattiaux 1966). The most remarkable feature of autophagy is the intracellular topology, the trafficking of a portion of cytoplasm as a sequestered vesicle into the lysosome/vacuole across the membrane. The molecular mechanism of autophagy remained totally unexplained for a long time; however, after the identification of *au*tophagy-related (*ATG*) genes in budding yeast (*Saccharomyces*

cerevisiae), the precise and stepwise mechanisms of autophagy gradually became more clear (for review, see Nakatogawa et al. 2009). Recent genome-wide analyses have revealed that the fundamental system of autophagy with homologs of yeast *ATG* genes is well-conserved in other eukaryotes, including plants (for review, see Levine and Klionsky 2004).

1. The Basics of Autophagy in Yeast

Autophagy is strongly induced under starvation conditions. The discovery of autophagy in yeast was based on the observation of vesicles, in which a portion of the cytoplasm was included, accumulating in the vacuole under nutrient-deprived conditions (Takeshige et al. 1992). These vesicles are called autophagic bodies. Thereafter, using mutants identified by their lack of autophagic bodies, the primary *ATG* genes were identified, which helped to shed light on the molecular mechanism of autophagy (Tsukada and Ohsumi 1993). Most *atg* mutants have similar phenotypes, namely, they lack autophagosome formation and are characterized by reduced survival under starvation conditions. Autophagy plays an essential role in cell survival under starvation conditions and is required for adaptation to nutrient limitation. In higher animals and plants, autophagy is responsible for recycling amino acids and cellular remodeling under nutrient limiting conditions.

Macroautophagy (hereafter referred to simply as autophagy), a major pathway of autophagy, is performed by the following steps commencing with induction in response to nutrient starvation. When starvation induces autophagy, a flat double-membrane structure called an autophagosome membrane appears in the cytoplasm. The autophagosome membrane elongates and sequesters bulk cytoplasmic constituents including organelles, preparing a full double-membrane vesicle called an autophagosome. After completion of autophagosome vesiculation, the outer membrane of the autophagosome fuses with the vacuolar membrane and the inner cargo is released into the lumen. The term 'autophagic body' designates the single-membrane autophagosome, inner membrane and its cargo, released into the vacuole, where it is degraded by vacuolar hydrolases. This autophagosome membrane formation is designated as the core machinery of autophagy. If any of *ATG* genes related to this core machinery is defective, the entire autophagy system is shut down.

2. The Advances of Autophagy Research in Yeast

In the past decade, great advances have been made in the field of autophagy, revealing its importance in many biological and physiological processes. Autophagy is now not only known to play a role in nutrient recycling during starvation, but also known to have various biological roles, e.g., protein/organelle quality control, prevention of the accumulation of abnormal proteins, digestion of micro-invading pathogens, programmed cell death, etc. Autophagy is recognized as bulk degradation, however recent advances have extended the concept of autophagy to more selective degradation. Selective autophagy targets specific proteins or organelles, e.g., pexophagy for peroxisome specific autophagy (Farré et al. 2008) and mitophagy for mitochondria specific autophagy (Lemasters 2005). Based on the same core machinery of bulk autophagy, these selective autophagy pathways require additional ATG proteins to label the targeted organelles and for recognition of the labeled organelle by autophagosomes. Moreover, autophagy is also related to protein biosynthesis called *c*ytoplasm-to-*v*acuole-*t*argeting (Cvt) pathway. In the Cvt pathway, a vacuolar hydrolase, aminopeptidase I (API), is first synthesized as an inactive precursor in the cytosol, then transported into the vacuole by the mechanism of macroautophagy (Shintani et al. 2002).

Based on the different dynamics of trafficking membranes, autophagy can be classified into two pathways, macroautophagy and microautophagy. In microautophagy, targeted cytoplasmic organelles are

directly engulfed in the vacuole by vacuolar membrane invagination. Consequently, to degrade the same organelle, these two pathways are regulated differently in the same cell. For example, methylotrophic yeast *Pichia pastoris* performs two types of pexophagy, macro-pexophagy and micro-pexophagy, under culture conditions with different carbon sources (Tuttle and Dunn 1995). During the process of microautophagy, a membrane structure called MIPA (*mi*cro*p*exophagy *a*pparatus), which is comprised of PAZ2/ATG8 protein, as also contained in the autophagosome membrane, appears around the invagination point of the vacuolar membrane, suggesting that MIPA is required to seal the membrane invagination and complete the engulfment forming a sub-compartment inside the vacuole (Mukaiyama et al. 2004).

3. Core Machineries of Yeast Autophagy: Two Ubiquitin-Like Systems

The center of the molecular machinery of autophagy is the formation of the isolation/autophagosome membrane. For all autophagy pathways, macro- or micro-autophagy, Cvt pathway, and bulk or selective autophagy, the set of *ATG* genes which are responsible for this membrane formation are commonly required. The core molecular mechanism of autophagosome membrane formation is often described as two ubiquitin-like systems, featuring two ubiquitin-like proteins, ATG12 and ATG8 (for the review see, Ohsumi 2001).

ATG12 is a hydrophilic protein without obvious amino acid sequence homology to the ubiquitin protein. In the ATG12 conjugation system, the carboxy-terminal glycine of ATG12 is ATP-dependently activated by ATG7, an E1-like enzyme of the ubiquitin system. The activated ATG12 is transferred to ATG10 (E2-like enzyme), and finally ATG12 is conjugated with ATG5, by an isopeptide bond between the activated glycine of ATG12 and the specific lysine residue of ATG5. The ATG12-ATG5 conjugate further associates with ATG16 to form ATG12-ATG5-ATG16 complexes. Furthermore, ATG16 has a coiled-coil region to form homo-oligomers, allowing ATG16 to crosslink two or more ATG12-ATG5 conjugates and form a large complex. The other ubiquitin-like protein, ATG8, is first processed by ATG4, a cysteine protease, resulting in the unveiling of the carboxy-terminal glycine residue. Sequentially, cleaved ATG8 is activated by ATG7 (E1-like enzyme) and transferred to ATG3 (E2-like enzyme), and finally conjugated with phosphatidylethanolamine (PE). The ATG8-PE conjugate functions as a constituent of both the inner and outer membrane of autophagosomes. ATG4 is also a deconjugation enzyme of ATG8, cleaving the amide bond between ATG8 and PE to liberate and recycle the ATG8 protein from the outer membrane of the autophagosome. The ATG12 conjugation system is required for ATG8-PE formation. The ATG12-ATG5 conjugate directly interacts with ATG3 and enhances its activity. ATG16 is also required for the ATG12-ATG5 conjugates to be localized to the *p*re-*a*utophagosomal *s*tructure (PAS), an assembly of ATG proteins and the construction site of the isolation membrane. The localization of ATG12-ATG5-ATG16 complex to the PAS mediates the conjugation of the ATG8-PE at the PAS and enhances the constitution of the isolation membrane.

B. ATG Genes in Plants

1. ATG Genes in Arabidopsis Genome

In the Arabidopsis genome, around 40 genes (*AtATG*) have already been identified as homologs of yeast *ATG* genes (for a reference to the list of identified *AtATG*s, see Yoshimoto et al. 2010). However, some of them are members of multigene families, and the present genomic information on *AtATG* genes does not fully cover all 33 kinds of yeast *ATG* genes thus far identified. Yeast *ATG* genes can be largely classified into two groups. One comprises of the genes encoding proteins which are related to the core machinery, and the other comprises of genes involved in selective autophagy pathways. A defect of any one of the core *ATG* genes

causes the complete dysfunction of the autophagy system. Conversely, a defect in one of the *ATG* genes required for selective autophagy affects the degradation of only the specific organelle or protein. For instance, in the specific autophagy pathway group, *ATG25*, *26*, *28*, *30* are exclusively essential for pexophagy (specific degradation of peroxisomes). In the Arabidopsis genome, all *AtATG* genes thus far identified belong to the core machinery group, with no specific *ATG* genes having been identified. Therefore, the degradation mechanism of autophagy in Arabidopsis may simply consist of only bulk macroautophagy, as is the case in other eukaryotes, such as mammals. The homologs of *ATG11* and *ATG32*, which are related to mitochondria specific autophagy, i.e., mitophagy, have not yet been identified in mammals, though the existence of mitochondrial specific autophagy has already been demonstrated (Narendra et al. 2008). Conversely, it is known that the *ATG* genes, which are not found in budding yeast, exist in other eukaryotes, e.g., *ATG101* (Hosokawa et al. 2009). The molecular mechanisms of Arabidopsis autophagy have not been as well characterized as those of yeast and mammals. As such, there are most probably other unidentified *ATG* genes, some of which are probably specific to plants.

2. The Homologs of the Core Machinery of Yeast Autophagy in Arabidopsis

In Arabidopsis, the molecular mechanisms of these two ubiquitin-like systems are very similar to those of the yeast system and performed by homologs of yeast *atg* genes, with some Arabidopsis specific features (processing of ATG8s and their deconjugation by ATG4s in Yoshimoto et al. 2004; ATG12s-ATG5 conjugation in Suzuki et al. 2005; dependency on ATG7 and ATG10 of ATG12-ATG5 conjugation, and on ATG7 and ATG3 of ATG8-PE conjugation in Fujioka et al. 2008; requirement of ATG12s for ATG8-PE conjugation in Chung et al. 2010). Some Arabidopsis *ATG* genes are encoded at multiple loci or are members of multigene families, the proteins of which are functionally identical. This may allow the autophagy system in Arabidopsis to be more precisely regulated with different genes expressed in different tissues and under different environmental conditions. *AtATG12*, for example, is encoded by two loci, *AtATG12a* (At1g54210) and *AtATG12b* (At3g13970). The amino acid sequences of AtATG12a and AtATG12b are 86% (83 of 96 amino acids) identical, and both the AtATG12a and the AtATG12b protein can identically react with ATG5 (Chung et al. 2010). Proving the biochemically redundant function between AtATG12a and AtATG12b, Chung et al. (2010) reported that *AtATG12a* and *AtATG12b* were expressed at different developmental stages. *AtATG12b* is expressed higher than *AtATG12a* in young seedling stage, conversely, *AtATG12a* is expressed in mature and senescent leaves. The expression of *AtATG12*s might be subfunctionalized in growth stages and may contribute to regulate autophagy at each growth stage. Arabidopsis has a multigene family of *ATG8*, which consists of nine genes (*AtATG8a-i*). All nine *AtATG8*s are expressed ubiquitously and show enhanced expression under nitrogen-starvation conditions (Yoshimoto et al. 2004). Sláviková et al. (2005) reported that five of the nine *ATG8*s (*AtATG8a*, *b*, *e*, *f*, *i*) were expressed at higher levels in roots compared to shoots under nutrient-starvation conditions, and that each *ATG8* showed a different GUS staining pattern in various regions of the root. It is suggested that these members of *AtATG8*s have non-redundant functions that can facilitate root development and protein turnover in different ways.

3. Distribution of ATG Genes in Plants

Autophagy is an evolutionary conserved system in eukaryotic cells found in fungi (yeast), animals and plants. In plants, the conserved degradation system of autophagy has already been identified in *Arabidopsis thaliana* (Yoshimoto et al. 2004), *Oryza sativa* (Su et al. 2006), *Zea mays* (Chung et al. 2009) and *Chlamydomonas reinhardtii*

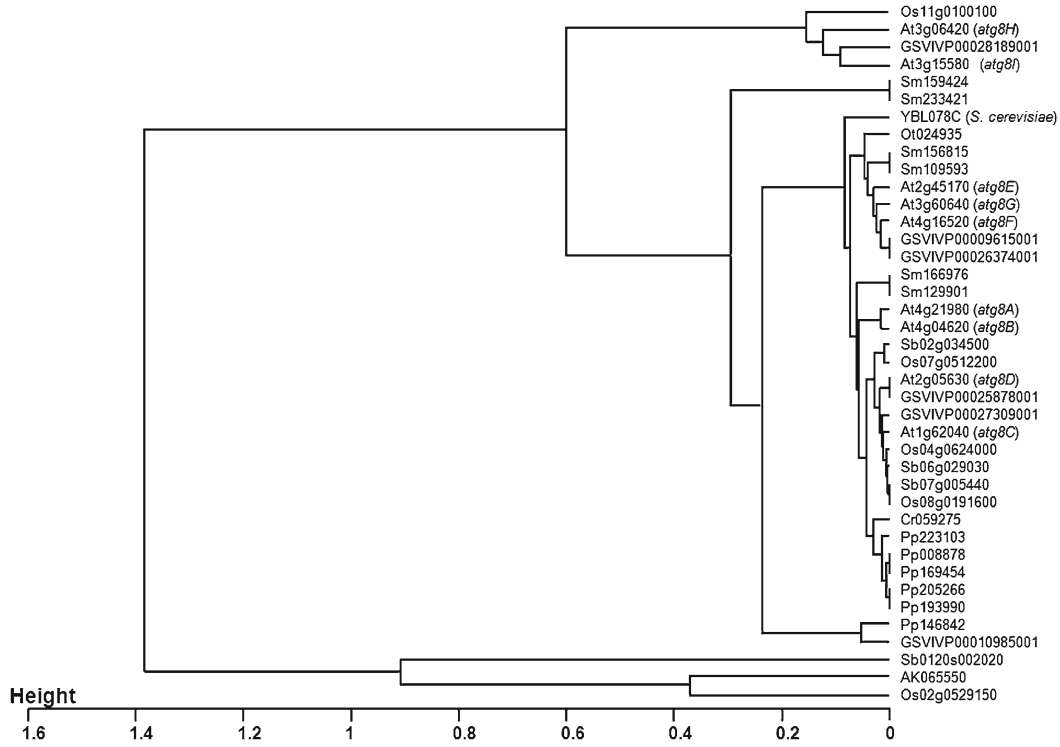

Fig. 19.1. Dendrogram of the amino acid sequence similarity of ATG8. This dendrogram was drawn based on the SALAD database containing genomic information of eight species of plantae (*Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Oryza sativa*, *Ostreococcus tauri*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Sorghum bicolor* and *Vitis vinifera*) and yeast (*Saccharomyces cerevisiae*). YBL087C indicates the locus tag of yeast ATG8. In other protein IDs, each initial alphabetical abbreviation indicates species, At (*Arabidopsis thaliana*), Cr (*Chlamydomonas reinhardtii*), Os (*Oryza sativa*), Ot (*Ostreococcus tauri*), Pp (*Physcomitrella patens*), Sm (*Selaginella moellendorffii*), Sb (*Sorghum bicolor*) and GSVIVP (*Vitis vinifera*). Exceptionally, AK065550 is one of the protein IDs of ATG8 in *Oryza sativa*.

(Pérez-Pérez et al. 2010). According to BLAST searches with the amino acid sequence of yeast ATG8, in genomic databases of various species in many groups of plants, highly homologous gene sequences are easily confirmed and thought to be related to autophagy, e.g. in dicots (*Nicotiana tabacum*, *Solanum lycopersicum*, *Vitis vinifera*), monocots (*Sorghum bicolor*), pteridophytes (*Selaginella moellendorffii*), mosses (*Physcomitrella patens*), chlorophytes (*Ostreococcus tauri*, *Micromonas pusilla RCC299*), and glaucophytes (*Cyanophora paradoxa*). Figure 19.1 shows a dendrogram of ATG8 proteins in the SALAD database referring to genomic information of nine species of plants and budding yeast (*Saccharomyces cerevisiae*) (http://salad.dna.affrc.go.jp/salad/). Only in *Cyanidioschyzon merolae*, a species of the rhodophyte group, no homologs of *ATG* genes including *ATG8* could be identified in its genome. Referring to the database of *Galdieria sulphuraria* genome project at Michigan State University (http://genomics.msu.edu/galdieria/index.html) also in the genome of the rhodophyte, *Galdieria sulphuraria*, no homologs of *ATG* genes were detected. The rhodophyte group species may have a set of *ATG* genes in their genome with lower similarity sequences; or, it may have lost the autophagy machinery during evolution. Based on evidence presented in this section, autophagy is expected to be functionally conserved in

many higher plants. It is interesting how autophagy functions in diverse species, especially focusing on different requirements of each nutrient among species and the induction of autophagy.

III. Chloroplast Autophagy

Chloroplast autophagy occurs during leaf senescence which is a highly ordered process during late development, regulated by internal and external signals (Guo and Gan 2005). The characteristic decrease of photosynthetic capacity during senescence results from chloroplast protein degradation. In that process, large amounts of nitrogen invested in chloroplast proteins are remobilized. This is considered as an important task for the plant. Most *ATG* genes are upregulated during leaf senescence, which suggests that autophagy is involved in the senescence associated nitrogen remobilization (Van der Graaff et al. 2006). The chloroplast/plastid is specific to photoautotrophs, and the relationship between chloroplast protein degradation and the vacuole is a long-held question. On the existence and the putative manner of plant specific chloroplast autophagy has been speculated (Krupinska 2007). Here, as one of the actual degradation pathways of chloroplast components and as the cause of the decrease in chloroplast size and number, chloroplast autophagy observed in Arabidopsis is described.

A. Rubisco-Containing Body (RCB): Partial Degradation of Chloroplasts

Chloroplast components are degraded in the vacuole via vesicle trafficking termed the RCB pathway. Exclusion of globules or vesicles from chloroplasts has been observed in some higher plants (Guiamét et al. 1999; Chiba et al. 2003; Otegui et al. 2005; Ishida et al. 2008; Martínez et al. 2008; Prins et al. 2008) and in Chlamydomonas (Park et al. 1999); however, only the formation of RCB reported by Ishida et al. (2008) has been identified to be dependent on autophagy and to be degraded in the vacuole. Guiamet and co-authors identified a similar subcellular compartment which they termed "senescence-associated vacuoles; SAVs" (Guiamét et al. 1999; Otegui et al. 2005; Martínez et al. 2008), but the SAV seems to be independent of *ATG7* and has some different characteristics from RCBs. e.g., RCBs transport only stromal proteins, while SAVs contain chlorophyll pigment (for details about the SAV, see Costa et al., Chap. 18). Here, the RCB pathway is introduced as a vesicular degradation mechanism of chloroplast components involving autophagy.

RCBs were first identified as vesicular bodies containing stromal proteins, which appeared in the cytosol and vacuole of senescing wheat leaves as shown by immunoelectron microscopy (Chiba et al. 2003). Thereafter, using the Arabidopsis transformants expressing stroma-targeted GFP protein, Ishida et al. (2008) visually demonstrated that stromal proteins including Rubisco were transported into the vacuole via RCBs. The mechanism of RCB formation from chloroplasts is not yet clearly understood. RCBs appear to occur more frequently in senescing leaves than in young leaves, which suggests that the formation of RCBs plays a role in chloroplast protein degradation for nitrogen remobilization (Ishida et al. 2008). The RCB pathway can be regarded as a mechanism for the partial degradation and the decrease in the size of chloroplasts, but it is most likely not responsible for the decrease in the number of chloroplasts. Considering the previously reported manner of Rubisco protein degradation during leaf senescence (Martinoia et al. 1983; Mae et al. 1984), the RCB pathway appears to be suitable for the degradation of Rubisco during early senescence preceding the decrease in the number of chloroplasts. The quantitative contribution of the RCB pathway to the degradation of chloroplast protein and to nutrient recycling in natural leaf senescence awaits future clarification that is difficult to obtain due to the early leaf senescence phenotype of *atg* mutants.

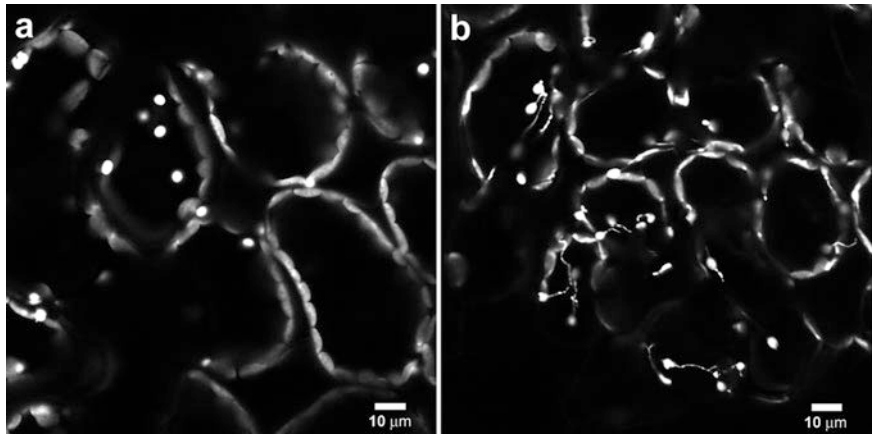

Fig. 19.2. The frequency of stromules on chloroplasts in *atg* mutants. Chloroplasts were visualized with stroma targeted GFP in wild-type (**a**) and in *atg5* (**b**).

1. ATG Gene Dependency

Regardless of the leaf age, RCBs are not detectable in *atg* mutants (*atg5*, Ishida et al. 2008; *atg4a4a-1*, Wada et al. 2009). Using dual observation of RCBs and autophagosome membranes, RCBs accumulating in the vacuole of wild type plants are inevitably attached to the autophagosome membrane visualized by GFP-ATG8 (Ishida et al. 2008). This supports the direct involvement of autophagy in trafficking of RCBs into the vacuole, and indicates that the trafficking is performed by the macroautophagy pathway. Chloroplasts of *atg* mutants are impaired in the formation of RCBs. Although the specifics are currently unclear in terms of the exact behavior of the autophagosome membrane and/or autophagy machinery in the process of the RCBs biogenesis, the formation of RCBs at the surface of the chloroplast requires ATG proteins or the autophagosome membrane to complete the vesiculation.

2. Stromules Are Altered in atg Mutants

The deletion of the RCB pathway might result in a lack of stromal protein export from the chloroplast, and in a lack of envelope dynamics for vesicle formation. *Atg* mutants seem to possess alternative or compensatory membrane dynamics as many stromules (stroma-filled tubule structures, see J. Gray, Chap. 9) protrude from the chloroplast surface (Fig. 19.2; *atg5,* Ishida et al. 2008; *atg4ab,* Wada and Ishida, unpublished). Stromules are characterized as a dynamic envelope extension observed in all kinds of plastids (for reviews see also Kwok and Hanson 2004; Natesan et al. 2005). Stromules are usually more abundant in tissues containing non-green plastids, and are relatively rare in mesophyll cells with chloroplasts except in *atg* mutants (Köhler and Hanson 2000). The primary function of stromules is still unknown. The frequent appearance of stromules emerging from the plastids/chloroplasts of *atg* mutants might be directly caused by the lack of RCB formation. Waters et al. (2004) suggested that the chloroplast membrane elasticity and amenability to distortion could be one factor contributing to stromule formation. The release of the terminal part of stromules, such release being called tip-shedding by Gunning (2005), would facilitate RCB budding. The lack of RCB formation in *atg* mutants might result in chloroplast membrane "margin" or "surplus" during leaf senescence, and contribute to membrane flexibility and stromule formation from chloroplasts. Alternatively, the "stromular phenotype" of the plastids/chloroplasts in *atg* mutants may represent an

intercellular stress response caused by the disruption of autophagy that contributes to plant survival under starvation conditions.

3. Trafficking Cargo of RCBs

To date, only some stromal proteins have been confirmed to be trafficked cargo of the RCB pathway. The stroma-targeted cargo selection of RCBs was observed both by immuno-electron microscopy (Chiba et al. 2003) and by live imaging of RCBs using laser-scanning confocal microscopy (Ishida et al. 2008). This study showed that the GFP-labeled Rubisco holoenzyme, which is thought to consist mostly of 8 RbcL, 7 RBCS and 1 RBCS-GFP, was transported via RCBs into the vacuole by autophagy. RCBs contained other stromal proteins besides Rubisco, such as glutamine synthetase (Chiba et al. 2003) and stroma targeted fluorescent proteins, GFP and DsRed (Ishida et al. 2008). By contrast, the transport of thylakoid components such as membrane proteins related to electron transport and chlorophylls has not been observed. In the Arabidopsis transformants accumulating the GFP-labeled $CF_1\gamma$ subunit of chloroplast ATP synthase bound to the thylakoid membrane, RCBs were not visualized during leaf senescence (Wada and Ishida, unpublished). It is unknown whether the RCB pathway carries out fine cargo selection of stromal proteins (e.g. oxidative or damaged protein selection) or performs bulk stroma protein degradation; however, the RCB pathway can be regarded as selective autophagy, targeting only stroma proteins in mesophyll cells.

4. RCB Induction Based on the Cellular Nutrient Condition

The RCB pathway can be considered as a part of plant autophagy involved in chloroplast degradation (Ishida et al. 2008; Wada et al. 2009). Autophagy is induced by nitrogen or carbon starvation in Arabidopsis (Yoshimoto et al. 2004; Thompson and Vierstra 2005; Xiong et al. 2005). For both situations of nutrient recycling, chloroplast components are the most abundant resource in the plant cell. To understand the significance of the RCB pathway in nutrient recycling, knowledge on the signals inducing RCB production is required. Izumi et al. (2010) revealed that RCB production was specifically affected by the leaf carbon status, but not induced under nitrogen starvation conditions. To investigate autophagy efficiency, Yoshimoto et al. (2004) monitored the accumulation of autophagosomes in Arabidopsis cells expressing *GFP-ATG8* and treated or not with concanamycin A. Concanamycin A is an inhibitor of the vacuolar H^+-ATPase, which causes a shift in vacuolar pH, and a repression of vacuolar proteases. Using similar procedures, Izumi et al. (2010) observed RCBs accumulating in the vacuole of excised leaves after incubation with concanamycin A in the dark. By applying various kinds of nitrogen and carbon nutrients to this monitoring system, Izumi et al. (2010) noted that the number of RCBs was decreased by the addition of metabolic sugars (glucose, fructose and sucrose), irrespective of the nitrogen status. Concomitantly to the decrease in RCB formation/abundance, the external sugar supply extensively suppressed autophagosome formation, indicating that metabolic sugars negatively regulate the entire autophagy system in Arabidopsis leaves. In excised leaves incubated with concanamycin A in the light, RCB production but not autophagosome formation was specifically suppressed. This specific suppression of RCBs, was certainly due to the production of carbohydrates by photosynthesis, since suppression did not occur when 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport, was added. These results show that the RCB pathway was regulated independently of the core autophagy pathway by photosynthetically-derived carbon. Photosynthesis indeed affected the RCB production during the regular life cycle. Comparing the production of RCBs in leaves sampled in different periods of time in a long-day life cycle, we showed that leaves sampled at the end of the night, when starch

content is the lowest, produced more RCBs than leaves sampled at the end of the day when starch content was highest (Izumi et al. 2010).

In the diurnal life cycle of Arabidopsis, the assimilated carbon source is stored in the chloroplast in form of starch granules during day time, and used as an energy source at night. In Arabidopsis starch metabolism mutants, RCBs appeared more in starchless mutants, such as *pgm-1* (Caspar et al. 1985) and *adg-1* (Lin et al. 1988), than in wild-type, while starch excess mutants, such as *sex-1* (Caspar et al. 1991) and *mex-1* (Niittylä et al. 2004), produced fewer RCBs than the wild-type (Izumi et al. 2010). It remains unknown whether the impact on RCB formation by diurnal cycle and the starch metabolism were caused independently of or dependently on the regulation of the entire autophagosome formation. However, in all these analyses, RCB formation seemed to be enhanced in carbon-depleted leaves, which indicates that the RCBs pathway might be responsible for the alternative energy supply from proteolysis in leaves. Additionally, under nitrogen starvation conditions, the amounts of Rubisco protein and leaf nitrogen were gradually decreased, despite the suppression of RCB production (Izumi et al. 2010). In nitrogen starved plants, starch and metabolite sugars accumulated in leaves, and might be responsible for the suppression of RCBs.

B. Degradation of Entire Chloroplasts

1. Evidences of Autophagy-Dependent Whole Chloroplast Engulfment into the Vacuole

During leaf senescence, the chloroplast population decreases in mesophyll cells. To date, the mechanism of chloroplast division and chloroplast development along with the cell proliferation and expansion have been well investigated by genetic analysis using chloroplast division defective mutants, e.g., *arc* mutants, and following up the maturation of mesophyll cells using multiple microscopic techniques (for reviews see, Osteryoung and Pyke 1998; Pyke 1999). Conversely, based on the decrease in number of chloroplasts, the existence of a mechanism of whole chloroplast degradation in senescing mesophyll cells has been presumed. There were however only suggestive evidences provided by electron microscopy, which showed whole chloroplasts engulfed in the central vacuole (Wittenbach et al. 1982; Minamikawa et al. 2001; Mulisch and Krupinska, Chap. 14). Decreases in the number of chloroplasts have been observed in the late stage of natural- (Kura-Hotta et al. 1990; Inada et al. 1998), dark induced- (Wittenbach et al. 1982) and nutrient starvation induced- (Mae et al. 1984; Ono et al. 1995) senescence. Whole chloroplast degradation has been indicated as one of the degradation pathways of chloroplast constituents during leaf senescence (Hörtensteiner and Feller 2002).

In Arabidopsis, rosette leaf senescence can be induced and accelerated by individual leaf darkness treatment (Weaver and Amasino 2001). In individually darkened leaves (IDLs), Keech et al. (2007) reported a sharp decline of photosynthetic activity and chloroplast size and number during a much shorter period than that of natural senescence. Wada et al. (2009) monitored the transport of chloroplast components, using Arabidopsis transformants having the DsRed fluorescent protein targeted to the stroma and IDLs as a model system. The vacuoles of IDLs were shown to be filled with many dotted RCBs and entirely stained with DsRed fluorescence, presumably diffused by the breakdown of RCBs. In the same leaf samples, the transport of "the whole chloroplast" into the isolated vacuoles of IDLs was also confirmed by including components (Wada et al. 2009). In the vacuole of IDLs, chloroplasts components were accumulated as small stroma fraction vesicles (RCBs) and as larger spherical bodies having only chlorophyll autofluorescence (whole chloroplasts). In the *atg4a4b-1*, none of these bodies were found inside the vacuole, showing that the transport of both RCBs and whole chloroplast is autophagy dependent.

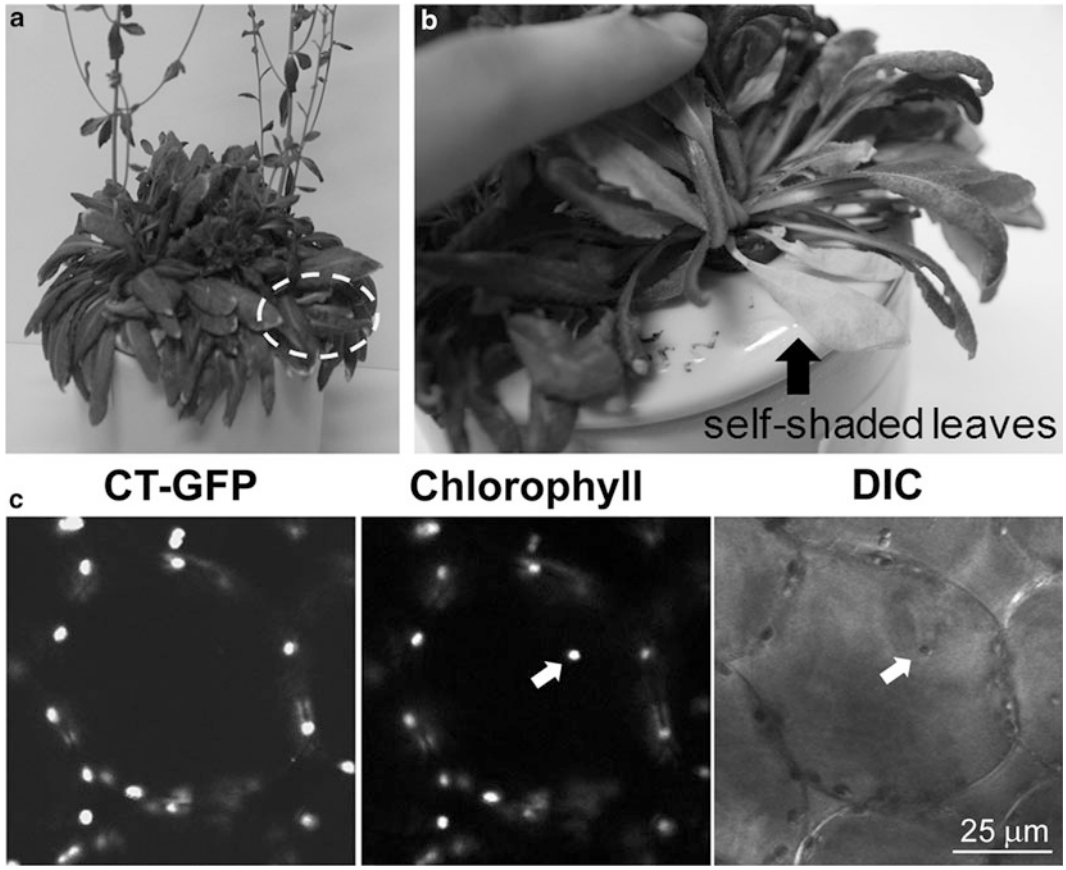

Fig. 19.3. The whole chloroplast autophagy in self-shaded leaves of Arabidopsis. The transformant of Arabidopsis plants expressing stroma targeted GFP were grown in short-day conditions (**a**), and the self-shaded leaves attached to the lower position in the *dashed-circle* area in (**a**) showed accelerated leaf senescence (**b**). The chloroplast autophagy was observed in mesophyll cells of self-shaded leaves by laser-scanning confocal microscopy (**c**). CT-GFP and Chlorophyll show the fluorescence of chloroplast stroma targeted GFP and chlorophyll autofluorescence. DIC is the image of differential interference contrast. In figure (**c**), the *white arrow* indicates the chloroplast, transported into the vacuole, which has only chlorophyll autofluorescence.

After 5 days of IDL treatment, chloroplasts in wild-type plants were shrunken by one-third while *atg4a4b-1* showed only a slight reduction in chloroplast size. The number of chloroplasts was decreased in the wild-type but remained stable in *atg4a4b-1*. These changes in chloroplast morphology and number strongly indicate that, during leaf senescence of IDLs, the content of chloroplasts is exported via RCBs causing chloroplast shrinkage, after which the shrunken chloroplasts are engulfed and undergo autophagy. The autophagy-dependent degradation of whole chloroplasts, demonstrated in the experimental model conditions of IDLs (Wada et al. 2009), was investigated under natural environmental conditions, on leaves shaded by upper leaves or neighboring vegetation. Actually, in Arabidopsis grown under a short-day life cycle, shaded lower leaves undergo accelerated leaf senescence (Fig. 19.3a, b). In these leaves, whole chloroplast engulfment was confirmed by observing whole chloroplasts exhibiting Brownian motion and showing chlorophyll autofluorescence in the vacuole (Fig. 19.3c). Whole chloroplast engulfment was observed more often at the late stage of leaf senescence, even in the IDLs.

2. How Is Whole Chloroplast Engulfed into the Vacuole?

The size of autophagic bodies observed in Arabidopsis vacuoles is around 1.5 μm (Yoshimoto et al. 2004), while RCBs are about 0.4–1.2 μm, within the capacity of autophagosomes. RCBs are speculated to be transported by the macroautophagy pathway (Ishida et al. 2008). The chloroplast, however, is a large organelle with a diameter around 5–10 μm on average. In fact, even when shrunken by RCB production, whole chloroplasts observed in the vacuole were distinctly bigger than RCBs (Wada et al. 2009). The size of the chloroplast could be over the extent of the cargo size of autophagosomes. The mechanism of whole chloroplast autophagy may be microautophagy by vacuolar membrane engulfment. However, *ATG* genes specifically required for microautophagy have not yet been found in the Arabidopsis genome and this remains speculative. Saito et al. (2002) reported a vacuolar sub-region named "bulb" in Arabidopsis cotyledon leaves. The role and morphogenesis of the bulb structure are not yet clearly understood. However, visualized by the vacuolar membrane markers, GFP-γ-TIP and GFP-RAB75, the bulb structure was found to be derived from vacuolar membrane invagination. Ultrastructural observation by electron microscopy showed that some cytoplasmic organelles such as mitochondria were engulfed between the interspace of the bulb-like structure (Saito et al. 2002). This suggests a phagocytic-like engulfment of cytoplasmic fraction by the vacuole, and the existence of the microautophagy pathway in plants.

3 Why Is the Whole Chloroplast Engulfed by the Vacuole?

Niwa et al. (2004) reported that abnormally shaped chloroplasts were transported into the vacuole in the cotyledon leaves of *AtTIC40* defective mutants, while Stettler et al. (2009) suggested that the dysfunction of chloroplast starch metabolism caused chloroplast degradation in the vacuole in a *mex-1* mutant. These reports indicate that one function of whole chloroplast autophagy is in the quality control of organelles. In yeast, dysfunctional mitochondria are selectively recognized and degraded by autophagy (Okamoto et al. 2009). Whole chloroplast autophagy may play another role such as the elimination of damaged and dysfunctional chloroplasts, not only during senescence but throughout the leaves' lifespan, in plants.

IV. Concluding Remarks

In this chapter, we introduced chloroplast degradation during leaf senescence by autophagy. The investigation of plant autophagy has been gradually developing, but our understanding of chloroplast autophagy is still limited. Up to now, we found that stromal proteins are transported in vesicles and are degraded in the vacuole by the RCB pathway (Ishida et al. 2008). And also, whole chloroplasts are engulfed and degraded in the vacuole by autophagy (Wada et al. 2009). These two chloroplast autophagy pathways are induced during leaf senescence, and might be involved in nitrogen remobilization. However, the Rubisco content of Arabidopsis *atg* mutants declines same rate as wild-type plants during leaf senescence. Therefore, multiple pathways are responsible for chloroplast protein degradation. In various protein degradation pathways, the quantitative contribution of autophagy to Rubisco degradation during leaf senescence and the advantage of chloroplasts autophagy compared to other proteolytic pathways remain to be revealed in the future. Additionally, the RCB pathway is down-regulated by photosynthetically-assimilated carbohydrates (Izumi et al. 2010). Under the light condition in which photosynthesis is active, while the macroautophagy pathway actively sequesters bulk cytoplasmic protein and organelles, autophagosome membranes do not recognize RCBs as their target. This substrate selectivity of the autophagosome membrane indicates that

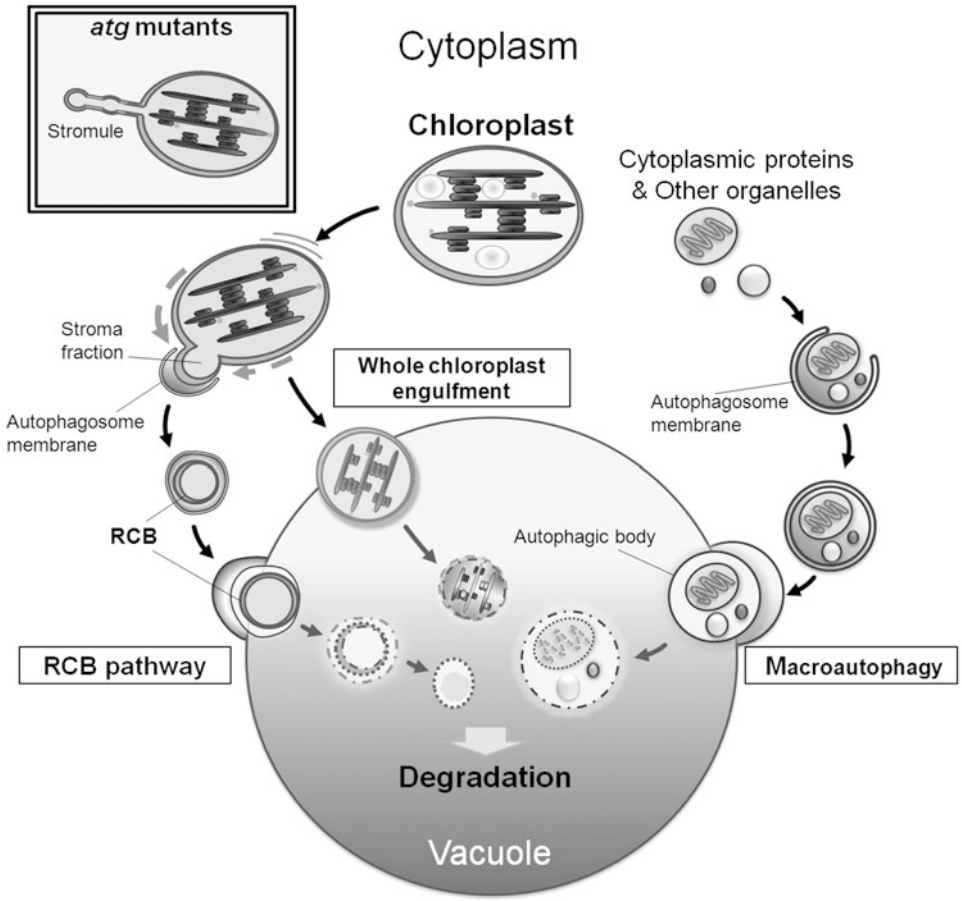

Fig. 19.4. Proposed model of chloroplast autophagy. In the general macroautophagy pathway, autophagosome membrane is produced in the cytoplasm depending on *ATG* genes. Autophagosome membrane sequesters and transports cytoplasmic organelles/proteins into the vacuole (**Macroautophagy**). The stromal fraction of chloroplasts is excluded as a vesicle named RCB by autophagy. RCBs are also transported into the vacuole by an autophagosome membrane (**RCB pathway**). Based on the common transport mechanism by autophagosome membrane, the RCB pathway is a kind of macroautophagy. However, the accumulation pattern of RCBs and other autophagic bodies in the vacuole is different depending on the leaf nutrient conditions. The RCB pathway is strongly regulated by photosynthetically assimilated carbohydrates. Chloroplasts of *atg* mutants exhibit stromule formation more frequently than chloroplasts in the wild-type. Whole chloroplasts are also transported into the vacuole, but the membrane dynamics of the transportation is still unclear (**Whole chloroplast engulfment**).

the RCB pathway has originally developed as a mechanism based on carbohydrate sensing within the entire macroautophagy system.

Autophagy is an evolutionarily conserved system for protein degradation under starvation conditions in eukaryotes. In plants, autophagy is uniquely applied to target chloroplasts as a substrate. The mechanisms of two pathways of chloroplasts autophagy can't be explained only by our knowledge of the core yeast *atg* system. There must be, however, also some specific mechanisms in plants. Identification of specific chloroplast-autophagy-related genes will certainly shed light on the unique machinery in plants and the relationship with the core *atg* system (Fig. 19.4).

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Chapter 20

Plastid Protein Degradation During Leaf Development and Senescence: Role of Proteases and Chaperones

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Summary

During leaf development, plastids undergo dynamic changes in morphology. Chloroplasts develop from proplastids during leaf growth: this process includes synthesis, import, and maturation of numerous chloroplast proteins. During leaf senescence, chloroplasts change gradually into a senescing form termed gerontoplast, with the breakdown of thylakoid membranes and the degradation of photosynthetic proteins. In these developmental processes, it is apparent that the proteolytic activity within chloroplasts is a key to control such remarkable morphological/functional changes. Processing and maturation of chloroplast proteins are very important since chloroplast development requires numerous proteins that are imported from the cytosol. Various efforts to elucidate the functions of chloroplast proteases have revealed the existence of signal peptidases (SPP, PreP, TPP, and PlsP1) that are involved in the processing and the maturation steps. In addition, the quality control of proteins is

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necessary for proper chloroplast development. Recent studies using Arabidopsis mutants have identified several important chloroplastic proteases (Clp, FtsH, Deg, and some intramembrane-proteases), which originated from bacterial homologs, in the quality control of proteins during chloroplast development. In contrast, studies on the degradation of chloroplast proteins during senescence implied that multiple pathways, not limited to chloroplast proteases, control protein degradation in this process. In addition to protein degradation inside the chloroplasts, degradation of engulfed whole-chloroplasts within the vacuole, and small spherical bodies like senescence-associated vacuoles (SAV), and Rubisco-containing bodies (RCB) that include chloroplast stromal proteins are known to occur during leaf senescence. The latter implicates that autophagy plays an important role in delivering chloroplast proteins into the vacuole. This chapter provides an integrated summary on the roles of chloroplast proteases during chloroplast development, and the current view of the chloroplast protein degradation during senescence.

I. Introduction

Along with protein synthesis, proteolysis is necessary for biological activities. Because, proteases are involved widely in various activities of living cells: they play important roles in proper protein maturation, quality control of proteins, and unnecessary protein degradation. In cellular organelles such as chloroplasts, proteases play crucial roles during change in the plants' morphology and function dynamically in response to leaf developmental status and environmental signals. During leaf development, rapid differentiation of proplastids into chloroplasts occurs; during leaf senescence, chloroplasts change gradually into a senescing form designated as gerontoplasts (Mulisch and Krupinska, Chap. 14; Lichtenthaler, Chap. 15). These conversions of chloroplasts accompany dynamic changes in the composition of chloroplast proteins.

To date, more than 16 kinds of proteases have been identified in chloroplasts using various experimental and in silico approaches (Kato and Sakamoto 2010); the proteases that have been identified in Arabidopsis chloroplast are listed in Table 20.1. Chloroplasts are evolved from a cyanobacterial ancestor by a primary endosymbiotic event 1 billion years ago (Archibald 2009). It seems reasonable to consider that the vast majority of these proteases are homologs of known bacterial proteases. Mounting evidence indicates that many of these proteases are actually involved in the processing and maturation of polypeptides, and in the quality control of proteins during plastid differentiation and chloroplast homeostasis. In contrast, the participation of these proteases in degrading chloroplast proteins during leaf senescence is poorly understood. During leaf senescence, in addition to protein degradation inside chloroplasts, protein degradation in other organelles is known to occur. Results of recent studies imply that multiple

Abbreviations: AAA – ATPases associated with diverse cellular activities; ATG – Autophagy-related gene; Cpn60 – Chaperonin 60; cpSec – Chloroplast secretory pathway; cpSRP – Chloroplast signal recognition particle; cpTat – Chloroplast twin-argnine translocation; GroEL – Hsp60 class oligomeric molecular chaperone; Hsc – Heat shock cognate protein; Hsp – Heat shock protein; LHC – Light-harvesting complex; MEP – Methylerythritol phosphate; OEC – Oxygen-evolving complex of photosystem II; PAO – Pheophorbide *a* oxygenase; Plsp – Plastidic type I signal peptidase; PPH – Pheophytin pheophorbide hydrolase; PreP – Presequence protease; PSII – Photosystem II; RCB – Rubisco-containing body; Rubisco – Ribulose 1,5-bisphosphate carboxylase-oxygenase; SAG – Senescence-associated gene; SAV – Senescence-associated vacuoles; SPaseI – Type I signal peptidase; SPP – Stromal processing peptidase; SREBP – Sterol-regulatory element binding protein; TIC – Translocon at the inner envelope membrane of chloroplasts; TOC – Translocon at the outer envelope membrane of chloroplasts; TPP – Thylakoidal processing peptidase

Table 20.1. Proteases in Arabidopsis plastid

Protease type	Clan	Family	Protein	Gene name	Location	Protein property	Mutant phenotype	References
Serine	PA	S1	**Deg protease**					
			Deg1	At3g27925	T (l)	PDZ domain	No knockouts reported (Lethal?)	Kapri-Pardes et al. 2007 Sun et al. 2010b
			Deg2	At2g47940	T (s)	PDZ domain	No phenotype	Huesgen et al. 2006
			Deg5	At4g18370	T (l)	–	High-light sensitive	Sun et al. 2007
			Deg7	At3g03380	T (s)	PDZ domain	High-light sensitive	Sun et al. 2010a
			Deg8	At5g39830	T (l)	PDZ domain	High-light sensitive	Sun et al. 2007
			Deg11[a]	At3g16540	–	PDZ domain	–	–
	SF	S26	TPP	At2g30440	T (l)	Membrane-bound	–	Chaal et al. 1998
	SF	S26	Plsp1	At3g24590	E/T	–	Seedling lethal	Shipman-Roston et al. 2010
	SJ	S16	Lon4	At3g05790	T (s)	AAA	–	Ostersetzer et al. 2007
	SK	S14	**Clp protease**[b]					
			ClpP1	AtCg00670	S/T (s)	Clp protease core	No knockouts reported (Lethal?)	–
			ClpP3	At1g66670	S/T (s)	Clp protease core	No knockouts reported (Lethal?)	–
			ClpP4	At5g45390	S/T (s)	Clp protease core	Yellow-heart chlorotic appearance (KD)	Zheng et al. 2006
			ClpP5	At1g02560	S/T (s)	Clp protease core	Embryo lethal	Kim et al. 2009
			ClpP6	At1g11750	S/T (s)	Clp protease core	Yellow-heart chlorotic appearance (KD)	Sjögren et al. 2006
			ClpR1	At1g49970	S/T (s)	Clp protease core (no catalytic triad)	Retarded-growth Chlorotic leaves	Kim et al. 2009 Koussevitzky et al. 2007 Stanne et al. 2009
			ClpR2	At1g12410	S/T (s)	Clp protease core (no catalytic triad)	Seedling lethal	Kim et al. 2009
			ClpR3	At1g09130	S/T (s)	Clp protease core (no catalytic triad)	–	–
			ClpR4	At4g17040	S/T (s)	Clp protease core (no catalytic triad)	Seedling lethal	Kim et al. 2009
			ClpT1	At4g25370	S/T (s)	–	–	–
			ClpT2	At4g12060	S/T (s)	–	–	–
			ClpS1	At1g68660	S/T (s)	–	–	–
	SK	S41	CtpA1	At3g57680	T (l)	PDZ domain	High-light sensitive	Yin et al. 2008

(continued)

Table 20.1. (continued)

Protease type	Clan	Family	Protein	Gene name	Location	Protein property	Mutant phenotype	References
	SK	S49	SppA	At1g73990	T (s)	–	Altered responses to high light acclimation	Lensch et al. 2001 Wetzel et al. 2009
	ST	S54	Rhomboid11	At5g25752	–	Intramembrane protease	–	Kimec-Wisniewska et al. 2008
Metallo	MA	M41	**FtsH protease**					
			FtsH1	At1g50250	T (s)	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003
			FtsH2	At2g30950	T (s)	AAA/membrane-bound	Yellow variegated	Chen et al. 2000 Takechi et al. 2000
			FtsH5	At5g42270	T (s)	AAA/membrane-bound	Yellow variegated	Sakamoto et al. 2002
			FtsH6	At5g15250	–	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003 Zelisko et al. 2005
			FtsH7	At3g47060	–	AAA/membrane-bound	–	–
			FtsH8	At1g06430	T (s)	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003
			FtsH9	At5g58870	–	AAA/membrane-bound	–	–
			FtsH11	At5g53170	–	AAA/membrane-bound Dual targeting to mitochondria and chloroplasts	Thermosensitive	Chen et al. 2006
			FtsH12	At1g79560	–	AAA/Membrane-bound	–	–
	ME	M16	SPP	At5g42390	S	–	Most antisense caused lethality to seedlings	Zhong et al. 2003
	ME	M16	PreP1	At3g19170	S	Dual targeting to mitochondria and chloroplasts	Double-knockout mutant shows a chlorotic phenotype	Bhushan et al. 2003, 2005 Moberg et al. 2003 Nilsson et al. 2009
	ME	M16	PreP2	At1g49630	S	Dual targeting to mitochonadria and chloroplasts		
	MM	M50	EGY1	At5g35220	–	Intramembrane protease	Pigmentation-deficient Defective in ethylene-stimulated hypocotyl gravitropism	Chen et al. 2005
	MM	M50	EGY2	At5g05740	–	Intramembrane protease	–	–
	MM	M50	AraSP	At2g32480	IE	Intramembrane protease	Impaired chloroplast development	Bölter et al. 2006

E envelope, *IE* inner envelope, *S* stroma, *T* thylakoid membrane, *s* and *l* stromal and lumenal side of the thylakoid membrane, respectively, *KD* knockdown mutant

[a]Personal communication by L. Zhang and X. Sun

[b]The Clp/HSP100 subunits (ClpC1, ClpC2, ClpB3, and ClpD) of Clp protease complexes are not included in here

degradation pathways of chloroplast proteins and their fine-tuning contribute to nutrient recycling, which is important for proper plant growth and possibly for reproduction as well. In this chapter, we first describe our knowledge of the chloroplast proteolytic machineries in leaf development, and subsequently we describe degradation mechanisms of chloroplast proteins in leaf senescence. For further background informations, we refer the readers to other recent reviews, some of which provide comprehensive information about plastid proteases and their physiological roles in chloroplast homeostasis (Adam 2000; Adam and Clarke 2002; Clarke et al. 2005; Richter et al. 2005; Adam et al. 2006; Sakamoto 2006; Kato and Sakamoto 2009, 2010; Chi et al. 2011; Olinares et al. 2011).

II. Plastid Protein Degradation During Leaf Development

During leaf development, proplastids are converted to mature chloroplasts in a light-dependent manner. Because chloroplast differentiation from proplastids occurs rapidly and dynamically, the synthesis and import of numerous proteins (see also Ling et al., Chap. 12) are necessary for this process. Concomitantly, synthesis of plastid-encoded proteins is activated. Finally the imported proteins from the cytosol and plastid-encoded proteins assemble into various protein complexes coordinately for the proper chloroplast function. Generation of abnormal peptides and proteins is unavoidable during these comprehensive protein dynamics. Therefore, quality control of proteins by proteases is a necessary feature of chloroplast maturation.

A. Proteases Involved in Protein Import

Proteins found in chloroplasts of mature leaves are encoded either by the chloroplast or by the nuclear genome. During differentiation of proplastids to chloroplasts, only a few chloroplast proteins are produced within the organelle; the larger share of chloroplast proteins are synthesized in the cytosol as precursors containing N-terminal transit sequences. These precursors are imported from the cytosol rapidly to developing chloroplasts through the general import machinery called the translocon at the outer envelope membrane of chloroplasts (TOC) and the translocon at the inner envelope membrane of chloroplasts (TIC) (Andres et al. 2010; Kovacs-Bogdan et al. 2010). Mutants lacking a component of the TOC/TIC protein import apparatus frequently show non-photosynthetic albino phenotypes, suggesting the necessity of protein transport into chloroplasts for chloroplast differentiation and plant viability (Andres et al. 2010; Kovacs-Bogdan et al. 2010). After import to the stromal space, their transit peptides are removed by stromal processing peptidase (SPP) (Richter et al. 2005). Originally, SPP was purified from pea as a member of soluble metalloprotease that contains an inverted zinc-binding motif HXXEH at the catalytic site (VanderVere et al. 1995). The cleavage of transit peptides by SPP proceeds in a two-step process. In the first step, SPP binds to the transit peptide of precursors and cleaves it from the mature form. In the second step, the transit peptide is further cleaved by SPP into subfragments and finally released from SPP (Richter et al. 2005). Studies using antisense SPP transgenic plants have elucidated the crucial role of SPP in chloroplast biogenesis. The SPP antisense transgenic tobacco plants display chlorosis and retardation of plant growth (Wan et al. 1998). Similarly, a large share of antisense transgenic plants in Arabidopsis cause seedling lethality (Zhong et al. 2003). Furthermore, the import capacity of precursors is markedly impaired in chloroplasts isolated from the antisense transgenic tobacco; N-terminal transit sequences fused to a reporter Green Fluorescent Protein (GFP) were not transported into chloroplasts but accumulated in the cytosol in these antisense transgenic plants (Zhong et al. 2003). These results verify experimentally that the loss of SPP function affects not only the removal of transit peptides in chloroplasts, but also a series of protein import mechanisms required for chloroplast development.

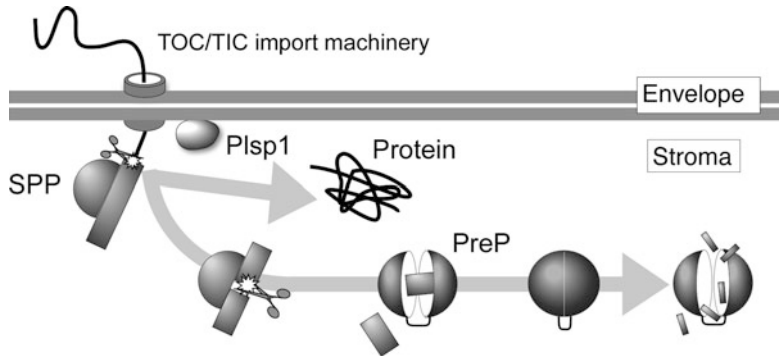

Fig. 20.1. Precursor proteins, imported via TOC/TIC import machinery, undergo processing in the stroma. This figure illustrates removal of transit peptides by peptidases in chloroplasts. To remove transit peptides, SPP binds to the transit peptide and cleaves it from the precursor. Simultaneously, SPP also cleaves the transit peptide into subfragments, which are further degraded by PreP.

Subfragments of transit peptides released from imported precursor proteins are potentially toxic for the integrity of plastid membrane structures and its proper function. Evidence was provided to suggest that the presequence protease (PreP) is responsible for degrading the released subfragments (Ståhl et al. 2005). An ATP-independent metalloprotease, PreP, contains an inverted version of the common zinc-binding motif HXXEH; it has been identified initially as a protease involved in degrading mitochondrial presequences in potato (mitochondrial proteins imported from the cytosol also undergo maturation, and transit peptide sequences of these precursors are termed presequences) (Ståhl et al. 2002). Two homologs, AtPreP1 and AtPreP2, are found in the Arabidopsis genome. Both are dual-targeted to chloroplasts and mitochondria (Bhushan et al. 2003, 2005; Moberg et al. 2003). The unique chamber structure by two halves of this protease connected by a hinge region appears proper for the degradation of the peptide substrates because the chamber size is suitable to hold a small peptide and to exclude larger proteins (Fig. 20.1) (Johnson et al. 2006). Actually, PreP has the capacity to degrade the cleaved precursor peptides that are between 10 and 65 amino acid residues without substrate specificity (Ståhl et al. 2005). Nilsson Cederholm et al. (2009) showed that Arabidopsis double mutants lacking both PreP homologs have a pale green phenotype and retarded plant growth, and, this was due to the presence of aberrant chloroplasts and mitochondria. These observations strongly imply that proper degradation of the subfragments by chloroplast protease is necessary for normal leaf development.

Chloroplast development accompanies biogenesis of thylakoid membranes where photosynthetic light reaction and ATP synthesis occur. Similar to stromal proteins, many proteins in protein complexes of thylakoid membranes and luminal proteins are also synthesized in the cytosol and then transported to other sites. After being moved into the stromal space, proteins targeting the thylakoid lumen are further transported by either the chloroplast secretory (cpSec) or the chloroplast twin-arginine translocation (cpTat) pathway. The proteins that are localized in thylakoid membranes are inserted by the chloroplast signal recognition particle (cpSRP) or via the spontaneous pathway (Cline and Dabney-Smith 2008). To be localized in the thylakoid membrane, many thylakoid proteins have a bipartite transit peptide, which is constituted by a plastid transit peptide and a thylakoid-targeting signal peptide. Proteases functioning for the removal of the thylakoid-targeting signal

peptide are called thylakoidal processing peptidases (TPP). Chaal et al. (1998) demonstrated that membrane-anchored serine-type proteases that belong to the type I signal peptidase (SPase I) family function as TPP. In the Arabidopsis genome, at least three SPase I homologs, plastidic type I signal peptidase (Plsp), exist. In Arabidopsis, Plsp3 was first identified as TPP based on in vitro signal peptidase activity assay (Chaal et al. 1998). Subsequently, Plsp1 has been regarded as a peptidase responsible for maturation of the component of the translocon at the plastid outer envelope membrane: Toc75 (Inoue et al. 2005). Further studies using *plsp1* mutants revealed that Plsp1 also contributes to the processing of several thylakoid luminal proteins (subunits of the oxygen-evolving complex of PSII [OEC33 and OEC23], and plastocyanin) (Shipman and Inoue 2009). These results are consistent with the character of Plsp1 localized not only in the envelope membranes but also in the thylakoid membranes. To date, the in vitro signal peptidase activity of Plsp1 has not been confirmed, but Plsp1 is likely to function as a TPP in vivo. Meanwhile, the result that the lack of Plsp1 caused an abnormal plastid membrane structure and a seedling lethal phenotype suggests, similarity observed in case of SPP, that the the proper processing of thylakoid lumenal proteins is necessary for chloroplast development during leaf growth (Shipman-Roston et al. 2010). Together with studies in SPP and PreP, these observations clearly indicate that a series of protease activities in protein maturation is involved in the early process of chloroplast development.

B. Protein Quality Control During Leaf Development

In addition to the significance of proteases in the protein maturation steps as described above, proteases are well known to play a crucial role in the quality control of proteins in chloroplasts. Presuming that the occurrence of the abnormal proteins that are caused by protein misfolding increases coincidentally with large-scale protein synthesis that is required during chloroplast differentiation, the quality control of proteins is expected to become more important in this step. Actually, the loss of chloroplast proteases often severely impairs chloroplast biogenesis and its homeostasis. In this section, we describe the function of chloroplast proteases that are involved in quality control.

1. Protein Quality Control in Stroma

a. Clp Protease

To date, several proteases have been identified in the chloroplast stroma (Kato and Sakamoto 2010). Among these stromal-localized proteases, Clp is considered to play a central role in quality control as a stromal housekeeping protease (Clarke et al. 2005). Actually, Clp protease is an ATP-dependent serine-type protease complex that is present in almost all bacteria and in mitochondria and chloroplasts (Porankiewicz et al. 1999; Yu and Houry 2007). The functional Clp is constituted of a chaperone that belongs to AAA+ATPases and a proteolytic core component. In *E. coli,* the chaperone components comprise a homogeneous hexameric ring of the Clp/Hsp100 subunits, either ClpA or ClpX containing two and one AAA domains, respectively (Grimaud et al. 1998). The central proteolytic core of the Clp protease in *E. coli* comprises two stacked homogeneous heptameric rings of seven identical ClpP with serine-type proteolytic active sites (Fig. 20.2). These heptameric rings form a barrel-like structure enclosing a large chamber containing exposed proteolytic sites (Wang et al. 1997). The chaperone component docks on one or both ends of the barrel-like core component (Kessel et al. 1995; Grimaud et al. 1998). The connected chaperone components recognize denatured protein substrates; then they unfold and translocate the substrates through the narrow axial pore into the proteolytic chamber. Moreover, a small adapter protein, ClpS, is associated with the Clp chaperone component to prevent accidental degradation of functional proteins

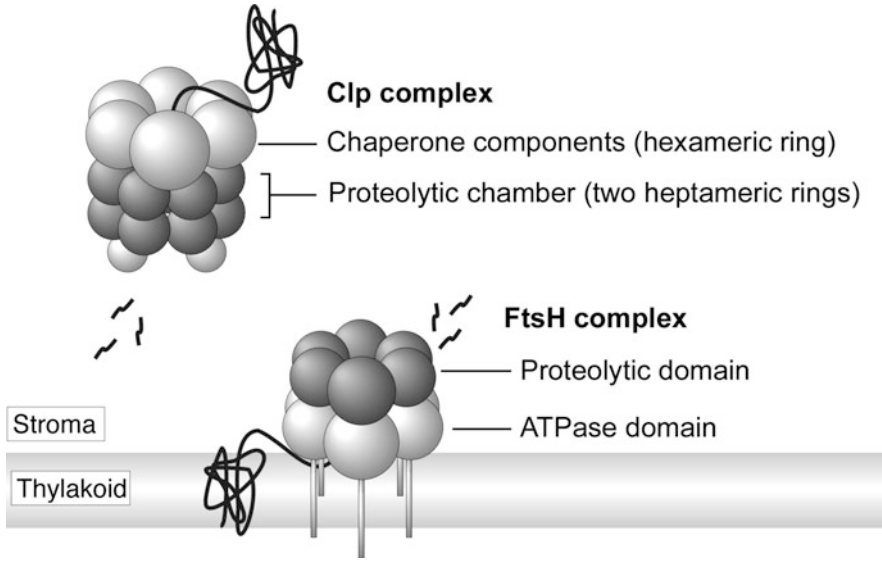

Fig. 20.2. Schematic representation of Clp and FtsH protease complexes in chloroplasts. Clp protease complexes are composed of the catalytic component with two stacked heteroheptameric rings and the chaperone component that comprises a homohexameric ring of the Clp/HSP100 subunits. One of the heteroheptameric rings in the catalytic component contains ClpP1 and ClpR1-4 and the other contains ClpP3-6. The chaperone components are likely to be constituted by ClpC1, ClpC2, and ClpD. In addition, ClpT proteins are associated to the peripheral surface of the proteolytic core complex. On the other hand, FtsH protease complexes constitute heterohexameric ring structure in thylakoid membranes. In contrast to the Clp protease complexes, the subunits of FtsH protease complexes harbor both its proteolytic and ATPase domains in a same polypeptide. Four subunits—FtsH1 and FtsH5 (type A), FtsH2 and FtsH8 (type B)—have been shown to comprise major isomers of chloroplast FtsH complexes.

(Erbse et al. 2006; Wang et al. 2008). These structural features of the Clp protease complex indicate that the Clp complex employs common principles with the 26S proteasome proteolytic machinery in eukaryotes because both Clp and 26S proteasome use the unfolding-coupled processive protein degradation system. Consequently, Clp protease complexes are regarded as the counterparts of eukaryotic proteasomes.

The ClpP subunits are diversified and exist as multiple copies in photosynthetic organisms, in contrast to most bacteria containing a single copy of the proteolytic subunit. In chloroplasts, the proteolytic chamber is present as a heterocomplex; the proteolytic core comprises five ClpP isomers (ClpP1 and ClpP3–P6) and four ClpP-like subunits (ClpR1–ClpR4) that lack the conserved amino acid of the catalytic triad (Peltier et al. 2001, 2004; Clarke et al. 2005; Sjögren et al. 2006). In cyanobacteria, Andersson et al. (2009) have demonstrated that ClpR is indeed proteolytically inactive. Compared with the complexity and the diversity of the proteolytic chamber subunits, the subunits of the chaperone component in chloroplasts are rather simple. Among the four homologs of Clp/Hsp100 subunits (ClpB3, ClpC1, C2, and ClpD) that are localized in chloroplasts (Moore and Keegstra 1993; Weaver et al. 1999; Peltier et al. 2004) with two distinct AAA+domains, three (ClpC1, C2 and ClpD) seem to form a complex with a proteolytic chamber (Peltier et al. 2004). In addition, a homolog of an adapter protein ClpS has been discovered in chloroplast Clp complexes. Along with these subunits, two novel plant-specific subunits, ClpT1 and ClpT2, which share homology with the N-terminal domain of Clp/Hsp100 proteins, have been identified (Peltier et al. 2001, 2004). Further studies showed that the

peripheral attachment of ClpT proteins to the proteolytic core components is likely to regulate the assembly of Clp protease complex (Sjögren and Clarke 2011).

The physiological importance of the Clp protease was initially demonstrated by studies showing that tobacco transgenic lines with significantly reduced levels of ClpC or ClpP1 could not survive (Shanklin et al. 1995; Shikanai et al. 2001). Additional studies using Arabidopsis mutants and antisense lines supported the results of early studies and revealed the indispensable function of the Clp protease (Rudella et al. 2006; Sjögren et al. 2006; Kim et al. 2009; Stanne et al. 2009; Zybailov et al. 2009). Knockout mutants showed that the defect of the ClpP subunit in protease complexes engenders a lethal phenotype in Arabidopsis. Therefore, the antisense strategy was employed for additional analysis of ClpP. The ClpP4 and P6 antisense plants showed leaf chlorosis and slow growth (Sjögren et al. 2006; Zheng et al. 2006). In particular, the phenotypes of mutants are severe in younger inner leaves. This impairment of chloroplast development by repression of Clp suggests a functional importance of Clp in the early phase of plastid differentiation. Of the *clpr* mutants, the *clpr1* null mutant can survive because the ClpR1 function is partially complemented by the closely related ClpR3 protein (Koussevitzky et al. 2007; Kim et al. 2009; Stanne et al. 2009). The loss of ClpR1 brings about leaf chlorosis and reduces photosynthesis rates; further, the more severe phenotype of the *clpr1* mutant in the younger leaves suggests the involvement of Clp function in chloroplast development. The proteomic analysis using isolated chloroplasts from ClpP6 antisense and *clpr1* mutant revealed some potential substrates for Clp protease, which are involved in general homeostatic roles such as protein synthesis, protein maturation, and RNA maturation (Sjögren et al. 2006; Stanne et al. 2009). Furthermore, large-scale comparative proteomics, using the null-mutant *clpr4* shows albino phenotype, that cannot survive beyond the seedling stage, but is able to grow under heterotrophic conditions, demonstrated that many chloroplast proteins had changed in the *clpr4* mutant (Kim et al. 2009). Especially, the nearly complete loss of photosynthetic proteins in the mutant is thought to be the reason for the severe phenotype in *clpr4*. Similarly, the large-scale comparative proteomics of the *clpr2* knockdown mutant and the wild-type showed that impaired protease activities are involved widely in various chloroplast protein accumulation including the methylerythritol phosphate (MEP) pathway proteins, which have been implicated in other reports as substrates of Clp protease (Zybailov et al. 2009). In addition, the loss of function of chaperone component subunit ClpC1 causes growth retardation and leaf chlorosis with reduced photosynthesis proteins (Constan et al. 2004; Sjögren et al. 2004; Kovacheva et al. 2005). Moreover, the lack of both ClpC1 and ClpC2 causes very early embryo lethality (Kovacheva et al. 2007). These results suggest important roles of the unfolding function of the Clp protease complex for plastid development and plant viability. Collectively, the studies of the Clp protease indicate that the derangement of protein degradation resulting from impairment of Clp causes the disruption of chloroplast development with disintegration of protein homeostasis.

b. Protein Degradation and Chaperones

We note that the loss of Clp subunits, not only Clp/Hsp100 subunits that are likely to function as a chaperone independently, but also Clp protease core subunits, causes accumulation of molecular chaperones (unfoldase ClpB3, chloroplast Hsp90, Hsp70, Cpn60 and so on) (Kim et al. 2009; Stanne et al. 2009; Zybailov et al. 2009). The molecular chaperones help in proper protein folding to prevent the generation of harmful polypeptides and aggregation of proteins. Apparently, the higher level of molecular chaperones results from the increased protein instability, which appears to be associated with the accumulation of undegraded

harmful polypeptides in *clp* mutants. Therefore, the increased level of chaperones in *clp* mutants suggest the presence of a close relation between protein degradation and the folding for protein quality control in chloroplast protein synthesis and protein transport from the cytosol. To date, many research groups have identified molecular chaperones in chloroplasts; many reviews describe their functions (Jackson-Constan et al. 2001; Levy-Rimler et al. 2002; Weiss et al. 2009). Here, we briefly explain their roles and involvement in chloroplast development.

One chloroplast Clp/Hsp100 subunit, ClpB3, seems to function as a chaperone independently of Clp protease complexes because it lacks the conserved domain for binding to ClpP (Clarke et al. 2005). The mutant of ClpB3 is reported as one of the *albino* or *pale-green* (*apg*) mutants, *apg6*, and it shows severe defects in chloroplast development (Myouga et al. 2006; Lee et al. 2007). Further, ClpC1 and ClpC2, also known as Hsp93-V and Hsp93-III, have been identified as proteins associated with the chloroplast protein import apparatus (Akita et al. 1997; Nielsen et al. 1997). Because a large proportion of ClpC is present in the stroma in a soluble form, the primary function of ClpC is thought to serve as the chaperone component of the Clp protease complexes; however, a role as an independent chaperone in chloroplast protein import also seems feasible. Another Hsp protein, chloroplast Hsp90, was originally identified through a chlorate-resistant mutant that shows an altered response to red light (Cao et al. 2003). The mutant of chloroplast Hsp90 also shows delayed chloroplast development, suggesting the involvement of chloroplast Hsp90 in the maturation of newly imported or synthesized proteins during chloroplast development (Cao et al. 2003). Regarding Hsp70 homologs, four Hsp70 systems have been detected within the different compartments of chloroplasts (Jackson-Constan et al. 2001). Two of them, Com70 and IAP70, both localized in the envelope membrane, are apparently involved in protein import to chloroplasts (Schnell et al. 1994; Kourtz and Ko 1997). The other two Hsp70, cpHsc70-1 and cpHsc70-2, which have been shown to accumulate in *clp* mutants, are apparently present in stroma (Su and Li 2008). An Arabidopsis T-DNA insertion mutant of cpHsc70-1 shows variegated cotyledons and malformed leaves, although a T-DNA mutant of cpHsc70-2 shows no visible phenotype (Su and Li 2008). Furthermore, attempts to obtain a double mutant and the approach of co-suppression of both genes using RNAi interference showed their redundant functions and their important role in chloroplast development and plant viability (Latijnhouwers et al. 2010). In addition to these homologs, the homologs of the bacterial chaperone GroEL, Cpn60s, are reported to be proteins that accumulate in *clp* mutants. Similar to Hsp70s, Cpn60, which interacts with a component of the inner membrane import apparatus Tic110, helps the imported protein to fold into its native conformation (Kessler and Blobel 1996). The results demonstrating that the *cpn60* mutants show abnormal development of embryos suggest the necessity of Cpn60 for plastid biogenesis and plant viability (Apuya et al. 2001). In addition to these typical molecular chaperones, the chaperone activity of Deg protease was recently reported as described below.

2. Protein Quality Control in Membranes

a. FtsH Protease

The quality control of membrane proteins is necessary for chloroplast biogenesis, especially in the formation of thylakoid membranes. Of the membrane proteases, FtsH protease is the best characterized one because the unique variegated phenotype of the *ftsh* mutants has been the subject of interest of many studies. FtsH protease is a membrane-bound ATP-dependent metalloprotease that was originally identified in *E. coli* as a necessary protein for growth (Tomoyasu et al. 1993). FtsH is a large complex that has a AAA+ATPases domain and a proteolytic core component (Krzywda et al. 2002; Niwa et al. 2002); however, in contrast to

the Clp protease complex, it has both domains on the same polypeptide chain (Fig. 20.2). Crystal structure studies have revealed that six identical FtsH subunits form a large complex with unique catalytic site(s) located at the peripheral region of the hexamer ring (Bieniossek et al. 2006; Suno et al. 2006).

We note that FtsH is highly conserved in all organisms. In plants, FtsH homologs have been isolated and characterized from, e.g., spinach, tobacco and peas (Lindahl et al. 1996; Seo et al. 2000; Yue et al. 2010). In Arabidopsis, among the enumerated 12 homologous genes, 9 homologs (FtsH1, 2, 5, 6, 7, 8, 9, 11, and 12) are located in the chloroplast (Sakamoto et al. 2003). Of these homologs, FtsH1, 2, 5, and 8 are identified by immunoblot and proteomic analyses of the isolated thylakoid membrane (Sakamoto et al. 2003; Friso et al. 2004; Sinvany-Villalobo et al. 2004; Yu et al. 2004). Immunoblot analysis showed that the proteolytic domain faces the stroma side of the membrane (Lindahl et al. 1996; Sakamoto et al. 2003). The four isomers, mentioned above, have been regarded as major isomers of chloroplast FtsH complexes. They are divided into closely related pairs of two types, FtsH1 and FtsH5 (type A), FtsH2 and FtsH8 (type B), which are likely duplicated (Sakamoto et al. 2003). Among them, FtsH2 is the most abundant isomer, followed by FtsH5, FtsH8, and FtsH1. Originally, FtsH2 and FtsH5 were reported as responsible genes of leaf-variegated mutants, which have long been known in Arabidopsis (Chen et al. 2000; Takechi et al. 2000; Sakamoto et al. 2002). The mutants of FtsH2 and FtsH5 are called *yellow variegated 2* (*var2*) and *var1*; *var2* shows severe variegated phenotype and *var1* shows weak leaf-variegation. On the other hand, mutants with a loss of FtsH1 and FtsH8 show no visible change in their phenotype. The difference in the degree of leaf-variegation among these mutants is considered to be dependent on the abundance of isomers in the FtsH heterocomplexes. The variegated phenotypes in *var1* and *var2* mutants are rescued, respectively, by overexpression of FtsH1 and FtsH8; this suggests functional redundancy of each type A and type B FtsH isomers (Yu et al. 2004, 2005). Furthermore, the results that *ftsh2 ftsh8* and *ftsh1 ftsh5* double mutants showing an albino-like phenotype underscore the possibility that the existence of both type A and type B isomers is required for the proper function of FtsH (Zaltsman et al. 2005b). Together with the studies by proteomics and the biochemical approaches, the construction of heterohexameric FtsH complex by type A and type B isomers in chloroplasts has been published.

Additionally, the observations that overall FtsH levels correlate with the degree of white sectors in leaves led to the proposal of a threshold model of leaf variegation; subthreshold amounts of the FtsH complex block thylakoid formation in leaf development, leading to the failure of chloroplast development (Yu et al. 2004). In this threshold model, it was expected that FtsH would have a decisive role at a particular stage of chloroplast development. The result that white viable sectors of *var2* mutants have undifferentiated plastids, and the observations that the variegated pattern is irreversible once developed, supports this expectation (Zaltsman et al. 2005a; Kato et al. 2007). Furthermore, recent careful observation of plastid ultrastructures during the early stage of leaf development demonstrated that the abnormal plastids in white sectors is formed as a result of the arrest of chloroplast development at its initial steps (Sakamoto et al. 2009). To summarize, these results demonstrate that the early stage of chloroplast development requires sufficient levels of FtsH for thylakoid formation.

Further studies have shown that several genetic defects cause the suppression of leaf-variegation. Most identified mutant genes are involved in the chloroplast translation (chloroplast rRNA processing and protein synthesis), which implies that the delay in chloroplast development, which results from the impairment of chloroplast translation, lowers the requirement of FtsH for protein quality control in chloroplast biogenesis (Park and Rodermel 2004; Miura et al. 2007;

Yu et al. 2008; Liu et al. 2010). Electron-microscopic observations of plastids revealed that the loss of FtsH in *var2* mutant causes slow progression of chloroplast development in the prospective green sectors (Sakamoto et al. 2009). In other words, the variegated mutant seems to avoid the serious dysfunction of chloroplasts by limiting the progression of chloroplast differentiation in early leaf development. Taken together, these results indicate that the balance between the speed of thylakoid development and the protein quality control by FtsH is an important point for proper chloroplast development and that it is regulated strictly by communication between the nucleus and the chloroplasts.

Using transgenic plants that ectopically expressed a proteolytically inactive FtsH2, Zhang et al. (2010) posed an interesting question for the FtsH function in leaf variegation; the result that expression of proteolytically inactive FtsH2 rescued not only leaf variegation in *var2*, but also seedling lethality in *ftsh2 ftsh8* double mutant suggests that not all proteolytic activities of FtsH heterocomplexes are necessary for their function in chloroplasts (Zhang et al. 2010). Further analysis of *var1 var2* mutants, with expression of proteolytically inactive FtsH2, and a study using an inducible FtsH2, show that the overall amount of FtsH complexes predominantly determines the threshold of chloroplast development when the protease activity is excessive (Zhang et al. 2010).

What is the substrate of FtsH in chloroplast development? Using mature chloroplasts, several studies suggest only a partial answer to this question. In vitro studies showed the involvement of FtsH in the degradation of unassembled Rieske Fe-S protein and in the degradation fragment of the PSII reaction center D1 protein (Ostersetzer and Adam 1997; Lindahl et al. 2000). Additional in vivo analyses using mutants lacking FtsH2 or FtsH5 also indicated the involvement of FtsH in D1 degradation (Bailey et al. 2002; Kato et al. 2009). Most of the available evidence point to the central role of FtsH in D1 degradation in the PSII repair cycle in mature chloroplasts. However, the study of the substrates of FtsH in the chloroplast developmental stage has scarcely been made. Consequently, although D1 protein might be a possible substrate in developing thylakoids as well as that in mature chloroplasts, the substrates of FtsH in the chloroplast developmental stage remain largely unknown.

b. Deg Protease

DegP protease is an ATP-independent serine type protease that was originally identified in *E. coli* as the protease necessary for survival at high temperatures (Skorko-Glonek et al. 1995). It has two domains: the proteolytic domain at the N-terminus and the PDZ domain(s), which are involved in protein–protein interactions, at the C-terminus (Clausen et al. 2002). It is noteworthy that DegP has not only a proteolytic activity, but also a chaperone activity to prevent the accumulation of abnormal proteins (Spiess et al. 1999). The switch between protease and chaperone function seems be regulated by a conformation change of the protein caused by a temperature shift. The chaperone activity is predominant at low temperatures; protease activity is increased at elevated temperatures (Spiess et al. 1999). In Arabidopsis, among the 16 DegP homologous genes, five homologs (Deg1, 2, 5, 7, and 8) have already been found in the chloroplast (Itzhaki et al. 1998; Haussühl et al. 2001; Peltier et al. 2002; Schubert et al. 2002; Huesgen et al. 2005; Sun et al. 2007, 2010a). Furthermore, the presence of Deg11 in the chloroplast stroma has also been suggested (L. Zhang and X. Sun, personal communication, 2011). These homologs are generally peripherally attached to the stromal (Deg2 and 7) or the lumenal sides (Deg1, 5, and 8) of thylakoid membranes. In *E. coli* DegP peripherally attaches to the plasma membrane. Early in vitro studies using recombinant proteins showed that Deg1 proteases are involved in the degradation of several thylakoid lumen proteins, such as plastocyanin and OEC33 (Chassin et al. 2002), and Deg2 functions in the initial endoproteolytic cleavage of the D1 protein

(Haussühl et al. 2001), although the in vivo contributions of these Deg proteases to the degradation of these proteins remain unconfirmed (Huesgen et al. 2006). Meanwhile, there is evidence that several Deg proteases (Deg1, 5, 7, and 8) play an important role in the in vivo PSII repair under high-light conditions (Sun et al. 2007, 2010a, b). The work of Kapri-Pardes et al. (2007) suggests the importance of Deg protease for plant viability since the authors failed to obtain homozygous knockout lines of Deg1. Furthermore, antisense lines with a reduced level of the Deg1 protein showed pale-green phenotypes, suggesting the requirement of Deg1 for chloroplast biogenesis and homeostasis (Kapri-Pardes et al. 2007). It is particularly interesting that Sun et al. (2010b) show the existence of chaperone activity of Deg1, like it is known for *E. coli* homolog DegP. However, the functions of Deg1 in chloroplast development are largely unknown, although the chaperone activity of Deg1 seems to be very important for proper protein assembly that is needed in thylakoid development.

c. Intramembrane Proteases

Chloroplasts have several intramembrane proteases to degrade membrane proteins for proper chloroplast biogenesis. Of these intramembrane proteases, the loss of the homologs of the sterol-regulatory element binding protein site 2 protease (SREBP S2P protease) causes the impairment of chloroplast biogenesis. One homolog of SREBP S2P proteases, ethylene-dependent gravitopism-deficient and yellow-green 1 (EGY1), is an ATP-independent metalloprotease with eight putative transmembrane helices in its C-terminus (Chen et al. 2005). The *egy1* mutant was originally identified as a mutant that shows abnormal gravicurvature in hypocotyls and a pale-yellow phenotype. The development of the thylakoid membrane system is severely impaired in the mutants. In particular, the remarkable decrease of the levels of the chlorophyll-binding proteins in the *egy1* mutants seems to cause the loss of grana stacks because it is considered that the LHCII-mediated physical connection contributes to the stability of grana stacking (Chen et al. 2005). The association of EGY1 with chloroplast membranes has been confirmed by immunoblotting analysis, but the detailed localization of EGY1 in chloroplasts has not yet been identified. However, another homolog of the SREBP S2P proteases, AraSP, is localized in the chloroplast inner envelope membrane (Bölter et al. 2006). The deduced amino acid sequence of AraSP indicates 4–5 transmembrane helices and a conserved catalytic motif, which is localized between the first two helices. The T-DNA knockout mutant of AraSP cannot germinate and the heterozygous T-DNA insertion mutant still shows severely impaired plant growth (Bölter et al. 2006). Together with the defective phenotype of chloroplast biogenesis in the AraSP antisense lines, these results underscore the important role of AraSP during leaf development (Bölter et al. 2006).

III. Plastid Protein Degradation During Leaf Senescence

In response to organ developmental status and various environmental signals, chloroplasts change their morphology and convert the plastid type from one form to another. This happens also in the final stage of leaf development, when photosynthesis is no longer required, but senescence is initiated. Chloroplasts in senescing leaves gradually shrink and transform themselves into gerontoplasts (Wise 2007). Breakdown of thylakoid membranes, degradation of photosynthetic proteins, and accumulation of a remarkable number of plastglobuli occur during leaf senescence (Krupinska 2007). Although the chloroplast-to-gerontoplast transition occurs in senescing leaves, it is an extremely important event for plant growth because the chloroplast nutrients that are generated by degradation of accumulated proteins during leaf senescence contribute to crop yields and biomass accumulation (Mae 2004).

Despite numerous studies on the degradation of chloroplast proteins and the change of chloroplast structure during senescence, the fate of chloroplasts and the degradation mechanism of chloroplast proteins during senescence remain poorly understood. In the following sections, degradation processes of chloroplast proteins mediated by the proteases are summarized. For additional information, we refer the readers to several reviews on chloroplast component degradation during leaf senescence (Hortensteiner and Feller 2002; Feller et al. 2008; Gregersen et al. 2008; Martinez et al. 2008b).

A. Degradation of Stromal Proteins

Available results suggest that different mechanisms exist for chloroplast protein degradation during leaf senescence (Minamikawa et al. 2001; Chiba et al. 2003; Kato et al. 2004; Otegui et al. 2005). Chloroplast protein degradation mechanisms may be divided broadly into two categories: the degradation of proteins inside the chloroplasts and the degradation of chloroplast proteins in other organelles such as lytic vacuoles (see also Wada and Ishida, Chap. 19). Chloroplast protein degradation pathways that interact with other organelles can be divided further into at least three categories: degradation of chloroplast proteins via chloroplast-derived vesicles, the degradation of engulfed whole-chloroplasts within the vacuole, and the degradation of chloroplasts as a result of tonoplast rupture. It is particularly interesting that studies of Arabidopsis mutants show multiple degradation pathways of chloroplast stromal proteins and their fine-tuning (Hortensteiner and Feller 2002; Feller et al. 2008; Gregersen et al. 2008; Martinez et al. 2008b). We provide below an overview of stromal protein degradation.

1. Protein Degradation Inside Plastids

The idea that proteins are degraded inside chloroplasts is supported by the finding that the protein level decreases rapidly in the early phase of leaf senescence prior to the decline of the chloroplast number (Friedrich and Huffaker 1980; Mae et al. 1984). In particular, stromal enzymes, known to be involved in carbon and nitrogen assimilation, such as glutamine synthetase and ribulose 1, 5-bisphosphate carboxylase-oxygenase (Rubisco), are lost in the early phases of leaf senescence. During this process, soluble proteins are gradually degraded as leaves age; then degraded products are exported to reproductive organs to salvage nutrients. Furthermore, experiments using isolated chloroplasts, which exclude contamination of proteases derived from other cellular components, showed that hydrolysis of Rubisco takes place inside the isolated organelles (Ragster and Chrispeels 1981; Mitsuhashi and Feller 1992).

Although the importance of proteolysis inside chloroplasts has been recognized, plastid proteases involved in protein degradation in senescent leaves remain poorly understood. Early biochemical approaches for the identification of plastid proteases, involved in degradation of Rubisco, suggest that a stromal metalloprotease, that has been partially purified from pea chloroplasts, is able to degrade the large subunit of isolated Rubisco to smaller polypeptides in vitro (Bushnell et al. 1993). A further study using isolated chloroplasts also showed that metalloprotease activities are involved in stromal protein degradation under dark or nitrogen-starvation conditions (Roulin and Feller 1998). However, the metalloprotease that actually functions in chloroplast protein degradation in vivo during leaf senescence remains unidentified.

Various proteases localized in chloroplasts are known to participate in proper organellar functioning through protein quality control. Of these chloroplast proteases, Clp and Lon are considered to participate in protein degradation in the stroma, and FtsH and Deg are known to be involved in protein degradation in thylakoid membranes (Kato and Sakamoto 2010). Of these proteases, one Clp chaperone subunit, ClpD, was first reported to show early response to dehydration (ERDs) at the mRNA level (Kiyosue et al. 1993); ClpD/ERD corresponds to

SAG15 (senescence-associated gene 15), whose transcripts have been known to accumulate during leaf senescence (Nakashima et al. 1997). Although several research groups have reported up-regulation of the mRNA levels of *clpD* during leaf senescence, the ClpD protein does not show any significant accumulation; instead, it declines during senescence (Nakabayashi et al. 1999; Weaver et al. 1999). In spite of up-regulation of genes corresponding to several other subunits of the Clp protease core complex (ClpP3 and ClpP5) has been reported, ClpP protein levels remained almost constant or declined during leaf senescence (Nakabayashi et al. 1999). Consequently, further research is necessary to examine if Clp participates in the degradation of stromal proteins during leaf senescence.

It is noteworthy that the aspartic protease CND41 has been localized in plastids and shown to be up-regulated in natural leaf senescence (Kato et al. 2004). In contrast to Clp, its up-regulation has been detected at both transcriptional and protein levels. This CND41 was originally isolated from chloroplast nucleoids, a large complex of chloroplast DNA and proteins in photomixotrophically cultured tobacco cells (Nakano et al. 1997). Unlike many other major chloroplastic proteases, CND41 that belongs to A1 aspartic protease family (pepsin-like family), appears to be of eukaryotic origin. Studies on the proteolytic activity of CND41, that had been purified from cultured tobacco cells, showed that CND41 can degrade denatured inactive Rubisco at physiological pH (Murakami et al. 2000). However, native active Rubisco appeared to be a poor substrate for it. Furthermore, characterization of transgenic tobacco lines overexpressing CND41 implied that there is a post-translational activation mechanism for CND41 to degrade chloroplast proteins during leaf senescence in vivo (Kato et al. 2005). Aside from tobacco, up-regulation of CND41 homologs during senescence has been observed in Arabidopsis and barley (Parrott et al. 2007; Diaz et al. 2008). It is particularly interesting that immunoblot analysis of Arabidopsis recombinant inbred lines, which were selected based on the differential leaf senescence phenotypes, revealed that the CND41 homolog appeared to accumulate in early senescing Arabidopsis lines, suggesting that the CND41 homolog is associated with senescence (Diaz et al. 2008). These results imply that CND41 contributes to protein degradation inside chloroplasts during leaf senescence in many plant species.

2. Protein Degradation in Other Organelles

The chloroplast number per cell decreases during leaf senescence (Ono et al. 1995; Inada et al. 1998; see also Mulisch and Krupinska, Chap. 14). This decrease was suggested to indicate the presence of a whole chloroplast degradation system, possibly conducted by other organelles. Furthermore studies revealed the presence of small spherical bodies containing chloroplast stromal proteins during leaf senescence (Chiba et al. 2003; Martinez et al. 2008a). We summarize below the degradation pathways of chloroplast proteins via chloroplast-derived vesicles and the degradation of engulfed whole-chloroplasts within the vacuole (see also Wada and Ishida, Chap. 19). These degradation pathways participate in leaf senescence, although chloroplast protein degradation after tonoplast rupture seems to occur mainly during programmed cell death.

a. Rubisco-Containing Bodies

Immunocytochemical detection of Rubisco in naturally senescing wheat leaves showed the existence of distinct small spherical bodies, termed Rubisco-containing bodies (RCBs), in the cytoplasm and in the vacuole (Chiba et al. 2003). In fact, RCB-like structures have been observed in senescent leaves of tobacco (Prins et al. 2008). They also contain another stromal protein, glutamine synthetase, but do not include thylakoid membrane proteins and chlorophylls. The RCBs are 0.4–1.2 μm in diameter and are surrounded by double membranes. Careful observation of RCBs revealed that they are

further surrounded by the other membrane structures, suggesting that an autophagy mechanism is involved in degrading chloroplast proteins (Chiba et al. 2003). Arabidopsis mutants that are defective in autophagy-related genes (*atg* mutant) were recently examined for the relevance of autophagy for RCB formation (Ishida et al. 2008). The analysis of visualized RCBs using GFP-labeled Rubisco shows that RCBs are not observed in leaves of *atg* mutant, although RCBs are observed in the lumen of the vacuoles in the wild-type. Furthermore, characterization of a GFP-linked ATG protein as an autophagy marker demonstrated that the GFP signals are co-localized in autophagic bodies with chloroplast stroma-targeted DsRed. These results show that transfer of stromal proteins to the central vacuole via RCBs pathway requires ATG-dependent autophagy. Another study using an *atg* mutant showed that the size of chloroplasts did not decrease in the *atg* mutant during leaf senescence, suggesting that ATG-dependent autophagy mediated degradation of chloroplast proteins is responsible for chloroplast shrinkage in the senescent stage (Wada et al. 2009). It is particularly interesting that the defect in the generation of RCBs in the *atg* mutant induced the increase of stromules as compared to the stromules in the wild-type (Ishida et al. 2008). The release of small vesicles from the ends of stromules has been observed, and the vesicle diameter is similar to that of RCBs (Gunning 2005). These observations imply the possible association between the release of small bodies from stromules and RCBs.

b. Senescence-Associated Vacuoles

Senescence-associated vacuoles (SAVs) are another type of vesicles that were originally identified in the senescent leaves of soybean and Arabidopsis using the SAG12-GFP fusion protein as a fluorescent marker (Otegui et al. 2005; see also Costa et al., Chap. 18). Distinguishable from the central vacuole with respect to their acidic pH, SAVs have intense proteolytic activity. A typical SAV is approximately 0.7 μm in diameter. In addition, the senescence specific cysteine protease SAG12 is localized selectively within SAVs. Despite the strong relationship of SAG12 and senescence, however, the Arabidopsis *sag12* mutant lacking SAG12 shows no altered senescence phenotypes and has SAVs, indicating that SAG12 is not directly required for SAV formation (Otegui et al. 2005). Direct evidence of the involvement of SAVs in chloroplast protein degradation was obtained by using tobacco transformants expressing chloroplast-targeted GFP (Martinez et al. 2008a). During leaf senescence, GFP that is targeted to the chloroplast stroma was relocalized to SAVs. Furthermore, apart from GFP, Rubisco and glutamine synthetase are contained in isolated SAVs and are degraded within SAVs. Although the D1 protein of the photosystem II reaction center and the light-harvesting complex II (LHC-II) are not contained in SAVs, some SAVs with chlorophyll autofluorescence have been detected (Martinez et al. 2008a). The SAVs are known to be surrounded by a single membrane in contrast to RCBs that have double membranes, but there are several known similarities between RCBs and SAVs. Although the possible relation between these two types of vesicles is currently unknown, SAVs appear to be formed in *atg7* (Otegui et al. 2005), which is defective in the ATG-gene-dependent autophagy and the formation of RCB. The selectivity of proteins that are carried in these compartments as substrates, is still an unanswered question.

c. Degradation Inside the Central Vacuole

In addition to the above-mentioned specific vesicles associated with leaf senescence, the central vacuole, the largest lytic compartment in mature cells, is an important compartment related to chloroplast degradation. An early study using electron microscopy showed the possible physical interaction between the outer envelope of the chloroplast and the tonoplast (Peoples et al. 1980); in this report, the outer envelope of some chloroplasts flanked by the tonoplast was apparently degraded and merged with the tonoplast, but the inner envelope of the chloroplasts appeared to

remain intact. However, another ultrastructural study showed that some chloroplasts observed in mesophyll cells appeared to move toward the center of the cell as leaf senescence proceeded (Wittenbach et al. 1982). This movement was concomitant with the decrease of chlorophylls and soluble proteins and the decline of chloroplast number per cell, suggesting degradation of engulfed whole-chloroplasts within the vacuole. These observations have been supported by an electron microscopic examination of dark-induced senescence of French bean leaves (Minamikawa et al. 2001). Electron microscopic observations indicates chloroplast internalization into vacuoles, and the disruption of the outer membranes of chloroplasts in vacuoles. In this study, the possible involvement of vacuolar cysteine proteases in degradation of chloroplast proteins was suggested. Although the debate about the degradation of proteins in vacuoles and the uptake of chloroplasts by vacuoles under natural leaf senescence continues, it seems that the degradation of whole chloroplasts inside vacuoles is a key step for chloroplast protein degradation. Apparently, the question arises of how chloroplasts are transferred into vacuoles. Wada et al. (2009) have provided a novel finding related to this question: Their observations showed that chloroplasts were found within some vacuoles that were isolated from individually darkened leaves of the wild type, but no chloroplasts were found in the vacuole of an *atg* mutant. Furthermore, the decrease in the chloroplast population was inhibited in *atg* mutant, whereas the loss of chlorophylls and the decrease of soluble proteins in the mutant were comparable to those in the wild-type. These results demonstrate that autophagy also plays a key role in the transport of whole chloroplasts into vacuoles during leaf senescence.

B. Degradation of Thylakoid Membrane Proteins

Thylakoid membranes contain multiple protein complexes (e.g., Photosystem I, Photosystem II, the cytochrome b_6f complex and the light-harvesting complex), which play an important role in light-harvesting and the light-dependent reactions of photosynthesis (see Joshi et al., Chap. 28 in this book). More than 30% of the chloroplast proteins are in the protein complexes of the thylakoid membranes. Therefore, the proteins in these complexes are also considered important as nutrient sources during leaf senescence (Krupinska 2007). During leaf senescence, the structure of thylakoid membranes changes dramatically. The progressive loss of grana stacking, disappearance of thylakoid membranes and massive accumulation of plastoglobuli occur at this stage (Krupinska 2007; see also Mulisch and Krupinska, Chap. 14). These structural changes are caused by the massive degradation of the protein complexes and the lipids in the thylakoid membranes. In this section, we describe the current knowledge of the degradation mechanisms of proteins in the thylakoid membranes and the participation of proteases during leaf senescence.

1. Degradation Mechanisms of Thylakoid Proteins

Of the chloroplast-protein degradation pathways described above, RCBs do not exhibit chlorophyll autofluorescence and SAVs do not contain thylakoid proteins, whereas chlorophyll *a* was detected in isolated SAVs (Ishida et al. 2008; Martinez et al. 2008a; see also Costa et al., Chap. 18). Consequently, the degradation of proteins inside chloroplasts and the uptake of chloroplasts by vacuoles are expected to become more important in the degradation of thylakoid membrane proteins. Of thylakoid membrane protein complexes, most thylakoid membrane proteins appear to bind chlorophylls and carotenoids. Accumulating evidence points to fine-tuned regulation between the breakdown of chlorophylls and degradation of apoproteins during leaf senescence (Hörtensteiner, Chap. 16). Loss of chlorophyll *b* reductase in Arabidopsis (NYC1 and NOL), which catalyzes the first step of chlorophyll *b* degradation, results in a non-functional *stay-green* phenotype that impairs chlorophyll catabolism but shows other senescence processes (Kusaba et al. 2007; Sato et al. 2009). In addition,

pheophorbide *a* oxygenase (PAO) and pheophytin pheophorbide hydrolase (PPH) deficiencies result in a non-functional stay-green phenotype (Pruzinska et al. 2003; Schelbert et al. 2009). These stay-green mutants, which retain chlorophylls in senescent leaves, show highly stable LHC proteins, suggesting the requirement of chlorophyll degradation for the full degradation of thylakoid membrane proteins, especially LHC proteins. These findings indicate a close relation between chlorophylls and apoproteins and suggest that the degradation of thylakoid membrane proteins is strictly controlled. Because free chlorophyll is potentially phototoxic, the degradation of chlorophylls during the degradation of thylakoid membrane proteins needs to be tightly controlled. In spite of rapid progress made towards the understanding of chlorophyll degradation (see Hörtensteiner, Chap. 16 in this book), elucidation of the degradation mechanism of thylakoid membrane proteins is lagging.

2. Proteases Involved in Degradation of Membrane Proteins

The best characterized protein-degradation mechanism in thylakoid membranes is the degradation of the PSII reaction center D1 protein in the PSII repair cycle. In higher plants, as described above, much evidence indicates that the thylakoid membrane-bound FtsH and Deg proteases play crucial roles in this process (Kato and Sakamoto 2009). Meanwhile, a study of the Clp protease suggested the cytochrome b_6f complex to be a putative substrate for Clp during nitrogen starvation in the green alga *Chlamydomonas reinhardtii* (Majeran et al. 2000). Lon protease, an ATP-dependent serine protease, and SppA protease, an ATP-independent serine protease, are closely associated with the stromal side of thylakoid membranes (Lensch et al. 2001; Ostersetzer et al. 2007). These proteases also seem to play roles in proteolysis of the thylakoid membrane (Wetzel et al. 2009), although their substrates are poorly understood. These reports specifically address protease functions in the mechanism of protein homeostasis, but do not explain the role of the protease during leaf senescence. We hope that further studies will reveal the role of the major chloroplast proteases in the degradation of thylakoid membrane proteins in leaf senescence.

IV. Concluding Remarks

Over the past two decades, much effort has been devoted to identify and characterize chloroplast proteases. Consequently, numerous chloroplast proteases that are homologous with known bacterial proteases have been identified. The analyses of Arabidopsis mutants has revealed, as described in this chapter, that some are necessary not only for chloroplast biogenesis but also for plant viability. However, the specific substrates of these proteases during leaf development remain unclear. Exploring the recognition mechanisms of substrates in these proteases constitutes an important area of future studies in this field. Additionally, communications between proteolytic activities and chloroplast development, like the delayed chloroplast development that is observed in the variegated mutants, is expected to become an interesting area of investigation. Our knowledge about chloroplast protein degradation during leaf senescence remains poor despite recent efforts in unravelling the mechanisms of protein degradation. The difficulty in elucidation of the mechanism of protein degradation during leaf senescence is apparently due to the complexity of leaf senescence that results from the involvement of multiple factors (e.g., light, nutrition, and the developmental stage of the plant). The contribution of chloroplast proteases in protein degradation during leaf senescence is an important question that should be examined critically in future studies. We hope that greater efforts in this field will answer these questions.

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Part V

Organellar Control of Development

Chapter 21

Cross-Talk of Mitochondria and Chloroplasts

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Summary

Mitochondria represent a key organelle in plant cells being involved in many aspects of the plant life: normal cell metabolism, stress response and programmed cell death regulation. In the last 40 years there have been many contributions to understanding those aspects of mitochondrial function in plants, but the availability of genome sequencing data and the development of GFP-based technologies have provided enormous improvements to these studies. Besides the specific molecular composition of the electron transport chain and the pattern of enzymatic pathways that distinguish plant from animal mitochondria, the presence of chloroplasts, with which they interact, contributes to the uniqueness of plant mitochondria and their evolution in the plant eukaryotic cell. Chloroplasts and mitochondria are traditionally considered to be autonomous organelles but they are not as independent as they were once thought to be. Here we will focus on the evidence that contributes to define the metabolic, functional and physical inter-connections between mitochondria and chloroplasts.

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I. Introduction

Mitochondria occupy a central place in the metabolic network of eukaryotic cells, with essential metabolic processes occurring within the organelle itself and several other pathways either emanating from or converging on mitochondria. The convergence of many metabolic pathways upon mitochondria reflects the evolutionary origins of these organelles. Derived from an engulfed α-proteobacterium, mitochondria brought to the ancestral eukaryotic cell a new arsenal of metabolic capabilities (Martin and Russell 2003). The transfer of the major part of the original endosymbiont genome to the nucleus has resulted in an integration of these metabolic pathways. The majority of the proteins encoded by mitochondria-derived genes are targeted back to the organelle via a dedicated import pathway (Chew and Whelan 2004). However, in many cases, duplication of enzymes with those derived from plastid genes or host genes (e.g., glycolysis), together with a lack of selective pressure of one form over another, has led to a spatial fragmentation of pathways in the cell (Liaud et al. 2000).

One of the key components of plant cell metabolism is the TCA cycle that, in addition to its catabolic role during respiration, provides precursors for a number of essential extra-mitochondrial biosynthetic processes, such as nitrogen fixation and biosynthesis of amino acids, tetrapyrroles and vitamin cofactors (Douce and Neuburger 1989; MacKenzie and McIntosh 1999; Sweetlove et al. 2007). Although, a great deal of the amino acid metabolism is localized within the plastids, mitochondria play a key role in the synthesis and degradation of these compounds (Regierer et al. 2002). Amino acid biosynthesis with oxaloacetate being used for aspartate synthesis and 2-oxoglutarate being used for nitrogen assimilation are the best established metabolic pathways (Lancien et al. 2000). The logic in such a spatial organization is fairly transparent, given that the TCA cycle exhibits an anapleurotic function and that plants use proteins and lipids (through degradation and respiration) in periods of carbohydrate deficiency (Buchanan-Wollaston et al. 2005; Sweetlove et al. 2007).

Another metabolic pathway in which mitochondria are involved is that of tetrapyrrole biosynthesis. It is highly important in plants since it provides essential molecules involved in light harvesting, energy transfer, signal transduction, detoxification and systemic acquired resistance (Grimm 1998; Molina et al. 1999). The most abundant plant tetrapyrroles are chlorophyll and heme, which are essential in the processes of photosynthesis and respiration, respectively. It has been found that even if enzymes catalysing steps up to protoporphyrin IX biosynthesis are plastid-targeted (Kruse et al. 1995), enzymes catalyzing the final steps of heme biosynthesis (ferrochelatase and protoporphyrinogen oxidase) are present in duplicate and are likely targeted to different organelles (Lermontova et al. 1997; Watanabe et al. 2001).

II. Characteristic Features of the Respiratory Chain of Plant Mitochondria

The respiratory chain of plant mitochondria is different in many aspects from the animal counterpart (Fig. 21.1; for review, see Millar et al. 2011). In plant mitochondria, in addition to Complex I, that utilizes matrix NADH as

Abbreviations: ΔΨ – Electrochemical gradient; $[Ca^{2+}]_{cyt}$ – Cytosolic calcium concentration; $[Ca^{2+}]_{mt}$ – Mitochondrial calcium concentration; ABI4 – Abscisic acid insensitive4; ANT – Adenine nucleotide transporter; AOX – Alternative oxidase; CsA – Cyclosporin A; Cyt c – Cytochrome c; Cyt f – Cytochrome f; dnOPDA – dinor OPDA; FIS1 – Fission1; *flu* – *Arabidopsis fluorescent mutant*; GFP – Green fluorescent protein; IDL – Individually darkened leaves; IMS – Mitochondrial intermembrane space; Ja – Jasmonate; MeJa – Methyl jasmonate; mtETC – Mitochondrial electron transport chain; $^1O_2$ – Singlet oxygen; OMM – Outer mitochondrial membrane; OPDA – Cyclopentenone 12-oxo-phytodienoic acid; PCD – Programmed cell death; PTP – Permeability transition pore; ROS – Reactive oxygen species; SA – salicylic acid; TCA cycle – Tricarboxylic acid cycle; UQ – Ubiquinone; VDAC – Voltage-dependent anion channel

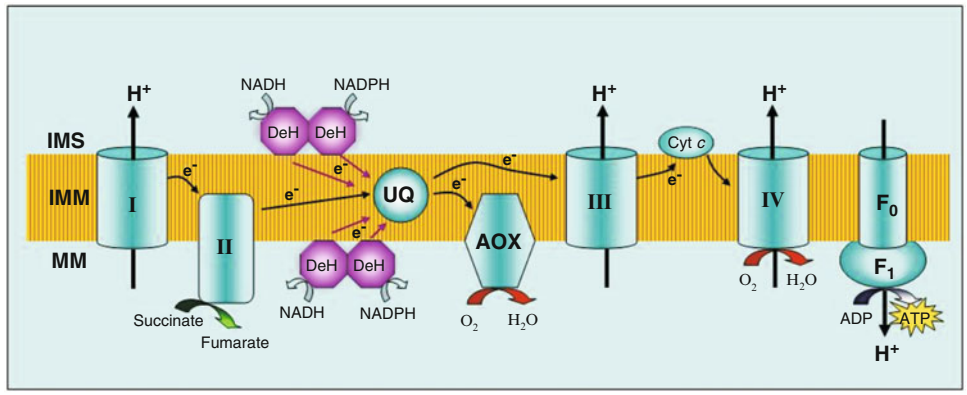

Fig. 21.1. Characteristic features of plant mitochondrial respiratory chain. The scheme shows the whole respiratory chain in animal and plant mitochondria. Apart from the ordinary electron respiratory transporters (complexes I to IV, UQ and Cyt c) and the ATP synthase (F_o and F_1 complex), common to plant and animal mitochondria, plants have accessory complexes. They are comprised of two external and two internal rotenone-insensitive DeHs that transfer electrons from NADH and NADPH to UQ. The plant respiratory chain also contains AOX that uses electrons derived from UQ to reduce oxygen, in a cyanide-insensitive mode. *IMS* intermembrane space, *IMM* inner mitochondrial membrane, *MM* mitochondrial matrix, *I, II, III, IV* respiratory chain complexes, *UQ* ubiquinone, *Cyt c* cytochrome c, *DeH* dehydrogenase, *AOX* alternative oxidase.

a substrate, other NAD(P)H dehydrogenases, insensitive to rotenone (the classical Complex I inhibitor), are present and that allow oxidation of both the matrix and cytosolic NAD(P)H (Rasmusson et al. 2008). These enzymes, called type II NAD(P)H dehydrogenases, are localized on the inner and outer surface of the inner mitochondrial membrane and transport electrons without pumping protons. Thus, they will not directly contribute to respiratory ATP production, nor be directly controlled by cellular adenylate status (Rasmusson et al. 2008).

Plant mitochondria, moreover possess cytochrome oxidase (Complex IV), an additional terminal respiratory oxidase called alternative oxidase (AOX) whose pathway branches from the UQ pool and consists of a single enzyme. AOX catalyzes the oxidation of ubiquinol and reduction of O_2 to H_2O (Vanlerberghe and McIntosh 1997). Differently from Complex I, III and IV, whose proton pumping activity results in formation of a proton gradient across the inner mitochondrial membrane used by ATP synthase to make ATP, AOX does not have a proton pumping activity. AOX was first identified in the Arum lily spadex where its activity results in a great increase in non-phosphorylating proton transport, thereby generating the heat required for releasing the volatiles and attracting pollinators (Raskin et al. 1989). In non-thermogenic tissues, AOX has been proposed to act as an energy-overflow that enables the TCA cycle to continue to function and provide intermediates for biosynthesis even under conditions in which the cytochrome pathway has become limiting (Mackenzie and McIntosh 1999). Cytochrome oxidase and AOX compete for electrons in plant mitochondria, and the inhibition of one pathway redirects flow to the other (Hoefnagel et al. 1995; Zottini et al. 2002), thus allowing the electrons to flow through the respiratory chain and preventing the mtETC components from becoming overly reduced, which can result in production of ROS (Møller 2001). The two pathways can be differentiated by inhibitors such as cyanide, antimycin A, myxothiazol or carbon monoxide (acting on the cytochrome pathway) and n-propyl gallate (nPG) or salicylhydroxamic acid (acting on AOX). Due to its structural characteristics, plant mitochondria can theoretically respire in a way that is not energy conserving and, therefore, beyond the

control of the proton-motive force. Because of this, one proposed function of the alternative pathway is to allow continued carbon metabolism in mitochondria when the ATP/ADP ratio is high. Furthermore, AOX acts also as an efficient ROS scavenging system in plant cells (Maxwell et al. 1999).

III. Mitochondria and Photosynthesis

A. Metabolic Interactions

The presence of additional respiratory chain components that are not seen in animal mitochondria, as well as a number of plant-specific metabolite exchanges between mitochondria and the cytosol, reflect the unique demands placed on plant mitochondria (Douce and Neuburger 1989; Picault et al. 2002). The cellular environment of plant mitochondria is also distinctive because of the presence of photosynthesis, which creates an environment rich in oxygen and carbohydrates. In this peculiar surrounding, plant-specific mitochondrial features appear of great relevance (De Gara et al. 2010). It has long been recognized that photosynthesis and respiration in plant cells must be intimately linked (for a review, see Nunes-Nesi et al. 2010). While the core reaction schemes of the pathways of photosynthesis and respiration – the major pathways of energy production which are confined to the plastid and mitochondria, respectively – are well defined and their coordinated regulation has been presumed for decades, it is only since the advent and widespread adoption of reverse genetic strategies that the high level of interaction between them has begun to be fully realized (Bauwe et al. 2010; Sweetlove et al. 2010).

Since the pathways of photosynthesis and respiration catalyze almost opposite reactions, and they share carbon dioxide and oxygen as substrate and/or product, it follows that the exact contribution of each pathway to energy status is dependent on the cell type and that their relative activities must be carefully regulated within the plant cell. In the illuminated leaf, photosynthesis directly provides substrate for mitochondrial reactions. In heterotrophic tissues and the darkened leaf, substrates from photosynthesis are provided indirectly via storage pools. While these facts have long been established, the converse impact of mitochondria on photosynthesis has only been demonstrated recently.

In some plant tissues, such as roots, the complete reliance on mitochondrial oxidative phosphorylation to meet the energy demands of the cell greatly simplifies matters. However, recent demonstrations of photosynthesis occurring in germinating seeds and the fact that some enzymes of the photorespiratory pathway are expressed in a root-specific fashion (Borisjuk and Rolletschek 2009) means that such generalizations should not be assumed.

Interactions between chloroplasts and mitochondria depend on exchange of metabolites such as ATP (energy), NAD(P)H (reducing equivalents) and carbon skeletons. The degree to which, in the light, mitochondrial ATP supply is required for optimal photosynthesis depends on the balance of ATP production and consumption in chloroplasts which, on the other hand, depends on the contribution from cyclic and pseudo-cyclic phosphorylation occurring in the chloroplasts (Hoefnagel et al. 1998). Energy metabolism is tailored to the specific demands of the cell and its environmental circumstances. There are over 40 different cell types in a typical plant, and the energy requirements of these differ substantially, as does the reliance on the different pathways of energy metabolism (Nunes-Nesi et al. 2010). If the chloroplast is unable to meet its ATP requirements, additional ATP must be imported from other compartments of the cell. The most likely source of additional ATP is mitochondrial phosphorylation. Mitochondria have a greater capacity for ATP synthesis than chloroplasts, producing up to 3 ATP per NAD(P)H compared to the 1.5/2 ATP per NAD(P)H in the chloroplast (Hoefnagel et al. 1998).

Another important role played by mitochondria in the light is their capability to remove excess photosynthetic reducing

equivalents, which could lead to over-reduction of chloroplast electron transport components and consequently damage the thylakoid membranes (Raghavendra and Padmasree 2003). It is therefore essential that chloroplasts export excess reducing equivalents to be either stored (e.g., as malate) or to be oxidised by respiration. The regulation of metabolite distribution as a means to balance cellular redox status is a long-established mechanism of metabolic and photosynthetic control and is based on the operation of the so-called "malate valve", which effectively operates as an indirect export system for reducing equivalents (Padmasree et al. 2002).

Another redox-related mechanism linking mitochondrial functionality to that of the plastids is that mediated by ascorbate. The terminal enzyme of ascorbate biosynthesis, L-galactono-1,4-lactone dehydrogenase, is in fact coupled to the cytochrome pathway (Bartoli et al. 2000) and can be engaged as an alternative electron donor to the mitochondrial electron transport chain (Nunes-Nesi et al. 2005; Nunes-Nesi et al. 2008; Dinakar et al. 2010).

B. Retrograde Signaling Pathways in Mitochondrion-Chloroplast Cross-Talk

The coordination of metabolic activity that involves different cell compartments, such as chloroplasts, mitochondria and nucleus, is tightly controlled by both anterograde and retrograde regulatory pathways (Pesaresi et al. 2007). Anterograde regulation is essentially a top-down regulation pathway from the nucleus to the organelles. Retrograde regulation describes the ability of organelles to modulate, through signaling molecules, nuclear gene expression, the products of which may or may not be located within the same organelle (Leister 2005). Anterograde regulation can take place at several levels, from transcriptional to posttranslational events, and the signals that control gene expression for this type of signaling can be developmental, hormonal, or environmental (Pesaresi et al. 2007). In contrast, retrograde regulation occurs in response to changing conditions and allows the cells to modulate anterograde regulatory pathways to affect the functioning of organelles (Rhoads and Subbaiah 2007).

The coordinated activity of chloroplast, mitochondrial, and nuclear compartments strictly controls photosynthesis (Raghavendra and Padmasree 2003). This, in turn, implies the existence of signaling pathways that serve to integrate nuclear and organellar gene expression (Rodermel and Park 2003). The preeminence of the nucleus in this interorganellar exchange is indisputable: nuclear proteins imported into plastids and mitochondria regulate the transcription and translation of organellar genes. Nevertheless, it is now well established that perturbation of a number of plastid processes, including tetrapyrrole biosynthesis, protein synthesis, and photosynthesis, influence the expression of nuclear genes encoding photosynthetic proteins (Leister 2005; Woodson and Chory 2008; Pfannschmidt and Munné-Bosch, Chap. 22). It has actually been demonstrated that alterations in the translation rate within plastids and mitochondria induce a marked and general down-regulation of nuclear photosynthesis genes implying synergistic roles for the two organelles in gene regulation. A mechanism that reduces the absorption capacity for light energy would serve to prevent the accumulation of ROS when the capacity for energy transformation is limited (Pesaresi et al. 2006). How these signals are transduced from the organelles to the nucleus and integrated at the molecular level remains, however, unknown. The identification of molecular links between mitochondrial and chloroplastic retrograde signaling pathways would provide a platform to understand how perturbation of photosynthesis or respiratory activity can affect each other.

The best-studied retrograde signal(s) in plant mitochondria involve increased expression of the nuclear-encoded AOX as an adaptive response to recover from mtETC inhibition. There might be several signals for AOX induction as different mutants for this protein do not all respond equally to different

mechanisms of mtETC inhibition (Zarkovic et al. 2005) but these separate signals appear to lead to different elements within the same 93-bp promoter region of AOX (Dojcinovic et al. 2005).

A great improvement in understanding this regulation mechanism comes from studies conducted on ABI4, a nuclear-localized APETALA2-type transcription factor, that acts as a negative regulator of the *AOX1a* nuclear gene (coding for the mitochondrial AOX), by binding to a specific region of the promoter (Dojcinovic et al. 2005; Giraud et al. 2009). ABI4 has also been shown to be involved in the inhibition of the light-induced expression of photosynthesis related genes, including *Lhcb* (Koussevitzky et al. 2007). ABI4 plays also a role in the regulation of genes that are responsive to retrograde signals from both the mitochondrion and the chloroplast. Thus, changes in chloroplast function when AOX1a is inactivated and conversely activation of AOX1a when chloroplast function is altered may in part be explained by perturbations in interacting regulatory pathways, rather than in terms of biochemical mechanisms alone (Giraud et al. 2009). ABI4 is also intimately involved in sugar and chloroplast retrograde signaling and thus integrates mitochondrial retrograde signaling and respiratory chain regulation into other cellular anterograde and retrograde regulatory pathways (Woodson and Chory 2008). A connection between mitochondrial and chloroplast retrograde signaling is a strong indication that signals from both organelles interact.

Additional strong evidence that mitochondrial performance influences the activities of plastids and nucleus was obtained in investigations on mutant plants that, altered at the level of mitochondria (see below), show a strongly impaired chloroplast development (in maize *ncs*; Newton and Coe 1986) or photosynthesis efficiency (in tobacco *cms*; Noctor et al. 2004).

In *ncs* plants, mitochondrial mutations give rise to sectoring in leaves that appear variegated green and light yellow and, for this reason, called *n*on *c*hromosomal *s*tripe (*ncs*) mutants. In the yellowish sectors, chloroplast development is abnormal because of still not-defined mitochondrial mutations (Newton et al. 1990; Gu et al. 1993). Leaves are variegated because they contain mitochondria with all or mostly mutated mitochondrial DNA in the light yellow sectors and normal mitochondria in the green sectors (Newton and Coe 1986). Different leaf sectors can develop from single cells, but not all cells contain the mutation or the plants would likely not survive. This is an interesting example of how mitochondria can deeply influence chloroplast development; however, the reason and the mechanism involved is still under investigation. In the *c*ytoplasmic *m*ale *s*terility (*cms*) tobacco mutant, a deletion in the mitochondrial gene *nad7* lead to the absence of functional Complex I, the major NADH sink in the plant cell (Gutierres et al. 1997). This mutation leads to a re-adjustment of whole-cell redox homeostasis and gene expression, affecting leaf metabolism (Noctor et al. 2004).

The expression regulation of the nuclear gene *Lhcb* is another example of interorganellar communication in retrograde signaling. *Lhcb* is repressed when the plastids are dysfunctional after photo-oxidation (Mayfield and Taylor 1984). In a screening for mutants with genetic defects disrupting the down-regulation of the *Lhcb* in photo-oxidizing conditions, various *g*enomes *u*ncoupled (*gun*) mutants were identified that displayed *Lhcb* gene expression despite their impaired plastids (Susek et al. 1993). *Lhcb* expression is de-repressed in these mutants. These mutants demonstrated for the first time that plastidial signals can be disrupted genetically, supporting the idea of a true signaling pathway. However, despite many years of research, it is still not understood how retrograde signaling is disturbed in these mutants. Among different hypothesis, one possibility involves the action of mitochondria (Pfannschmidt 2010). During the first steps of seedling development, the plant depends solely on storage compounds, and mitochondria are essential in providing the necessary ATP during this heterotrophic growth phase. At later stages of plant growth, this dependency still applies to the developing

meristematic tissues that represent a metabolic sink. With the light-induced initiation of chloroplast development, the seedling or young tissues changes to an autotrophic lifestyle; however, this requires the establishment of metabolic interactions between plastids and mitochondria, which is disturbed if plastidial development is prevented. The shift from heterotrophic to autotrophic metabolism might create a mitochondrial signal that is sensed by the nucleus and indirectly communicates the developmental stage of the plastids. *Gun* mutations might interfere with mitochondrial processes during the early heterotrophic stage, which usually results in a shift of the nuclear response towards autotrophic growth, including a derepression of *Lhcb* expression (Pfanschmidt 2010).

1. Signaling Molecules in Inter-organelle Interactions

Chloroplasts function as environmental sensors which, together with mitochondria, control stress responses and environmental acclimation in a manner largely independent of photoreceptors. Despite the pronounced metabolic interdependence of plastids and mitochondria and the ability of the latter to influence photosynthesis and chloroplast development, little is known about the signals that mediate communications between mitochondria and plastids or about the coordination of the activities of the two organelles by signals other than carbon metabolites.

Many factors have been suggested to be involved in the retrograde signaling control. These include: ROS (both plastid and mitochondria), cellular carbohydrate status (Yoshida and Noguchi 2009), release of mitochondrial Ca^{2+} (Subbaiah et al. 1998), and the reduction state of plastoquinone and UQ (Yoshida et al. 2010).

There is ample evidence that upon a stress stimulus, the cellular redox status is compromised and mitochondria act as a target/sensor generating and/or amplifying the molecular response both within the organelle and also across the cell. ROS are generated in the mitochondrion as a consequence of over-reduction of the respiratory complexes in the mitochondrial electron transport chain causing leakage of single electrons to molecular oxygen and superoxide formation (Møller 2001).

Although ROS accumulation is buffered by the presence of a dedicated antioxidant enzyme network, this system can quickly become saturated and ROS will be free to react with other mitochondrial molecular components. The resulting oxidative changes effectively provide the mitochondrion with information about the redox poise of the respiratory chain and are used to initiate molecular responses, such as uncoupling mitochondrial proteins, induction of alternative pathways and modulation of respiratory bioenergetics (Noctor et al. 2007).

Mitochondria play a key role in redox signaling in photosynthetic cells even though chloroplasts and the peroxisomes generate far greater quantities of ROS. This incongruity is explained by the fact that it is not just the rate of production of ROS that is important, but rather the balance between the rate of ROS production and the antioxidant capacity (Foyer and Noctor 2003). In this context, the levels of ROS that are free for signaling in the chloroplast and peroxisome may in fact be lower than in mitochondria because of the greater antioxidant capacity in the former two organelles.

Ca^{2+} is a general second messenger involved in many physiological and stress responses of plants. Cytoplasmic Ca^{2+} levels can increase due to the opening of plasma membrane Ca^{2+}-channels or by release from cellular stores. Knowledge about the role of mitochondria in the regulation of Ca^{2+} homeostasis in the plant cell is still fragmentary. A well-described phenomenon in which Ca^{2+} is involved in mitochondria-to-nucleus retrograde signaling is the plant response to hypoxia (Subbaiah et al. 1998). It has been described that mitochondria play a key role in the hypoxia-associated signaling inducing anoxic gene expression changes. Cold, osmotic stress, and touch also result in increased $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mt}$ in Arabidopsis leaf cells (Logan and Knight 2003), indicating

that Ca^{2+} level changes in various cellular compartments which may be stress-specific. Further, ruthenium red, an inhibitor of Ca^{2+} flux from mitochondria, and lanthanum chloride, an inhibitor of plasma membrane Ca^{2+} fluxes, both partially inhibit the induction of AOX capacity in tobacco cells due to mtETC inhibition (Vanlerberghe et al. 2002). These data strongly suggest that Ca^{2+} fluxes into and out of mitochondria could be a key component of the cytosolic "Ca^{2+} signature" generated during stress responses and this can be considered part of the mitochondrial retrograde response to stress in plants.

IV. Programmed Cell Death Regulation by Organelle Interactions

A. Mitochondria

Programmed cell death (PCD) is an essential cellular component of several developmental processes defined by a sequence of events that lead to the controlled and organized destruction of the cell (Lockshin and Zakeri 2002). It can occur in response to environmental stressors and is often a part of the defence mechanism against pathogen attack via the hypersensitive response activation to restrict the spread of pathogens (Dangl and Jones 2001).

Mitochondria-regulated PCD is a shared feature of all eukaryotes and has already been shown to be a feature of all metazoan, yeast and higher plant cell-death programmes (Vianello et al. 2007). Upon a death stimulus, cells can undergo PCD, resulting in a distinctive sequence of events. An early event in the PCD process is the release from the IMS into the cytoplasm of Cyt c that, in animal systems, triggers PCD through a caspase-dependent process that ultimately leads to cell dismantling (van Gurp et al. 2003). Although Cyt c release from mitochondria has been frequently observed in plants (Balk et al. 1999; Balk and Leaver 2001; Zottini et al. 2002), plant homologs of animal caspases have not been identified, despite reports on the inhibition of PCD by specific inhibitors of caspase activity (Lam and del Pozo 2000; Zuppini et al. 2009). It has been suggested that more distantly related metacaspases or other proteases may play a central role in plant PCD (Woltering 2004).

Alternatively, a caspase-independent pathway may involve Cyt c to amplify the death process. Cyt c is a component of the electron transport chain and one effect of its release is the disruption of the electron transport chain that results in generation of ROS. ROS, on the other hand, cause a feedback loop that amplifies the original PCD-inducing stress signal. The chloroplast is also a source of ROS and most probably contributes to this feedback loop (Noctor et al. 2007).

Other caspase-independent pathways have been suggested to exist, involving release of *a*poptotic *i*nducing *f*actors (AIF) into the cytosol, during the early events of PCD. AIF are flavoproteins that show similarities with bacterial, plant and fungal oxidoreductases and, like Cyt c, they are normally found in the mitochondrial IMS but can translocate to the nucleus upon induction of PCD. Balk et al. (2003) employing a cell-free system showed that, the mitochondrial IMS fraction could induce chromatin condensation and fragmentation of DNA into 30 kb fragments, consistent with an AIF-like apoptotic activity. Five homologs of AIF were identified in Arabidopsis as monodehydro-ascorbate reductases (MDAR). However, a purified recombinant MDAR (At1g63940; Ball et al. 2004), did not show any nuclease activity. It is instead involved in the ascorbate–glutathione cycle, which is one of the major antioxidant systems involved in protection against ROS damage and therefore crucial for plant defence against oxidative stress. Of special interest in the context of plant PCD regulation is the finding that MDAR is dually targeted to chloroplasts and mitochondria, sustaining a role of the chloroplast in plant PCD (Reape and McCabe 2010). On the other hand, it has been reported that Ca^{2+}/Mg^{2+} nuclease activity, localized to the nucleus of dying nucellar cells, is capable of triggering DNA fragmentation in both plant and human nuclei, demonstrating that similar features of nucleus degradation could be shared between plant and animal cells (Dominguez and Cejudo 2006).

Following induction of PCD, early decreases of the inner mitochondrial $\Delta\Psi$, preceding cell shrinkage and DNA degradation, have been detected in plants as in animals. This has been reported in Arabidopsis protoplasts treated with MeJa or ultraviolet-C overexposure (Gao et al. 2008; Zhang and Xing 2008), in Arabidopsis suspension cultures treated with harpin and acetylsalicylic acid (Krause and Durner 2004), and in carrot suspension cultures treated with nitric oxide donors (Zottini et al. 2002). Loss of the inner mitochondrial membrane potential, osmotic swelling of mitochondria, disruption of the OMM and subsequent release of IMS proteins are associated, in animal systems, with the mitochondrial membrane permeabilization (Kroemer et al. 2007) through the activation of the PTP. PTP is a polyprotein complex formed at the contact sites between the inner and outer mitochondrial membrane and thought to act through the interaction between VDAC on the outer membrane, ANT from the inner membrane, and cyclophilin D in the matrix (Rasola and Bernardi 2007). The PTP can be formed following cellular stress such as build up of Ca^{2+}, changes in phosphate and/or ATP levels or ROS production. However, it can be inhibited by CsA, which acts by displacing the binding of cyclophilin D to ANT (Crompton 1999). CsA has been used in plant PCD models to provide pharmacological evidence for the existence of the PTP. CsA has been shown to inhibit Ca^{2+} induced swelling of potato mitochondria (Arpagaus et al. 2002), betulinic acid induced PCD in tracheary element cells of *Zinnia elegans* (Yu et al. 2002) and nitric oxide induced PCD in *Citrus sinensis* (Saviani et al. 2002). In addition, CsA has been shown to protect against loss of mitochondrial $\Delta\Psi$ and Cyt c release after protoporphyrin IX treatment of Arabidopsis protoplasts (Yao et al. 2004) and to prevent mitochondrial swelling by ROS following MeJa treatment of Arabidopsis protoplasts (Zhang and Xing 2008).

Among the signaling molecules regulating PCD, an important role is played by NO that is produced by mitochondria under stress conditions (Heyno et al. 2008; Gupta and Kaiser 2010) and can play a role in both triggering PCD (De Michele et al. 2009) and activating a protective mechanism (Zottini 2005). NO can act at the mitochondrial level by binding to cytochrome oxidase, the terminal enzyme of the respiratory chain, thus inhibiting respiration (Zottini et al. 2002). This interaction is thought to be responsible for the pro-apoptotic action of NO, since in NO-treated cells various processes are observed, including the release of Cyt c, production of ROS, decrease in ATP concentration, and opening of the PTP. However, NO-induced inhibition of respiration may also play a protective role. In fact, under limiting concentrations of O_2, NO plays a major role in reducing plant cell metabolism in order to decrease the consumption of O_2 and avoid anoxia. It has been suggested that NO may provide a sensing mechanism for O_2, allowing the cell to perceive variations in O_2 tension at O_2 concentrations well above limiting concentrations for mitochondrial electron transport (Zottini 2005).

B. Chloroplasts

The central regulatory role of mitochondria in integrating PCD signals in plant and animal PCD processes is well established, as well as the participation of the endoplasmic reticulum in soybean stress-induced PCD (Zuppini et al. 2004; Costa et al. 2008). It is likely that, in addition to the putative PCD regulators, conserved throughout the animal and plant kingdoms, there are plant-specific mediators of PCD, likely involving chloroplasts. Studies that test chloroplast involvement in plant PCD vary widely in both experimental protocols and ultimate conclusions as to the nature of that involvement.

1. Cytochrome f Release

It has been recently demonstrated that treatment of eggplant cells with palmitoleic acid induced cell death via a release of Cyt f (an essential component of the major redox complex of the thylakoid membrane) from the thylakoids into the cytosol (Peters and

Chin 2005), in a parallel mechanism resembling release of Cyt c from mitochondria. It has been also reported that in response to heat shock (HS) in the psycrophile chlorophyte *Chlorella saccharophila* the chloroplasts undergo deep alterations of the thylakoid membrane structure, accompanied by the release of Cyt f into the cytosol. In addition, it was suggested that Cyt f fulfils its role through modulation of its transcription and translation levels, together with its intracellular localization (Zuppini et al. 2009). The meaning and the mode of action of Cyt f in the PCD pathway, however, remain to be elucidated (Gray 1992): it is not clear if the involvement of Cyt f in the PCD process is related to its loss of function or if the protein by itself is an important regulatory factor in HS-induced PCD.

2. PCD Triggered by Light-Induced ROS Generation

A number of studies have shown that plant PCD is affected by light (Gray et al. 2002; Zeier et al. 2004) and that the hypersensitive response is accelerated by the loss of chloroplast function associated with ROS production (Seo et al. 2000; Mur et al. 2008), thus giving strong evidence that chloroplasts may be involved in the regulation of plant PCD (Mullineaux and Karpinski 2002). Mittler et al. (1997) revealed that changes in chloroplast ultrastructure occurred after hypersensitive response progression, Coffeen and Wolpert (2004) suggested that chloroplast localized proteases could degrade Rubisco as part of the PCD pathway, and Yao and Greenberg (2006) suggested that chlorophyll breakdown products could induce PCD, possibly by contributing to ROS production. In addition, research by Chen and Dickman (2004) on the transgenic expression of animal anti-apoptotic Bcl-2 family members in tobacco have implicated chloroplast involvement in oxidative stress-induced PCD. Bcl-2 family proteins were found to localize to chloroplasts and suppress PCD induction by herbicides that generate oxidative stress, suggesting that chloroplasts are involved in plant PCD regulation.

There is an increasing interest in understanding the role of the chloroplast in plant PCD, since chloroplast modifications and a cross-talk between chloroplasts and mitochondria seem to be involved in the response to various stressors. In a study performed on cultured cells, Doyle et al. (2010) showed that heat shock produced significantly higher levels of PCD in dark-grown compared to light-grown cultures. It has been suggested that, in light-grown cells, high level of chloroplast-produced ROS, during heat shock, may lower PCD levels by switching death towards necrosis. Antioxidant treatment did not protect cells from death but increased levels of PCD in light-grown cultures and decreased necrosis. In addition, light-grown cultures lacking functional chloroplasts due to treatment with norflurazon (a herbicide which results in early arrest of plastid development) also responded to heat treatment with much higher levels of PCD than untreated light-grown cells. These data suggest that the chloroplasts play a key role in PCD regulation.

a. Singlet Oxygen-Signaling and PCD

Since ROS act as signals, their biological specificities should exhibit a high degree of selectivity that could be ascribed to their chemical identities and/or the intracellular sites at which they are produced. One of the difficulties in studying the biological activity of a particular ROS originates from the fact that several chemically distinct ROS are generated simultaneously in cells under stress, therefore making it almost impossible to link a particular stress response to a specific ROS. However, to study the specific activity of singlet oxygen ($^1O_2$), this problem has been overcome by using the conditional *fluorescent* (*flu*) mutant (Meskauskiene et al. 2001) of Arabidopsis, which allows the induction of only $^1O_2$ within plastids in a non-invasive and controlled manner. In wild-type plants, upon illumination, excited porphyrin molecules such as chlorophyll (Chl) may transfer the excitation energy directly to oxygen, thus leading to the formation of highly reactive $^1O_2$ that can have detrimental effects.

Angiosperms use a very efficient strategy to prevent the accumulation of such intermediates by tightly controlling the biosynthetic pathway leading to chlorophyll formation (Meskauskiene et al. 2001). When angiosperms grow under dark conditions, chlorophyll biosynthesis halts at the stage of protochlorophyllide (Pchlide), the immediate precursor of chlorophyllide (Chlide). Once a threshold level of Pchlide has been reached, 5-aminolevulinic acid synthesis is halted. Only after illumination is Pchlide converted to Chlide and the block in 5-aminolevulinic acid synthesis released (Beale and Weinstein 1990). FLU is a nuclear-encoded protein that is tightly associated with plastid membranes. It regulates the Mg^{2+} branch of tetrapyrrole biosynthesis by interacting directly with glutamyl tRNA reductase (Meskauskiene et al. 2001; Meskauskiene and Apel 2002). In contrast to the wild-type, etiolated *flu* seedlings are no longer able to restrict the accumulation of Pchlide, which is a potent photosensitizer generating $^1O_2$ in the light (Meskauskiene et al. 2001; op den Camp et al. 2003). As a result, when these seedlings are transferred from the dark to the light they rapidly accumulate $^1O_2$. Immediately after the release of $^1O_2$ plants stop growing and activate several stress-response pathways also associated with the triggering of a cell death program (Danon et al. 2005). These stress responses do not result from physicochemical damage caused by $^1O_2$, but are attributable to the activation of a genetically determined stress response programme depending on the plastidial proteins Executer1 and Executer2 (Laloi et al. 2006; Lee et al. 2007). However, it has also been shown that $^1O_2$ is necessary, but not sufficient, to trigger the cell death response of the *flu* mutant and also requires a second light-dependent reaction requiring the activation of the UVA/blue light absorbing photoreceptor CRY1 (Danon et al. 2006). $^1O_2$-mediated stress responses at the transcriptional level necessitate a retrograde transduction of signals from the chloroplast to the nucleus that activate distinct sets of genes different from those that are induced by superoxide/hydrogen peroxide (op den Camp et al. 2003), thus suggesting that the biological activity of $^1O_2$ exhibits a high degree of specificity that differs from that of other ROS (Kim et al. 2008).

Among the genes that were up-regulated by $^1O_2$, several were identified that encode proteins involved in the biosynthesis or signaling of SA, Ja, or ethylene (Danon et al. 2005). It has been demonstrated that ethylene, SA, and Ja promote cell death, whereas other oxylipins such as OPDA and dnOPDA seem to suppress this response (Danon et al. 2005).

3. Lipid Signaling in PCD

A model that explains the mechanism of interaction between chloroplasts and mitochondria in PCD regulation can be inferred from the action mechanism of Ja. Ja is a well-known plant hormone that is produced at the chloroplast envelope by the action of LOX2 (Böttcher and Pollmann 2009). Ja and its volatile methyl ester MeJa are key signaling molecules, well-known for their roles during plant development, as well as plant defence, senescence promotion, and stress responses (Reinbothe et al. 2009). Ja and MeJa exert two major effects on gene expression in detached leaves of barley and other species, and in whole plants: first, they induce novel abundant proteins designated Ja-induced proteins (JIPs); second, they repress the synthesis of photosynthetic proteins (Reinbothe et al. 2009). Both nuclear and plastid photosynthetic genes are repressed under the control of Ja. Within the chloroplast, rapid MeJa-induced changes in the expression pattern of *rbcL*, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, are superimposed by later effects on plastid transcription and RNA stabilities. Together, these effects lead to a rapid cessation of ribulose-1,5-bisphosphate carboxylase/oxygenase LSU synthesis and cause a drastic drop of photosynthesis and carbon dioxide fixation rates (Reinbothe et al. 1993).

Upon exposure to stress, MeJa is accumulated and activates PCD and defence mechanisms through the production of ROS (Zhang and Xing 2008). The primary effects of MeJa action are the induction of a series of

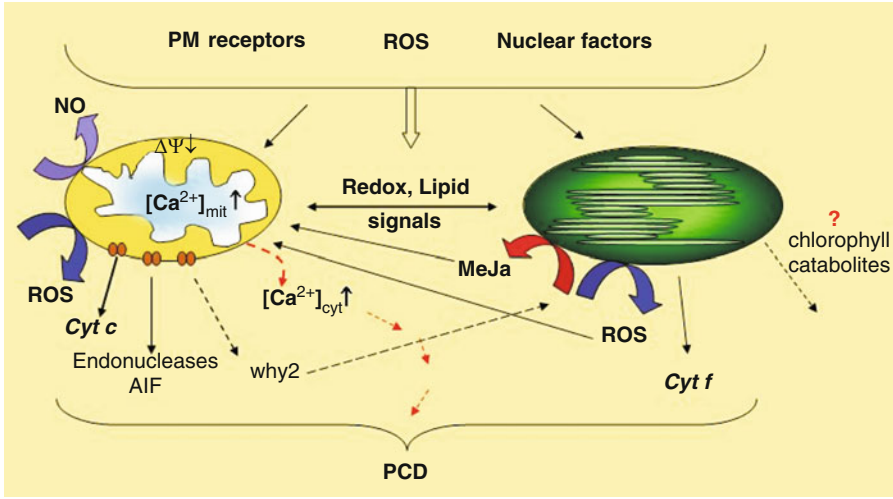

Fig. 21.2. Schematic representation of chloroplast and mitochondria involvement in PCD activation in plant cells. Having perceived a death stimulus from the plasma membrane (PM receptors) or secondary cytosolic (ROS) or nuclear (nuclear factors) signals, plant cells can undergo PCD activating a distinctive response pattern associated with mitochondria and chloroplasts. Cyt c is released rapidly from mitochondria in the early stages of PCD. Cyt c release will disrupt the electron transport chain resulting in generation of ROS, which causes a feedback loop that amplifies the original death signal, inducing PTP opening and further release of Cyt c Mg^{2+}/Ca^{2+}-dependent nuclease which also normally resides in the IMS can be released, causing DNA cleavage. Why2, AIF and NO are also released from mitochondria, but their role in PCD regulation is still not fully understood. The chloroplast is also a source of ROS and most probably contributes to the ROS feedback loop. Chloroplasts contribute to PCD regulation through Cyt f and pigment catabolite release into the cytosol, with mechanisms that are still far from completely understood. There is growing evidence that lipid signaling (MeJa, Ja, etc.) plays important roles in regulating inter-organellar communication and orchestrating PCD processes.

alterations at the mitochondrial level including changes in mitochondrial dynamics, loss of mitochondrial ΔΨ, morphological transition and aberrant distribution within the cell. Thereafter, photochemical efficiency dramatically declines before distortion in chloroplast morphology. Treatment of protoplasts with ascorbic acid or catalase prevents ROS production, organelle change, photosynthetic dysfunction and subsequent cell death. Moreover, the permeability transition pore inhibitor CsA gives significant protection against ΔΨ loss, mitochondrial swelling and subsequent cell death. These results suggest that MeJa induces ROS production and alterations of mitochondrial dynamics, as well as subsequent photosynthetic collapse, which occur upstream of cell death and are necessary components of the cell death process (Zhang and Xing 2008). The picture emerging is the following: besides plant PCD can occur independently of chloroplasts, however, when functional chloroplasts are present, they tend to play a major role in determining the severity and the quantity of cells undergoing PCD, and this effect seems to be driven by chloroplast ROS production that amplifies the process (Fig. 21.2; see also Sabater and Martin, Chap. 23). Therefore, chloroplasts, like mitochondria, not only provide life-giving energy for the cell but also act as sensors for external signals to the cell.

4. Senescence

Some evidence exists indicating chloroplasts as key regulators of senescence/PCD in green tissues. As plants mature, the number of available sinks for photosynthate decreases, consequently increasing overall sugar levels. Elevation in sugars results in reduced photosynthetic capacity and trigger senescence

(Rosenwasser et al. 2011). The extent to which sugar metabolism regulates age-related senescence remains unclear and, in general, the mechanism by which age-related senescence is initiated is largely unknown.

Chloroplasts could regulate the onset of leaf senescence by increasing the reduction state of electron transporters and by generating ROS production and accumulation (Zapata et al. 2005). Subjecting leaves to extended darkness causes premature senescence. For this reason, detached rosettes or leaves, in which postharvest conditions are induced, have been commonly used to investigate senescence processes (van der Graaff et al. 2006).

An important contribution to the comprehension of the role of reciprocal chloroplasts – mitochondria interactions in senescence regulation, comes from studies on dark-induced senescence in Arabidopsis (Keech et al. 2007), where the senescence of leaves from whole darkened Arabidopsis plants was compared to senescence of individually darkened leaves (IDLs) attached to the plant kept in the normal light/dark photoperiod. It has been observed that when the senescence process is activated in IDL, mitochondria increase their respiratory rates to supply the ATP needed for remobilization of nutrients from decaying darkened leaves to new developing organs. This idea is supported by the increase of the ATP/ADP ratio, whereas the decrease in the pool of adenylates most probably reflects the loss of functional chloroplasts. In addition, active respiration could also supply carbon skeletons needed for efficient redistribution of nitrogen. On the other hand, in whole darkened plants, senescence/PCD is not triggered and mitochondria switch to a "standby mode" determining a generally low cellular metabolic rate. This may be a strategy to maintain both a basal cellular functionality and a functional photosynthetic machinery to allow the leaf to resume photosynthesis when light becomes available. The landscape that results is that mitochondria actually regulate, directly or indirectly, the senescence/PCD onset.

Despite substantial evidence that chloroplasts and mitochondria do interact in the PCD regulation process, little is known about the signaling molecules involved. In recent years, Whirly proteins, belonging to a small family of transcription factors, have been studied as putative upstream regulators of cell death and senescence. Due to their peculiar subcellular localization, Whirly proteins are ideal candidates for mediating organelle control of cell death. While Whirly1 is located both in plastids and the nucleus, Whirly2 is a mitochondrial protein and may be also translocated to plastids (Krause et al. 2005). According to the model proposed by Krause and Krupinska (2009) Whirly1 would repress *Whirly2* transcription, regulating the onset of senescence until a pro-senescence signal, inducing a conformational change in Whirly1, de-represses *Whirly2*. The *Why2* gene would then be transcribed, leading to accumulation of Whirly2 in mitochondria where it likely acts as a pro-apoptotic factor, through a still unknown mechanism. It has been suggested that close contact among organelles envelopes could favour protein movement from one compartment to another (Krause and Krupinska 2009), underlining the possible importance of physical interactions among organelles in the facilitation of inter-compartmental trafficking.

V. Do Physical Interactions Exist Between Mitochondria and Chloroplasts?

Due to the metabolic inter-dependence of mitochondria and chloroplasts, the question arises regarding whether these interactions are mediated through physical interactions. As early as the 1960s, it was reported that numerous membrane continuities appeared between chloroplasts and mitochondria in spinach (Wildman et al. 1962), fern and tomato leaves (Crotty and Ledbetter 1973). At that time, when the idea of an endosymbiotic origin of eukaryotic cell organelles was still not a strongly accepted theory, the membrane connections were often cited as evidence of origin of one organelle from the other. However, Crotty and Ledbetter

suggested that those membrane confluences, also reported among other compartments, such as nucleus and endoplasmic reticulum, could be better related to peculiar physiological conditions or developmental situations. Later, experiments by Wellburn and Hampp (1976) indicated that a direct transport of metabolites from mitochondria to plastids occurs during chloroplast morphogenesis, suggesting that those metabolites were transported in a continuity compartment existing between perimitochondrial and perichloroplastic space. Hampp and Schmidt (1976) also demonstrated that the permeability of the inner membrane of both plastids and mitochondria changes during greening.

Studies conducted in characean internodal cells showed that shape, size, number, and distribution of mitochondria and chloroplasts vary according to the growth status and the metabolic activity within the cell. The motile properties of mitochondria probably ensure that these organelles are transported to and retained at regions with high metabolic activity and guarantee close contact with chloroplasts, a prerequisite for inter-organellar exchange of metabolites during photosynthesis (Padmasree et al. 2002). It has actually been demonstrated that mitochondria accumulate at regions with high rates of photosynthesis, whereby the ratio of mitochondria to chloroplasts increases with the rate of photosynthesis (Wang et al. 2003). Mitochondrial metabolism is actually not only essential for the continuous supply of metabolites for photoassimilation, but oxidative electron transport also protects chloroplasts against the ROS produced during photosynthesis (MacKenzie and McIntosh 1999; Padmasree et al. 2002). The differential positioning of mitochondria in the cortex could therefore also be an important strategy against photodamage. The chloroplasts of characean internodes are immobilized in the cortex and are not able to escape detrimental light intensities by relocation (Foissner 2004). In order to compensate for the lack of chloroplast avoidance movements, the members of *Characeae* likely evolved another mechanism and minimize photodamage by accumulating detoxifying mitochondria at photosynthetically active regions. The described motility of cortical mitochondria suggests an important role for the cytoskeleton in optimizing the interaction between chloroplasts and mitochondria.

The analysis and the characterization of the physical interactions between mitochondria and plastids were initially established by means of conventional and phase-contrast microscopy analyses. Those studies mainly focused on highly dynamic membranous structures extending from the surface of plastids, the stromules. These stroma-filled tubules allow the exchange of protein molecules between plastids and often appear to contact the plasma membrane and nuclear envelope, surround other organelles, and sometimes even pass through nuclei (Kwok and Hanson 2004; Gunning 2005; Natesan et al. 2005; J. Gray, Chap. 9).

By the same technical approach, Gunning (2005) reported that mitochondria, as plastids, can sometimes be seen to form transient tubular extensions and that mitochondrial equivalents of stromules interacted with the streaming cytoplasm to develop a stromule-like protrusion, then retracted and started to produce another along a different stream. As seen in the case of plastids, this mitochondrial equivalent of a stromule emerged along a pathway of cytoplasmic streaming that was revealed by the direction of movement of particulate material.

The introduction of green fluorescent protein (GFP) and its detection by fluorescence microscopy has revolutionized the detection and visualization of subcellular structures in plant cells. GFP is in fact an excellent in vivo marker for gene expression and protein localization studies and is particularly useful for real-time spatio-temporal analyses of organelles and membrane structures *in planta*.

The application of this advanced technology allows the recognition of a complex organization of mitochondria in a dynamic, continuous network of discrete organelles, whose number, size and shape are determined by a dynamic equilibrium between rates of fission and fusion events (Logan 2010).

Frequent inter-mitochondrial fusion, enabling the mixing and recombination of mitochondrial DNA, ensures that the plant mitochondria functions, at least genetically, as a discontinuous whole.

Mitochondria undergo continuous cycles of fission and fusion processes and these concerted activities control the maintenance of mitochondrial number during cell division, and the mitochondrial distribution and morphology within living plant cells (Logan 2006) and during plant development (Zottini et al. 2006). As a result of these fission-fusion events, mitochondrial shape and size are continually changing. A great heterogeneity in mitochondria dynamics and morphology has been reported in different cell types of the same organism, and in different organisms (Logan and Leaver 2000). On the other hand, extensive heterogeneity has been observed in the mitochondrial population with different protein composition and functionality in the same tissue upon stress exposure (Subbaiah et al. 1998) or during development, thus suggesting a well-defined division of labour among different mitochondrial sub-populations. The existence of a pattern of structure-function-coordinated gross heterogeneity among mitochondria was demonstrated by Dai et al. (1998) who, using density gradient fractionation, identified four mitochondrial populations in rapidly developing mung-bean seedlings: regular-sized organelles, poorly-respiring morphologically distinct mitochondria, and two populations of small, non-respiring low-density organelles. Studies by Thompson et al. (1998) on the biogenesis of mitochondria during barley leaf development led to the identification of significant heterogeneity in the biogenesis of major mitochondrial protein complexes, suggesting that the protein composition of mitochondria changes significantly during leaf development. The authors were able to identify three groups of mitochondrial proteins according to their developmental profile (Thompson et al. 1998).

Many papers, focused on the molecular mechanisms of fission-fusion processes, report the identification of components of the mitochondrial division apparatus in higher plants. In Arabidopsis two dynamin-related proteins (DRPs) have been described as homologs of proteins involved in mitochondrial division in mammals (Drp1) and yeast (Dnm1p). Disruption of one or the other of two Arabidopsis dynamin-like genes, DRP3A or DRP3B, results in an aberrant mitochondrial morphology that is characterized by an increase in the size of individual mitochondria and a correlative decrease in the number of mitochondrial per cell. The mutant mitochondria also have frequent constrictions along their length suggesting that they are arrested at the stage of membrane scission (Logan et al. 2004). Moreover, in *drp3a* mutants, the presence of individual narrow protrusions extending the mitochondria has been reported. These mitochondrial protrusions were named “matrixules”, following the name given to stromules (Scott and Logan 2008). Thus, matrixules are actually elongated mitochondria that represent the dynamic intermediates of the fission process, although their function in the context of metabolite transfer was not discussed (Foyer and Noctor 2007). Growing evidence of a close relationship between energy production and mitochondrial network organization exists, even if the physiological significance of the various morphological forms of mitochondria is still an open question. For instance, the pioneering observations performed in human cells by Hackenbrock (Hackenbrock 1968; Hackenbrock et al. 1971) revealed a rapid and reversible change of the mitochondrion from the so-called ‘orthodox’ to ‘condensed’ conformations upon activation of ATP synthesis suggesting a key influence of ATP levels on mitochondria conformations. Moreover, Benard et al. (2007) demonstrated that in mammal cells there is a bidirectional relationship between mitochondrial network organization and bioenergetics. The tight dependence of mitochondrial fusion on mitochondria functionality was also confirmed in plant cells where mitochondrial membrane potential or ATP level may serve as mitochondria quality control mechanisms in a cell (Twig et al. 2008).

It has actually been demonstrated that mitochondrial fusion is much more dependent on ATP levels in plants than in yeast and mammals (Wakamatsu et al. 2010). Apart from the link between mitochondria morphology and functionality, what is still not extensively investigated is the relationship between mitochondria dynamics and metabolite trafficking among mitochondria and other organelles, in particular plastids.

A. Inter-organelle Connections

Another story relates to peroxisomes that, in response to oxidative stress, have been shown to form short tubular tails that rapidly expand becoming longer than the original organelle (Mathur et al. 2010). This structural formation is considered a fast, local and transient response, leading to stress alleviation. Since peroxisomes play a major role in detoxification of high concentrations of hydrogen peroxide (Nyathi and Baker 2006), the formation of tubular peroxisomes and their subsequent dissolution could signify a cellular attempt to combat global stress by increasing the peroxisomal population (Sinclair et al. 2009). On the other hand, the "peroxules" also have a physiological role in metabolite transport. In fact, in sublethal mutants of *PEX10* (which is involved in peroxisome biogenesis) a loss of physical contact between chloroplasts and peroxisomes has been linked to photorespiratory defects, indicating that the shuttling of metabolites is aided by the juxtaposition of organelles (Schuman et al. 2007).

Short-term increases in the surface area of all three organelles through the formation of tubules, perhaps even transient physical interactions between the respective tubules, might increase the efficiency of inter-organelle metabolite transfer. There are, however, molecular indications that a physical connection exists among the membranes of different organelles, corroborated by the existence of a few proteins that localize to the external membrane of different organelles.

This model has been suggested for a few plant proteins such as cytochrome *b5* (Maggio et al. 2007) and the FIS1-type plant orthologues (Scott et al. 2006). FIS1-type plant orthologues, that can be classified as tail-anchored proteins, are suggested to be involved in the mitochondrial division machinery, however they also localize at the level of peroxisomes (Zhang and Hu 2008) and chloroplasts (Logan 2006; C. Ruberti and M. Zottini unpublished data). Moreover, it has recently been reported that a complex endomembrane network exists, frequently forming transient contacts, gliding over each other and often producing tubules and rounded vesicles (Mathur et al. 2010).

During the evolution of subcellular compartmentalization – and therefore the passage from prokaryotes to eukaryotes – intracellular membranes were formed to envelop the new organelles, mitochondria and chloroplasts. This ancient cellular inner membrane actually evolved in the outer membrane of mitochondria and chloroplasts and the ability of some tail-anchored proteins to insert directly in the lipid bilayer without the help of translocons and molecular machinery could be a feature of ancestral proteins that were able to recruit lipids, contributing to the formation of inner membranes.

Taken together, these data may suggest that a well-organized inner membrane system exists, establishing contact between the inter-membrane spaces of mitochondria and chloroplast, as well other compartments, in a dynamic, regulated process that functions as a preferential way for transduction of signal macromolecules, ions and radicals. It is thus important to consider that the knowledge about stromules, peroxules, matrixules and other membrane protrusions will improve our understanding not only of basic cellular compartmentalization, but also of plant biochemistry, organelle genetics, and of the mechanisms by which plants interact with their environment.

VI. Conclusions

The last few years have seen considerable advances in the understanding of the interaction between mitochondrial and extra-

mitochondrial metabolism. In particular it has been seen how important is the interdependence between mitochondria and chloroplast in maintaining the functionality of vital processes of plants. However, our knowledge on the identification of the key players orchestrating these interactions remains far from complete, compromising our understanding of signal transduction cascades and of retrograde signaling from the mitochondria and/or chloroplast. A major challenge to understanding the coordination of the different subcellular compartments will be not only to identify proteins that are involved, but to determine how their functions or levels are controlled. Our understanding of retrograde signal mechanisms remains limited, in part because the identity of many of the proteins involved is not known, even if promising data are emerging on the role of putative signaling proteins. It has also to be considered to carefully investigate on the role of Ca^{2+} and ROS as signaling molecules possibly involved both in anterograde and retrograde signaling.

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Chapter 22

Plastid Signaling During the Plant Life Cycle

Plastid Function as Developmental Reporter and Environmental Sensor in Plant Growth and Acclimation

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Summary

Plastids are semi-autonomous organelles of endosymbiotic origin. They possess their own DNA and a complete machinery to express the encoded information on it. However, the genome size is limited to about 120 genes encoding mainly components of the gene expression and

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photosynthesis machineries. For complete functionality, therefore, plastids largely depend on the import of cytosolic proteins since all plastid protein complexes are comprised of a mosaic of plastid and nuclear encoded components. Proper development and function of plastids, thus, requires a tight coordination of gene expression in the genetic compartments of a plant cell. This coordination is obtained by (1) nucleus-to-plastid signals which guarantee an appropriate establishment of the plastid type according to the tissues context of the plant cell, and by (2) plastid-to-nucleus signals which report the actual developmental and functional stage of the plastids to the nucleus. This mutual communication controls the expression of appropriate genes providing the right gene products required for the respective condition. Plastidial signals can be distinguished into distinct classes covering signals from (1) plastid gene expression, (2) pigment biosynthesis pathways, (3) pools of reactive oxygen species, (4) redox states of photosynthetic components and (5) metabolic intermediates such as sugars. This classification is mainly focused on the experimental system in which the respective plastid signal has been analyzed rather than describing the signal itself. In this review we follow a different strategy and summarize the current knowledge on plastid signaling according to the developmental stage of the plastids. We distinguish between signals from early plastid development, from mature plastids and from plastids being degraded during senescence. This also includes the action of three important plant hormones synthesized partly in the plastids, jasmonic acid, salicylic acid and abscisic acid. By this way we follow the plant's life cycle and put the roles of plastidial signals into a functional and developmental context which provides novel insights into the fascinating research field of intracellular signaling.

I. Introduction

A. The Flexible Appearance of Plastids

Plastids are plant cell-specific organelles of endosymbiotic origin which are surrounded by a double-membrane and display a high flexibility in their functional and morphological constitution (Lopez-Juéz and Pyke 2005; Hoober 2006; Pogson and Albrecht 2011). They are inherited as an undifferentiated precursor form, the so-called proplastid, which develops into a mature form during the development and growth of the plant. Like their cyanobacterial ancestors, plastids multiply by fission which, however, is coordinated with the life cycle and division of the cell in which they reside. This guarantees that all cells during plant growth will be provided with their own set of plastids. The precise structural and functional characteristics of the plastids are defined by the tissue context of the respective host cell. The most prominent representative of these organelles is the chloroplast in the aerial parts of the plant which is the site of photosynthesis and many other important metabolic reactions involved in, e.g., nitrate or sulfate reduction, purine and pyrimidine biosynthesis or amino acid production. Other forms are colourless amyloplasts or elaioplasts in the roots, which serve as starch or oil storage compartments or coloured chromoplasts in fruits or flowers (Buchanan et al. 2002).

The appearance of a respective plastid type in a certain tissue is not fixed and may change depending on developmental or environmental influences. During germination and seedling development colourless proplastids may develop into yellow etioplasts with a characteristic prolamellar body as long as the seedlings do not perceive light. Upon light perception the photomorphogenic programme is initiated, which includes the etioplast-to-chloroplast transition. Later on, in plant life cycle green chloroplasts may turn into yellow or red chromoplasts during fruit ripening or flower development or amyloplasts become

Abbreviations: ABA – Abscisic acid; ALA – Amino levulinic acid; GSH – Reduced glutathione; JA – Jasmonic acid; Mg-proto-IX – Magnesium-protoporphyrin IX; NF – Norflurazon; ROS – Reactive oxygen species; SA – Salicylic acid

green chloroplasts when roots reach the soil surface. Furthermore, chloroplasts may be transformed into senescing gerontoplasts at the end of the vegetative phase of the plant life cycle serving as source for carbon or nitrogen for seed filling or resource allocation towards the roots or stems in perennial plants. In summary, the function and morphology of plastids is tightly linked to the developmental and environmental context of the host cell and tissue. The morphology and function of plastids therefore represents (1) a reliable reporter for the developmental stage of a plant and its tissues and (2) an environmental sensor in plant growth and acclimation (Bräutigam et al. 2007).

B. The Concept of Plastid Signaling

Plastids are derived from an endosymbiotic event in which a cyanobacteria-like ancestor had been engulfed into a eukaryotic host cell (Martin et al. 2002; Stoebe and Maier 2002; Timmis et al. 2004). Remnants of this prokaryotic origin are the surrounding double membrane of the organelle and a plastid-own genome, the so-called plastome. In vascular plants this plastome contains a highly conserved set of around 120 genes, which encode mainly components of the photosynthetic apparatus and of a plastid-own transcription and translation machinery including an *E. coli*-like RNA polymerase, 70S ribosomes and a set of tRNAs and rRNAs (Sugiura 1992). Although plastids possess their own gene expression system their functions are highly dependent on the import of cytosolic proteins. Current estimates range from approx. 2,500–4,500 different proteins to be active in the plastids indicating that only a very small part of the plastid protein complement is encoded by the plastome (Abdallah et al. 2000; Kleffmann et al. 2004; Van Wijk and Baginsky 2011). Plastids therefore are regarded as semi-autonomous cell organelles. At the same time the permanent import of cytosolic translation products represents a flow of information from the nucleus to the organelle. Since virtually all protein complexes consist of nuclear and plastid encoded subunits the nucleus obtains control over all essential steps in development and function of plastids. Because of its dominant appearance, it became fashionable to call this direction of information flow "forward" or anterograde signaling. However, in the last three decades it became also apparent that there exist a number of signals originating from the plastids which inform the nucleus about the developmental or functional state of it representing a "backward" flow of information called retrograde signaling (Rodermel 2001; Papenbrock and Grimm 2001; Gray et al. 2003; Strand 2004; Beck 2005; Nott et al. 2006; Strand et al. 2006; Jarvis 2007; Bräutigam et al. 2007). This terminology is widely accepted, but it is ambiguous. The term "anterograde" implies that the nucleus is the initiator of the signaling events while "retrograde" suggests that the plastids send the responding signals. However, as we will see in the following sections the respective roles of the two compartments in their communication can be quite variable and it depends on the physiological and developmental context which one is the initiator and which one the responder. We, therefore, will instead use the term "plastid" or "organellar" (if mitochondria are also included) signaling throughout this article.

Plastids are an important and integral compartment in the plant cell's biochemistry and are involved in many biosynthetic pathways. At the same time plastids are highly susceptible to changes in the environment (e.g. changes in illumination, temperature or water availability) or cellular changes (e.g. changes in the developmental stage of the tissue) which often result in dramatic changes of plastid function and activity. Plastid signals are a way to transmit information about any change in developmental stage, age or metabolic activity of plastids to the nucleus and to induce appropriate changes in the expression of genes for nuclear-encoded plastid proteins which help to adapt plastid functions to the respective conditions. By this means plastids act as sensors for tissue and plant cell development and as receptors for changes in the environment. Thus, these

signals are highly important for plant growth and development (Lopez-Juez and Pyke 2005; Hoober 2006; Pogson and Albrecht 2011).

Several classes of signals have been identified. These cover signals originating from plastid gene expression (Gray et al. 1995), pigment biosynthesis pathways including carotenoids and tetrapyrrols (Oelmüller and Mohr 1986; Taylor 1989; Papenbrock and Grimm 2001; Beck 2001; Surpin et al. 2002), various reactive oxygen species (Mullineaux and Karpinski 2002; Apel and Hirt 2004; Baier and Dietz 2005), photosynthetic electron flow and pools of several metabolites (Pfannschmidt et al. 2003; Mullineaux and Rausch 2005; Pesaresi et al. 2007). This classification, however, mirrors mainly the experimental approaches chosen to analyse the respective plastid signals rather than characterizing the signals itself. Despite great effort in numerous studies the true nature of plastid signals remained largely unresolved and, therefore, the basic concept has been critically discussed (Pfannschmidt 2010). A reasonable alternative for classification of plastid signals is an arrangement according to the developmental stage of the tissue investigated (Pogson et al. 2008). Since the development of plastids in a cell always corresponds to the respective one of the tissue to which the cell belongs (Lopez-Juez and Pyke 2005; Hoober 2006; Pogson and Albrecht 2011) this provides a simple and distinct possibility of classification. Furthermore, since the various developmental stages of tissues are clearly separated in time this avoids overlaps or ambiguities concerning the signal in discussion. Therefore, also this book chapter follows this way of classification.

II. Signals from Plastids in Early Seedling Development: Biogenic Control

A. Signals Generated by Changes in Plastid Gene Expression

Historically the existence of plastid signals was first postulated from experiments in which plastid gene expression within young seedlings was disturbed. The typical observation was that the expression of nuclear genes for plastid proteins (usually *RbcS* encoding the small subunit of the ribulose-bisphosphate-carboxylase/oxygenase and *Lhcb1* (formerly called *cab* for chlorophyll a/b binding protein) encoding a major protein of the light harvesting complex of photosystem II (LHCII)) was repressed when plastid development in seedlings was disturbed or blocked either genetically or by inhibitor treatments. This was first reported for the barley mutant *albostrians* which has a genetic defect resulting in ribosome-deficient plastids and giving rise to white stripes in the leaves of the mutant (Bradbeer et al. 1979). In these white tissues expression of the small subunit of Rubisco was repressed although cytosolic ribosomes were intact. This observation was confirmed by studies using antibiotics which selectively inhibit 70S ribosomes such as chloramphenicol, streptomycin, erythromycin or lincomycin (Oelmüller et al. 1986; Gray et al. 1995; Yoshida et al. 1998; Sullivan and Gray 1999). In all cases the expression of *RbcS* and/or *Lhcb* genes was repressed and, therefore, the expression of these genes became a reporter for the action of plastid signals. Interestingly, it turned out that the translation inhibitors were effective only when applicated within the first 36–72 h after germination. Afterwards the antibiotics could not repress the nuclear genes suggesting that the action of the plastid signal might be restricted to an early time span in plastid development (Oelmüller and Mohr 1986; Gray et al. 1995). In principle, this could include even embryogenesis. Consistent with this are some more recent observations obtained with the model organism *Arabidopsis thaliana*. The *snowy cotyledons 1* (*sco1*) mutant has a defect in the gene for the plastid elongation factor G resulting in a diminished plastid translation (Albrecht et al. 2006). This causes the development of un-pigmented cotyledons and a decreased transcript accumulation of some nuclear encoded photosynthesis genes. However, the phenotypic effects are lost in the following leaves suggesting that reduction

of plastid translation is reported to the nucleus only in early stages, when photomorphogenesis (including the build-up of the photosynthetic apparatus) requires highly active translation. Another Arabidopsis mutant called *albino or pale green 3* (*apg3*) lacks the chloroplast ribosome release factor 1 and displays an albino phenotype in cotyledons and in all following leaves (Motohashi et al. 2007). This mutant is able to grow only on sucrose-supplemented medium and, interestingly, 21-day-old plants do not accumulate D1, RbcL and RbcS proteins while the expression of nuclear transcripts of photosynthesis genes appeared to be only slightly repressed. Again this suggests that defects in plastid translation have a repressive effect on nuclear gene expression only in early stages of plastid development. On the other hand, it clearly indicates that defects in plastid translation *per se* are likely not the cause for repression of nuclear gene expression. The latter conclusion is further substantiated by the observation that likewise the repressive effects on *Lhcb* and *RbcS* expression can be induced by inhibition of plastid transcription with respective inhibitors (tagetitoxin, nalidixic acid or rifampicin) (Lukens et al. 1987; Rapp and Mullet 1991; Gray et al. 1995; Pfannschmidt and Link 1997). Other nuclear genes remained largely unaffected by these treatments. Again this repressive effect could be observed only when the inhibitors were applied early in seedling development. The involvement of transcription, however, is not astonishing, as this level of gene expression is tightly connected to the level of translation via the expression of plastid encoded components of the translational machinery such as tRNAs or rRNAs. In summary, all these early data suggest that plastid gene expression at the very early stage of seedling development plays an important, yet not defined, role in the expression of nuclear genes. In addition, recent data indicate that there exists a connection to mitochondrial translation, which suggests that signals from both organelles act in a coordinated and synergistic manner (Pesaresi et al. 2006; M. Zottini, Chap. 21). For a more detailed description of the role of mitochondria in this context refer to Chap. 21 by Zottini in this issue.

B. Signals from Damaged Plastids

Other early experiments suggested the involvement of carotenoid biosynthesis in plastid signaling besides plastid gene expression. In seedlings of maize mutants with defects in carotenoid biosynthesis, *Lhcb* transcript accumulation was reduced while that of other nuclear encoded genes was not affected (Mayfield and Taylor 1984). The same could be observed if seedlings were treated with norflurazon, an inhibitor of the enzyme phytoene desaturase resulting in a block of carotenoid biosynthesis (Oelmüller and Mohr 1986; Chamovitz et al. 1991). Both genetic and chemical impairment of carotenoid biosynthesis lead to reduced photosynthetic efficiency and increased photo-oxidative effects in the thylakoid membranes. The latter are mainly caused by defective non-photochemical quenching (NPQ) mechanisms which require carotenoids for de-excitation of excessive light-energy (Niyogi 2000). An obvious explanation for the observed effects, therefore, is that proplastids or very young plastids which perceive photo-oxidative stress cannot perform the transition into mature plastids resulting in a repression of *RbcS* and *Lhcb* (Mayfield and Taylor 1984; Mayfield et al. 1986; Oelmüller et al. 1986; Oelmüller 1989). This led to the conclusion that intact plastids are required for correct expression of *Lhcb* and *RbcS* genes and that this must be mediated by a "plastid signal" or "plastid factor" (Oelmüller 1989; Taylor 1989). About the nature of this factor, however, one could only speculate. Further insights came from a mutant screen with a *Arabidopsis thaliana* population in which individuals were identified that were able to uncouple *Lhcb* expression from NF-mediated destruction of plastids (Susek et al. 1993). These *genomes uncoupled* (*gun*) mutants demonstrated that the *Lhcb* expression behaviour in presence of NF can be genetically disrupted and led to the conclusion that

damaged plastids send a repressive signal, which down-regulates *Lhcb* transcription. Six different *gun* mutants could be identified from this screen. The genetic defects in the mutants *gun2–gun5* were all mapped to the tetrapyrrole pathway, while in *gun1* the defect could be attributed to a PPR protein involved in plastid gene expression (Surpin et al. 2002; Koussevitzky et al. 2007). In a recent gain-of-function screen another *gun* mutant named *gun6-1D* was identified. It was found to over-express the plastid ferrochelatase, a key enzyme in the haem branch of the tetrapyrrole pathway (Woodson et al. 2011) (for more details refer to Sects. III, III.B, and III.C). This suggests that signals depending on plastid gene expression and carotenoid and tetrapyrrole biosynthesis converge at least under conditions in which plastids become damaged by NF. It is important to note that the tetrapyrrole biosynthesis starts with the formation of δ-amino levulinic acid (ALA) from glutamyl-tRNA (Rüdiger and Grimm 2006; Tanaka and Tanaka 2007). The latter is encoded in the plastid genome by the *trnE* gene and is expressed by the plastid transcription machinery (Hanaoka et al. 2003) providing a metabolic link between gene expression and pigment synthesis. Apparently, the different inhibitor treatments induce the same effect by affecting directly or indirectly the same processes.

C. The Role of Plastid Signals in "Biogenic Control"

All inhibitors mentioned above produce either a block of plastid development or severe plastid damage and because of their functional connection induce the same response in the cell nucleus. However, it is not understood how this nuclear response results in a decrease of *Lhcb* transcript accumulation and how this can be interrupted in the *gun* mutants. It appears plausible that damaged plastids send a repressive signal which is de-repressed in the *gun* mutants. However, other scenarios are also possible, and it is equally likely that, e.g., damaged plastids are unable to activate processes required for the build-up of the photosynthetic apparatus (Pfannschmidt 2010). Further research will be necessary to understand the complex interactions during the early phases of plastid development and the respective communication with the cell nucleus. However, it becomes increasingly clear that the signaling from young and mature plastids differs in many aspects. The concept of "biogenic control" clearly focuses on the biogenesis and establishment of functionally fully competent plastids (Pogson et al. 2008). It is important to note that the development of plastids is tightly connected to the developmental program of the cell and both are integral parts of the morphogenic program regulating the respective tissue development (Lopez-Juez and Pyke 2005). In other words, during seedling development the nucleus provides the right protein composition to generate the plastid type appropriate to the respective tissue. This is well studied in the proplastid-to-chloroplast transition in cotyledons during photomorphogenesis (Lonosky et al. 2004; Blomqvist et al. 2008), but also accounts for plastids in non-green tissues such as amyloplast development in the root (Enami et al. 2011). During these early stages, plastid signals likely report the progress of the organelle biogenesis to the nucleus. Therefore, it is conceivable to assume that any inhibitor treatment repressing either plastid gene expression or pigment biosynthesis pathways during the build-up of the organelle will lead to a de-synchronization between the developmental progress of the plastid and the corresponding developmental program of the nucleus. This de-synchronization likely causes a loss or a disturbance of communication between the genetic compartments and subsequently a down-regulation or shut-off of genes encoding plastid proteins (Pfannschmidt 2010). The effects of the inhibitor experiments, however, do not reflect natural condition in a plant's life and, thus, create artificial situations which have only limited informative value about plastid signals under normal physiological conditions. Nevertheless, mutants with defects in inhibitor-mediated repression of nuclear genes are valuable tools to understand the

incorporation of plastid development into cellular morphogenic programs. Recent investigations using norflurazon identified an interesting novel candidate protein for plastidial signals named PTM for plant homeodomain (PHD) type transcription factor with transmembrane domains (Sun et al. 2011). PTM is a PHD transcription factor tethered to the chloroplast outer envelope. Treatment of Arabidopsis plants with either high light or NF or lincomycin promotes a proteolytic cleavage and subsequent release of the N-terminal part of the protein from the membrane. It could be shown that this smaller protein localizes within the nucleus where it is required for proper expression of ABI4 (abscisic acid insensitive 4), a transcription factor shown to be responsive to plastid signals from damaged plastids. PTM, thus, could be the missing functional link in the so-called *gun* signaling pathway. Interestingly, PTM expression and processing during stress was decreased in the *gun1* mutant suggesting that GUN1 is important for PTM expression. The principle that PTM is released from the chloroplast in response to stress appears comparable to the signaling mechanism of Whirly1, which is proposed to be released from chloroplasts and to translocate to the nucleus in response to still unknown stimuli (Isemer et al. 2012). This identifies a signaling principle which corresponds largely to the original idea of a so-called "plastid factor" which proposed that "something" leaves the plastid (Oelmüller 1989; Taylor 1989). Whether PTM or Whirly 1 are also involved in other plastid signaling pathway (e.g. during operational or degradational control) requires further investigations, but these novel observations could be key findings for our understanding how plastid signals are transduced to the nucleus.

III. Signals from Plastids in Mature Tissues: Operational Control

In contrast to young or damaged plastids mature plastids are fully functional and very susceptible to environmental cues such as light, temperature or drought. They provide permanent signals to the nucleus which indicate the actual functional state and any change in it. By this means plastids can report environmentally induced changes in their biological activities which might have adverse effects on its function. This induces counteracting responses in the nucleus, which help to acclimate the affected plastid process by delivery of suitable proteins. Due to the multiplicity of environmentally induced variations the number and types of plastid signals are numerous and very different, but can be summarized under the term "operational control" (Pogson et al. 2008).

A. Redox Signaling

1. Signals from Photosynthetic Electron Transport

Chloroplasts are the dominant plastid type in green tissues and the site of photosynthesis. In photosynthesis the balance of light- and dark reactions provides a very sensitive perception system which detects even slightest changes in the environment, mainly by changes in the redox states of the electron transport components or electron end acceptors or by the accumulation of various reactive oxygen species. These trigger acclimation responses aimed to maintain photosynthetic electron transport efficiency to counteract the environmental changes. Many of these responses involve targeted changes in nuclear gene expression and require a mutual communication of chloroplasts and the nucleus (Pfannschmidt 2003; Apel and Hirt 2004; Mittler et al. 2004; Baier and Dietz 2005; Foyer and Noctor 2005; Walters 2005).

The influence of redox signals from photosynthetic electron transport (PET) on nuclear gene expression could be first demonstrated in unicellular algae but was soon also identified in vascular plants (Escoubas et al. 1995; Maxwell et al. 1995; Yang et al. 2001). Most of the early studies used light treatments in combination with electron transport inhibitors DCMU (blocking electron transport at the Q_B binding site of the D1

protein) and DBMIB (blocking the electron transport at the Q_O site of the Cytb_6f complex) to induce redox signals in the PET. Furthermore, a complementary response to the inhibitors indicated the involvement of the plastoquinone pool as specific origin of the redox signal studied (Pfannschmidt et al. 2009). As read-out again the expression of the nuclear *Lhcb* gene (sometimes in combination with the corresponding LHCII protein) was monitored and both the redox states of the plastoquinone pool and of electron acceptors have been proposed as regulatory signals (Pursiheimo et al. 2001). Alternative studies investigated the influence of signals from PET in the expression of reporter gene constructs in transgenic tobacco confirming that redox signals from PET are transduced to the nucleus (Pfannschmidt et al. 2001; Sullivan and Gray 2002). Furthermore, it became obvious that not only transcription, but also ribosome loading and possibly other levels of gene expression, were affected by plastid redox signals suggesting that the PET dependent redox signaling could be more complex than controlling transcription of nuclear photosynthesis genes as initially anticipated (Petracek et al. 1997, 1998; Sherameti et al. 2002a). That was confirmed by further reports which demonstrated connections to sugar signaling, to high light induced stress signaling as well as to ABA responses and leaf transpiration rates (Karpinski et al. 1997, 1999; Oswald et al. 2001; Fryer et al. 2003). Furthermore, it could be shown that also non-photosynthesis genes responded to redox signals from PET (Sherameti et al. 2002b). Although these first studies provided just a glimpse of the extent to which plastid redox signaling is implemented into cellular signaling networks, it became already evident that this class of plastidial signals is of major importance for maintenance of functionally active chloroplasts.

With the invention of array technologies it became possible to observe the effects of redox signals from PET on a genome-wide scale. Using a physiological approach with a combination of light quality-induced redox signals, counteracted by half-inhibitory concentrations of DCMU and a macroarray with probes for genes encoding proteins with predicted chloroplast localization, it was analyzed how many genes in Arabidopsis were controlled by PET redox signals (Fey et al. 2005). Two hundred and eighty six genes covering all major gene groups such as photosynthesis, gene expression, signal transduction and especially metabolism were identified. As an additional observation it turned out that the use of DCMU caused some side effects which interfered with a conclusive assignment of the origin of the responsible redox signal for many genes exacerbating the interpretation of the results. Another study using a different array with probes for about 8,000 randomly chosen genes from the Arabidopsis genome came to the conclusion that light induced redox control of nuclear genes is mainly induced by redox changes in stromal components on the acceptor side of PSI, e.g. thioredoxins or related redox buffers (Piippo et al. 2006). This apparent contradiction could be resolved by a systems biology approach measuring the impact of light quality induced redox signals on nuclear gene expression and on metabolism in parallel. It turned out that both redox signals from the plastoquinone pool and from stromal redox components contribute to the plastid signaling (Bräutigam et al. 2009). Surprisingly, it could be observed that the redox balance between these two redox pools did not always correspond, but could be also regulated in opposite ways giving rise to a hypothesis of four redox signal signatures controlling both metabolism and nuclear gene expression in an integrated way (Bräutigam et al. 2010). Furthermore, this study was also designed as a kinetic experiment describing for the first time the time-dependent impact of redox signals on nuclear gene expression. It could be demonstrated that the light-induced redox signals significantly affect nuclear transcription within less than 30 min suggesting that signaling pathways of plastid redox signals are likely not induced but permanently active. It was also observed that the response pat-

terns in the nucleus were very dynamic indicating a sequence of primary, secondary and tertiary regulated genes. The parallel impact on plastid and cytosolic metabolism suggests the existence of two so-called adjustment loops, which re-direct both photosynthetic and metabolic functions in a coordinated manner (Bräutigam et al. 2009). In another study a genetic approach was employed in which the transcript profiles of three different Arabidopsis mutants with defects in state transitions were compared to that of wild-type plants (Pesaresi et al. 2009). It could be demonstrated that the mutants *stn7* (lacking the thylakoid associated kinase STN7), *psae1-3* (lacking the PSI subunit E1) and *psad1-1* (lacking the PSI subunit D) are also defective in the long-term response to light quality shifts and, therefore, might be affected in the same redox signaling pathway to the nucleus. The array analysis uncovered 56 genes, which are jointly mis-regulated in the mutants, when compared to the wild-type. These include genes for post-transcriptional or post-translational regulatory processes as well as a number of genes for metabolic functions in plastids including the redox-controlled AGPase (Pesaresi et al. 2009). Thus, these genes potentially represent target genes of plastid redox signals. However, in contrast to the kinetic experiment mentioned above (Bräutigam et al. 2009) these array analyses were performed with samples obtained under stable growth conditions, but in different genetic backgrounds. This implies that the differentially genes are more likely controlled by long-term mechanisms compensating the genetic defect rather than by short-term redox signals. Intriguingly, among the up-regulated genes is one encoding the chloroplast-localized kinase FLN1 (Arsova et al. 2010), which has been identified as a permanent subunit of the plastid-encoded RNA polymerase (Steiner et al. 2011) providing a putative regulatory link to the plastid transcription machinery. Thus, further analyses which combine experimental systems providing inducible redox signals and genetic approaches blocking such signals are required to define the true set of redox-regulated nuclear genes (Pfannschmidt et al. 2009).

While a number of redox-regulated nuclear genes could be identified, the way how redox signals from photosynthesis leave the plastids, pass the cytoplasm and enter the nucleus is not understood yet. Early studies in *Dunaliella tertiolecta* suggested the involvement of a phosphorylation cascade from the plastid to the nucleus controlling a putative repressor protein (Escoubas et al. 1995; Durnford and Falkowski 1997). This idea is supported by inhibitor experiments demonstrating that in transgenic tobacco a photosynthesis (*PsaF*) promoter: GUS reporter gene construct can be regulated by cytosolic kinase and phosphatase activities even in the absence of functional plastids (Chandok et al. 2001). In addition, a number of plastid-localized kinases have been identified which affect nuclear gene expression if the respective gene is knocked-out, e.g. STN7 or STN8 (Bonardi et al. 2005; Tikkanen et al. 2006; Bräutigam et al. 2009; Pesaresi et al. 2009). So far, it appears likely that photosynthetic redox signals are converted into phosphorylation signals at the thylakoid membrane, but the remaining signal transduction steps towards the nucleus are still elusive and require further experimentation.

2. Redox Signals Mediated by Reactive Oxygen Species

Photosynthesis produces oxygen as a by-product at the water-splitting complex of PSII. This creates a potentially dangerous situation within the chloroplast since the close proximity of oxygen generation and electron redox chemistry promotes the generation of reactive oxygen species (ROS) which might damage the chloroplast. However, in recent years it turned out that ROS have also an important role as signaling molecules (Allen and Raven 1996; Noctor et al. 2000; Apel and Hirt 2004; Mittler et al. 2004). Hydrogen peroxide (H_2O_2) is produced mainly at PSI under stress conditions which lead to over-reduction of the electron transport chain such as high light or low

temperature. This can lead to a transfer of excessive electrons from ferredoxin to oxygen generating superoxide, which is subsequently detoxified by the enzyme superoxide dismutase to hydrogen peroxide. This is further reduced to water by the enzyme ascorbate peroxidase which uses ascorbate as reductant. The resulting monodehydroascorbate is then replenished to ascorbate by reduction through the glutathione pool. This reaction sequence in summary is called water-water cycle and serves as a safety valve for excessive electrons under stress conditions (Baier and Dietz 2005; Foyer and Noctor 2009). Under certain conditions of persistent or very strong stress the capacity of this process, however, might get saturated leading to accumulation of H_2O_2. Since it can freely diffuse through the envelope membrane it might act as a direct signal. In Arabidopsis it could be shown that high-light induced accumulation of H_2O_2 in the plastid correlates with a concomitant activation of the nuclear genes *apx1* and *apx2* (encoding plastid ascorbate peroxidases) (Karpinski et al. 1997, 1999). Array analyses in Arabidopsis uncovered that 1–2 % of all genes in the genome responded to H_2O_2 signals including many genes related to stress or defense responses (Desikan et al. 2001; Vandenabeele et al. 2004; Davletova et al. 2005; Vanderauwera et al. 2005). It is proposed that the H_2O_2 diffusing out of the plastid might activate cytosolic mitogen-activated protein kinase (MAPK) cascades linking the stress condition in the plastid to gene expression in the nucleus (Kovtun et al. 2000; Apel and Hirt 2004). However, how different types of stress resulting all in H_2O_2 production can be distinguished from each other is unclear in the moment, and the involvement of further factors must be assumed. This includes also the formation of H_2O_2 signatures and ROS waves transmitted within and between cells contributing to a systemic signaling within the whole plant (Mittler et al. 2011).

Singlet oxygen ($^1O_2$) is another ROS involved in plastid and stress signaling. It is a non-radical ROS which is permanently generated as an unavoidable side reaction at PSII by energy transfer from triplet state P680 (the special chlorophyll pair in the reaction center of PSII) to oxygen. It is a highly reactive, very short half-lived molecule and the main cause for photodamage of the D1 protein in PSII. Under conditions of over-excitation it is produced in higher amounts and initiates a number of distinct stress responses in the nucleus (op den Camp et al. 2003). This could be clearly demonstrated in the Arabidopsis *fluorescent* (*flu*) mutant which accumulates free protochlorophyllide (Pchlide) in the dark and specifically generates high amounts of singlet oxygen upon re-illumination by energy transfer from the Pchlide to oxygen leading to growth inhibition and cell death. Under permanent illumination, however, Pchlide does not accumulate and the mutant develops undistinguishable from wild-type (Meskauskiene et al. 2001). Transcript profiling uncovered a number of nuclear genes responding specifically to the singlet oxygen in the mutant (op den Camp et al. 2003). Interestingly, the phenotype could be uncoupled from singlet oxygen generation by second site mutations indicating that it is induced by a response programme rather than by the oxidative damage. By this means, two proteins called EXECUTER 1 and 2 were identified which appear to be necessary and essential for the singlet oxygen signaling to the nucleus. Both are chloroplast-localized proteins with still uncharacterized properties (Wagner et al. 2004; Lee et al. 2007). Thus, it remains to be elucidated how the signaling cascade from these two proteins extends to the nucleus.

Plastid ROS signaling in some cases appears to be mediated indirectly via the counteracting antioxidant systems. Using the redox-sensitive promoter of the nuclear 2-cysteine-peroxiredoxin (2CPA) fused to the luciferase gene, a screen in Arabidopsis was performed which identified redox-imbalanced (*rimb*) mutants (Heiber et al. 2007). These provide tools to identify components which activate the expression of plastid antioxidant enzymes in response to ROS stress in the plastid. This signaling

cascade might involve reduced glutathione (GSH) which is required to replenish the primary ROS scavenger ascorbate. Under high light stress the content of GSH declines (Karpinski et al. 1997), and the expression of stress-related genes was found to correlate with this change (Mullineaux and Rausch 2005). This was confirmed by genetic analyses with a mutant exhibiting a decreased GSH content and a concomitant increased expression of the *apx2* gene (Ball et al. 2004). Recent analyses suggest that at least in Arabidopsis the glutathione itself could be the corresponding plastid signal. Glutathione is synthesized in two consecutive steps by the enzymes glutathione synthetase 1 and 2 (GSH1 and 2) which first generate γ-glutamylcysteine from glutamate and cysteine, and in a second step glutathione by addition of glycine. GSH1 which catalyses the first step is mainly active in the plastid while GSH2 catalysing the second step is mainly active in the cytoplasm. For complete GSH synthesis a shuttling of the intermediates between plastid and cytosol is required which could exert a signaling function especially under stress conditions when increased GSH synthesis is required (Mullineaux and Rausch 2005).

In summary, ROS mediated signals are an important part of plastid signaling which are involved in many responses to abiotic and biotic stresses. They are connected to many other signals from e.g. pigment biosynthesis or plastid development. The PPR protein GUN1 was proposed to be a potential convergence point for such connections and the PTM protein appears to be also involved in this pathway (Koussevitzky et al. 2007; Sun et al. 2011), however, the molecular details are still largely unknown and require more experimental efforts for a complete understanding.

B. Signals from Tetrapyrrole Biosynthesis

The tetrapyrrole biosynthesis pathway provides the molecular building-blocks for the generation of chlorophylls, haem, and phytochromobilin and, therefore, is absolutely essential for plants. The pathway is entirely localized within the chloroplast, while the enzymes performing the catalyzing steps are all encoded in the nucleus. The metabolic fluxes through the different branches of this pathway need to be highly controlled and, therefore, require a coordination of nuclear gene expression with the respective activities in the pathway branches. Furthermore, all chlorophylls and their precursor are phototoxic and need to be assembled into the corresponding apoproteins or protein complexes immediately after synthesis. This requires additional coordination with the expression of chlorophyll binding proteins (Rüdiger and Grimm 2006; Tanaka and Tanaka 2007).

First evidence for plastid signals originating from the tetrapyrrole biosynthesis pathway came from feeding experiments with the iron chelator dipyridil, which led to a down-regulation of the *Lhcb* mRNA level in *Chlamydomonas reinhardtii*. The depletion of iron caused a block in the haem branch of the pathway and a subsequent accumulation of Mg-Protoporphyrin-IX (Johanningmeier and Howell 1984). Other feeding experiments using Mg-Proto-IX directly induced the nuclear heat-shock proteins HSP70 in Chlamydomonas (Kropat et al. 1997). This correlated to the finding that several *gun* mutants in Arabidopsis could be mapped to the tetrapyrrole pathway. The *gun2* mutant is defective in the haem oxygenase and the *gun3* mutant in the phytochromobiline synthase (Surpin et al. 2002). Both defects cause an accumulation of haem which in turn represses the entry enzyme of tetrapyrrole biosynthesis, the *trnE*-reductase (HEMA) resulting in down-regulation of Mg-Proto-IX levels. The *gun4* mutant is defective in an activator of the Mg-chelatase, a 22 kDa protein which binds either the chelatase substrate Proto-IX or the product of the reaction, Mg-Proto-IX (Larkin et al. 2003; Strand 2004). The *gun5* mutant is affected in the Chl-H subunit of the Mg-chelatase which provides the Proto-IX to the Chl-L:Chl-D complex in which the Mg chelation takes place (Mochizuki et al. 2001). Any defect in the Mg-chelatase results in a down-regulation

of Mg-Proto-IX and chlorophyll levels. A working model proposed that upon NF treatment Mg-Proto-IX directly leaves the plastids and represses *Lhcb* transcripts in wild-type while the down-regulation of Mg-Proto-IX levels in *gun* mutants causes the de-repression of the *Lhcb* transcript accumulation under NF treatment (Strand et al. 2003; Ankele et al. 2007; Woodson and Chory 2008). However, this model was heavily debated because (1) Mg-Proto-IX is exceptional phototoxic making it unsuitable as signaling molecule, and (2) a number of other tetrapyrrole pathway mutants with low Mg-proto-IX levels (e.g. *chl-I*, *ch42*, *cs* and *chl27*) did not display *gun* phenotypes (Koncz et al. 1990; Tottey et al. 2003; Kleine et al. 2009). Two recent studies performed detailed Mg-Proto-IX measurements and could demonstrate that changes in Mg-Proto-IX levels did not correlate to gene expression changes, indicating that this molecule does not work as a direct plastid signal (Mochizuki et al. 2008; Moulin et al. 2008). Two alternative models, however, still suggest a role of tetrapyrrole biosynthesis in plastid-to-nucleus signaling. The first one proposes that the metabolite fluxes through the haem and chlorophyll branches could act as signal (Larkin et al. 2003; Wilde et al. 2004), while the second model proposes that haem and/or its precursor Proto-IX which are exported from plastids to mitochondria represent the active signal (von Gromoff et al. 2008; Woodson et al. 2011). These two models have the advantage that they may function under physiological conditions reporting true functional states to the nucleus. It remains to be shown, how such kind of signals could be mechanistically linked to nuclear gene expression.

C. Signals from Carotenoid Biosynthesis

Carotenoid biosynthesis occurs in plastids of all photosynthetic organisms. Carotenoids are usually synthesized *de novo* in differentiated plastids of leaves, roots, flowers, fruits and seeds, accumulating mostly in chloroplasts, but also in chromoplasts, amyloplasts, leucoplasts, etioplasts and elaioplasts (Cazzonelli 2011). Carotenoid biosynthesis proceeds via the methylerythritol phosphate (MEP) pathway in plastids leading to the formation of the essential building blocks of all isoprenoids, isopentenyl diphosphate. The addition of several C5 blocks to the pathway leads to the formation of geranylgeranyl diphosphate (GGDP, C20). Phytoene synthase condenses two molecules of GGDP to give rise to phytoene, the first carotenoid in the pathway that after several steps will lead to the formation of *trans-cis*-lycopene. Then the pathway is diverted to either α- or β-carotene, which ultimately lead to the formation of xanthophylls and other signaling compounds that, as will be discussed here, indirectly might function as signaling molecules informing the cell about the biochemical and physiological status of the plastid (Figs. 22.1 and 22.2).

Guard cells are the unique cells among the epidermis of plant leaves that contain chloroplasts. A direct role for plastid-localized carotenoids, and zeaxanthin in particular, in signaling toward the cytosolic compartment has been proposed for blue light-stimulated stomatal opening. This response, which shows blue–green reversibility, responds to the expression of two inter-convertible forms of a blue light photoreceptor, an inactive one absorbing in the blue and an active form that, upon absorption of green light, reverts to the inactive form (Frechilla et al. 2000). After some dispute in the scientific community of whether carotenoids or phototropins could be responsible for the blue light-stimulated stomatal opening, much evidence supports the contention that zeaxanthin acts as a blue light photoreceptor in the opening of stomata at early morning in a reversible way. When *trans*-zeaxanthin absorbs blue light it is converted to the *cis* isomer, which has a large absorption in the green returning to the *trans* form (Milanowska and Gruczecki 2005). It has been shown that stomata from intact leaves exhibit the same blue–green reversibility

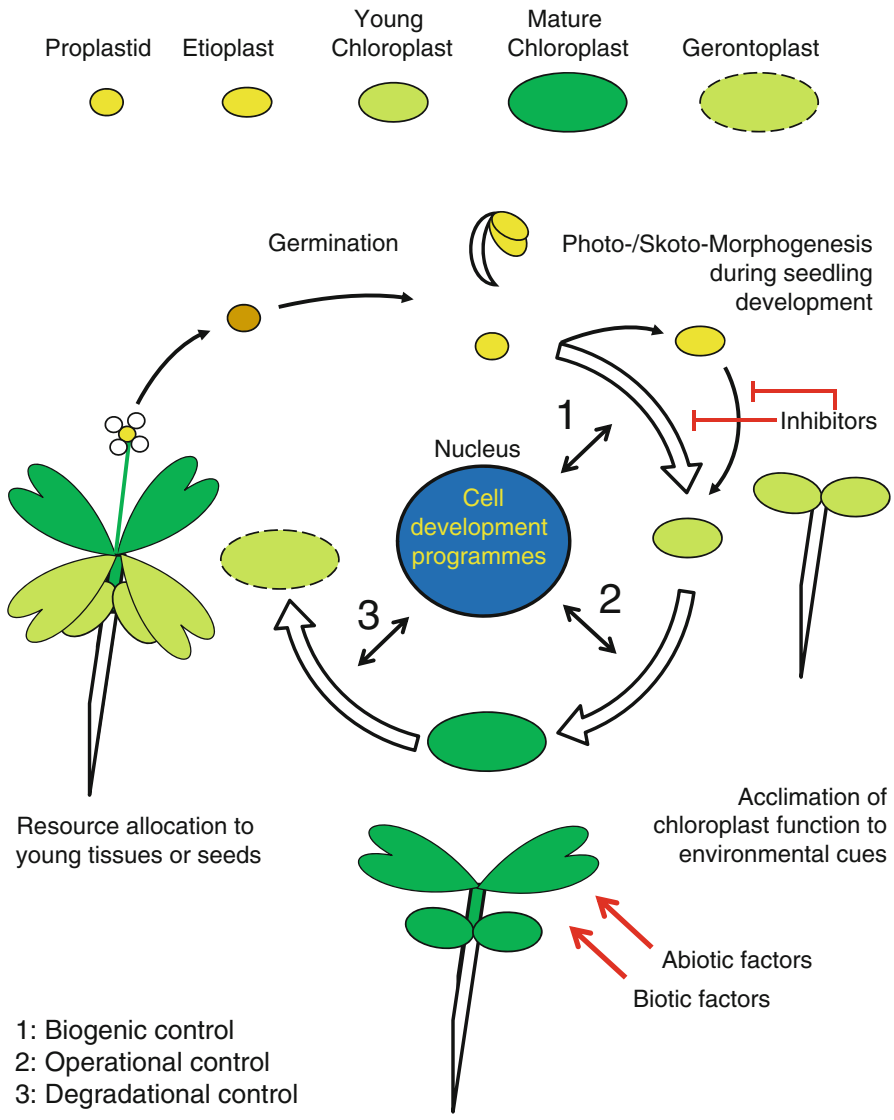

Fig. 22.1. **Overview on the different types of plastid signaling during a plant's life cycle**. The scheme depicts the different stages of plant development starting from germination of a seed (*brown oval*) as a continuous cycle around the nucleus. The developmental stage of plastids (exemplarily given for chloroplasts) corresponding to the developmental stage of the leaves is given in between (plastid types are indicated on *top*). Proplastids develop either into etioplasts during skotomorphogenesis or into young chloroplasts during photomorphogenesis. Disturbance of proper plastid development in this stage by inhibitors (indicated in *red*) interferes with "biogenic control". Young chloroplasts, which develop into mature chloroplasts represent sensors for environmental changes and report abiotic or biotic influences on their function to the nucleus via "operational control". During senescence of a tissue or a complete plant, resources from plastids are allocated to younger parts or seeds requiring a "degradational control".

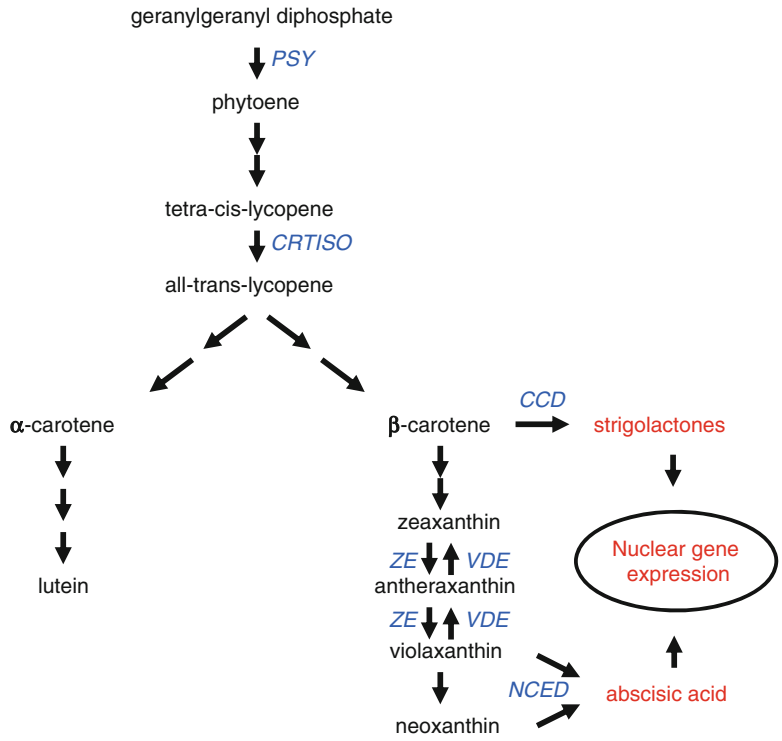

Fig. 22.2. **Signals derived from carotenoid biosynthesis**. Abscisic acid and strigolactones, key signals in abiotic stress resistance and shoot branching regulation, result from the cleavage of carotenoids by specific 9-*cis* epoxycarotenoid dioxygenase (NCED), which uses ascorbate as a cofactor, or carotenoid cleavage dioxygenase (CCD) enzymes. Violaxanthin de-epoxidase (VDE), essential for the formation of zeaxanthin, a blue-light photoreceptor in guard cells, also uses ascorbate as a cofactor. Zeaxanthin epoxidase (ZE), which reverts the reaction, is needed to provide the substrate (violaxanthin or neoxanthin) for abscisic acid biosynthesis. Carotenoid isomerase (CRTISO), which catalyzes the isomerization of tetra-*cis*-lycopene to all-*trans*-lycopene, and phytoene synthase (PSY) are two additional key regulatory steps in the carotenoid biosynthetic pathway leading to the plastid-derived signals, abscisic acid and strigolactones.

response, thus confirming previous evidence obtained with isolated stomata (Talbot et al. 2006). *npq1* stomata, which lack violaxanthin de-epoxidase (VDE) activity and therefore cannot synthesize zeaxanthin, are devoid of a specific blue light response. In contrast, stomata from intact leaves of *phot1 phot2* double mutants, which lack the blue-light photoreceptor phototropin, had a reduced but readily detectable response to the removal of green light and to blue and green pulses. Ascorbate acts as a cofactor of VDE playing an indirect role in stomatal opening through zeaxanthin formation, but at the same time controls H_2O_2 levels, which have been shown to be required for ABA-induced stomatal closure (Zhang et al. 2001). Other carotenoids have also been proposed to serve in blue-light stomatal opening, such as retinal. Indeed, retinal and two rhodopsin-like retinal-binding proteins have been suggested to be involved in stomatal response to blue light in *Commelina communis* (Paolicchi et al. 2005). However, further research is needed to confirm these studies in other species and its relationship and possible overlap with zeaxanthin function in blue-light photoreversible stomatal opening. Whether the zeaxanthin mediated signaling also involves expression changes of nuclear genes, is not known yet.

In addition, carotenoids serve as substrates for the production of phytohormones

and other important signaling molecules such as (1) abscisic acid (ABA), whose biosynthesis is induced under water deficit and plays a key role in stomatal closure, (2) strigolactones, which have recently been described as important regulators of shoot branching in several species, as well as (3) other signaling molecules (e.g. blumenin and mycorradicin), which are involved in plant-fungus interactions in the soil.

ABA mediates responses to environmental stresses, notably the control of stomatal aperture and transpiration during drought, and modulates several developmental processes such as seed maturation and dormancy (Seo and Koshiba 2002; Nambara and Marion-Poll 2005; Chinnusamy et al. 2008). But, how can carotenoids mediate blue light-mediated opening of stomata in the early morning, when the light spectrum is enriched in this wavelength, and indirectly stimulate ABA mediated stomatal closure during drought? First, it should be considered that drought-stressed plants open stomata at early morning and close when the evaporative demand is increasing, particularly at midday and during the afternoon. Second, ABA is a cleavage product of 9-*cis*-violaxanthin and/or 9′-*cis*-neoxanthin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) in mesophyll cells, which was first identified in the maize *viviparous14* (*vp14*) mutant (Schwartz et al. 1997; Tan et al. 1997; Seo and Koshiba 2002; Chinnusamy et al. 2008); while blue light-stimulated stomatal opening is mediated by zeaxanthin in guard cells (Zeiger et al. 2002; Talbot et al. 2006). It appears, therefore, that different cellular locations account for different processes. While ABA stimulates stomatal closure in guard cells, ABA derived from carotenoids appears to be formed in mesophyll cells of transpiring leaves. It should also be noted that zeaxanthin is needed for photoprotection in mesophyll cells (Demmig-Adams and Adams 2000). Therefore, how is violaxanthin de-epoxidized to zeaxanthin if violaxanthin is also the substrate for ABA biosynthesis? Here, both processes can occur in the same cellular location (mesophyll cells), but it should be considered that mesophyll cells can also receive a substantial part of ABA from the xylem of roots and from the vascular tissue (Christmann et al. 2005; Zhang et al. 2009). In addition, the violaxanthin (or neoxanthin) needed for ABA biosynthesis is at least two orders of magnitude lower than that used for photoprotection, where zeaxanthin acts as a photosynthetic pigment. Therefore, the synthesis of ABA by carotenoids does not really limit the photoprotective function of zeaxanthin in mesophyll cells and can occur simultaneously with the function of this blue-light photoreceptor in guard cells. In addition, zeaxanthin could exert an antioxidant role in thylakoid membranes of mesophyll cells, thus participating together with other lipophilic antioxidants (such as β-carotene and tocopherols) in the control of ROS signaling from plastids. Finally, it also appears that ABA and zeaxanthin interact in the control of stomatal opening. It has been shown that enhanced ABA levels can inhibit the blue light–stimulated stomatal opening. ABA inhibits proton pumping and potassium uptake by guard cells, which are central to blue light-induced stomatal opening (Parvathi and Raghavendra 1997). Therefore, ABA and zeaxanthin seem to share the same signal transduction pathway to modulate stomatal opening. Zeaxanthin may be responsible for stomatal opening during the early morning, while ABA helps closing stomata when the evaporative demand increases during midday and the afternoon, or in plant responses to water deficit. We are still, however, far of identifying the effects of zeaxanthin in nuclear gene expression in guard cells. Another interesting interaction in plastid-derived signals is suggested by the recent finding that the Chl-H subunit of Mg-chelatase (CHLH), which is a key component in both chlorophyll biosynthesis and plastid-to-nucleus signaling (*gun5* mutants), can act as an ABA receptor involved in stomatal closure as well as a regulator of ABA-responsive genes via control of WRKY transcription repres-

sors (Shen et al. 2006; Wu et al. 2009; Shang et al. 2010). Although this observation is debated since other ABA receptors have been reported (McCourt and Creelman 2008; Tsuzuki et al. 2011), it demonstrates the high connectivity between different types of plastid signals. Another example for this notion with respect to carotenoids is the observation that the expression of zeaxanthin epoxidase and beta carotene hydroxylase is correlated to the redox state of the plastoquinone pool. In addition, a block in photosynthetic electron transport was reported to down-regulate the violaxanthin de-epoxidase (Woitsch and Römer 2003). Furthermore, it could be shown that the AP2-transcription factor ABI4 (ABA insensitive 4) recognizes specific boxes in the promoter regions of *RbcS* and *Lhcb* genes supporting the idea that ABA-mediated signals interact with the *gun* signaling pathway at the promoter level (Acevedo-Hernandez et al. 2005; Koussevitzky et al. 2007).

Carotenoids can play additional roles in signaling in other plant locations with other specific and important functions for the plant. For instance, in the synthesis of strigolactones, which in turn inhibit shoot branching, presumably by inhibiting bud outgrowth (Gómez-Roldán et al. 2008; Umehara et al. 2008), influence mycorrhizal hyphal branching in order to stimulate a symbiotic relationship in the root rhizosphere (Akiyama et al. 2005) and may also promote germination of parasitic plant seeds such as Striga (Matusova et al. 2005). Strigolactones are presumably derived from β-carotene through the action of 9-*cis* epoxycarotenoid dioxygenase (NCED), which uses ascorbate as a cofactor, or carotenoid cleavage dioxygenase (CCD) enzymes (Matusova et al. 2005; López Ráez et al. 2008; Rani et al. 2008). It has been shown that the maize mutant *vp14*, mutated in an *NCED* gene, induced less germination of the seeds of the parasitic plant *Striga hermonthic* (Matusova et al. 2005). It has also been demonstrated that the tomato mutant *notabilis*, with a null mutation in the gene *LeNCED1*, produces 40 % less strigolactones, suggesting that the enzyme NCED1 is involved in the biosynthesis of strigolactones (López Ráez and Bouwmeester 2008). However, these authors did not observe any increase in expression of *LeNCED1* under phosphate starvation, which led them suggest that NCED1 is neither a regulatory step in the biosynthesis of strigolactones nor is regulated post-transcriptionally. Since NCEDs are known to be involved in the biosynthesis of ABA, and indeed *notabilis* contains 40 % less ABA than the corresponding wild-type (Burbidge et al. 1999), it has been proposed that a reduced production of strigolactones by this mutant may therefore also be due to a reduced ABA content. The other class of carotenoid cleavage enzymes that has been hypothesized to be involved in the biosynthesis of strigolactones is the CCD family (Auldridge et al. 2006; Bouwmeester et al. 2007). CCDs are involved in the formation of flavour volatiles such as cyclohexenone derivatives and mycorradicin, which are involved in fungus colonization of roots during micorrhiza formation (Booker et al. 2005). Furthermore, mutants of SDG8 (*ccr1*) and CRTISO (*ccr2*) show an increase in shoot branching, which may be partly explained by limited synthesis of strigolactones (Cazzonelli et al. 2009, 2010). Carotenoid isomerase (CRTISO) catalyzes the isomerization of tetra-*cis*-lycopene to all-*trans*-lycopene while CRTISO is regulated by the histone methyltransferase, SET DOMAIN GROUP8 (SDG8). Therefore, studies in these mutants further support a role for carotenoids in strigolactone biosynthesis. It should be, however, also considered that CRTISO is affecting both β-carotene (the presumed strigolactone precursor) as well as violaxanthin and neoxanthin levels (the ABA precursors), which does not exclude the possibility of an additional indirect effect of ABA on the observed effect on stigolactone biosynthesis in the *ccr1* and *ccr2* mutants.

Finally, blumenol (a C13 cyclohexenone derivative), and mycorradicin (a C14 apocarotenoid), play important roles in arbuscular

mycorrhizal fungi symbiosis (Giuliano et al. 2003; Akiyama et al. 2005; Walter et al. 2010). Also, by using the bypass (*bps*) mutant of *Arabidopsis thaliana*, a still unidentified graft transmissible β-carotene-derived signal has been reported to be required for root and shoot development. However, the nature of this novel compound remains elusive, although it is known that it does not require the activity of any CCD (Van Norman and Sieburth 2007).

D. Metabolites as Signals

Plastids not only represent a highly important compartment for energy metabolism, they are also connected to most metabolic pathways in the cell. Besides the various precursor molecules mentioned above they do exchange numerous metabolites with the cytosol via specific transporters in the envelope (Weber and Linka 2011). Any functional change in the plastid is reflected in the metabolite fluxes within the organelle and, in principle, should be also mirrored in the exchange rates. It is, thus, possible that each environmental situation creates a specific metabolite signature which could serve as a plastid signal (Pfannschmidt 2010). This hypothetical scenario, however, requires that the metabolites do not only affect biosynthesis pathways in the cytosol, but that they act as active signals having a direct impact on gene expression. Precisely this could be just recently shown for the phosphonucleotide 3′-phosphoadenosine 5′-phosphate (PAP) (Estavillo et al. 2011). PAP accumulates in plastids in response to stresses such as high light and drought, moves to the nucleus and regulates stress-responsive genes by inhibition of nuclear exoribonucleases. PAP accumulation, in turn, is counteracted by the phosphatase Sal1 which localizes both to chloroplasts and mitochondria. It is yet not known whether and how Sal1 functions as a stress sensor, but the study demonstrates for the first time that a metabolite pool size in the plastid has a direct regulatory impact on nuclear gene expression.

IV. Signals from Plastids During Senescence: Degradational Control

Leaf senescence is an orderly, active process in which nutrients and photoassimilates in a leaf are mobilized through the phloem to other parts of the plant (e.g. the youngest leaves, flowers or fruits) involving dramatic changes in gene expression (Smart 1994; Dangl et al. 2000; Munné-Bosch 2008). The loss of chloroplast integrity can be observed at the early stages of senescence associated with such remobilization, so that one of the most remarkable events during leaf senescence is the disassembly of the photosynthetic apparatus, which consequently leads to a decrease in photosynthetic activity and consequent changes in plastid metabolism and signaling (Dangl et al. 2000). This also includes the targeted degradation and breakdown of chlorophylls (for details refer to Chap. 16 by Hörtensteiner in this volume). Such catabolites are exported from the senescing chloroplast in order to reallocate the nitrogen and carbon resources, but are also shown to be able to induce cell death (Mur et al. 2010; Hörtensteiner and Kräutler 2011). Thus, they represent a potent plastid signal indicating the process of plastid aging. Whether such catabolites could be also active in tetrapyrrole dependent plastid signaling in mature plastids remains to be elucidated, but provides an interesting possibility for resolving many contradictions concerning the tetrapyrrole signaling pathways (compare Sect. III.B). Although senescence generally leads to leaf death and it sometimes is regarded as an inevitable and negative consequence of stress, senescence of mature leaves may be essential for maintaining the function of young leaves and therefore for plant survival during prolonged periods of stress (Munné-Bosch and Alegre 2004). Phytohormones and other regulators derived at least in part from plastid metabolism can play an important role in senescing leaves. ABA biosynthesis starts from NCED activity in chloroplasts and continues later in the cytosol to affect several nuclear genes in senescing leaves. Other plant growth regu-

lators are also derived from plastid signals, such as jasmonic acid (JA), which is primarily formed as a result of lipid peroxidation in plastids, or salicylic acid (SA) which can be entirely synthesized in plastids in the so-called bacterial isochorismate synthase pathway. The role of ABA, JA and SA as plastid signals during leaf senescence has been studied in detail in the model plant *Arabidopsis thaliana* using mutants that show a defect in hormone biosynthesis or signaling, as well as in other plant species through correlation studies.

A central role for ABA in senescing leaves in plants exposed to abiotic stresses such as water deficit, salt, heat or cold stress has been long recognized. Stomatal closure under abiotic stress-induced senescence is triggered by an ABA-induced increase in guard cells (Pei et al. 2000). Growth arrest, which is a general response of plants to stressful conditions and a pre-requisite for senescence to occur, is considered as a strategy to sustain metabolism under an impaired photosynthetic energy supply. ABA induces the expression of *ICK1* (inhibitor of cyclin-dependent kinase 1), which will lead to the inhibition of cyclin-dependent kinases (CDKs) and therefore of cell division (Zhou et al. 2003). Furthermore, ABA has been shown to induce several senescence-associated genes, thus confirming the role of ABA in the regulation of this process (reviewed in Lim et al. 2007). It should, however, be considered that ABA is involved in the progression of leaf senescence, but it does not appear to trigger the process.

In higher plants, it is well established that SA derives from the shikimate-phenylpropanoid pathway (Métraux 2002). Two routes from phenylalanine to SA have been described in plants, one cytosolic and the other operating in plastids. Wildermuth et al. (2001) were the first to map the SA-induction-deficient *sid2* mutation to a gene (*ICS1*) encoding isochorismate synthase found in plastids. The level of SA after infection in *sid2* mutants is only 5–10 % of the wild-type levels and resistance to fungal or bacterial pathogens is reduced, thus suggesting that plants produce significant amounts of SA from isochorismate, a biosynthetic pathway typical for bacteria. It was also shown that this pathway is also important for SA biosynthesis in seeds and in leaves, both under salt stress and during whole-plant and leaf senescence (Abreu and Munné-Bosch 2009; Asensi-Fabado and Munné-Bosch 2011). It has been shown that enhanced SA levels accelerate the developmental program in *A. thaliana* at the expense of reducing plant growth and seed production, so that rapid reproduction is achieved at the cost of reducing seed yield in this short-lived species (Abreu and Munné-Bosch 2009). SA mediates inhibition of cell division and elongation in *siz1* mutants of *A. thaliana* (being impaired in SUMO E3 ligase) through a down-regulation of expression of XTH8 and XTH31 genes, which encode xyloglucan endotransglycosylase/hydrolases necessary for cell wall loosening and reorganization (Miura et al. 2010). Furthermore, it has been shown that this compound has a role in the regulation of nuclear gene expression during leaf senescence (Morris et al. 2000; Buchanan-Wollaston et al. 2005) and that SA accelerates the latest stages of senescence inducing cell death (García-Heredia et al. 2008). Comparison of changes in gene expression patterns during natural, dark-induced or starvation-induced senescence in *A. thaliana* has shown not only similarities but also considerable differences. For instance, it was found that developmental (natural) leaf senescence, but not dark-induced senescence, is delayed in plants defective in the SA pathway.

JA effects on senescing leaves results also from degradational processes in plastids, since JA is synthesized as a result of lipid peroxidation in chloroplasts. Lipid peroxidation starts by generating a radical, generally an alkyl radical from an (poly)unsaturated fatty acid (PUFA) by the action of activated oxygen (non-enzymatic lipid peroxidation) or by the action of lipoxygenases (enzymatic lipid peroxidation). The resulting alkyl radical formed is highly reactive and combines with molecular oxygen to give rise to peroxy radicals. These can abstract the hydrogen

from PUFAs and give rise to lipid hydroperoxides and new alkyl radicals, which most importantly will propagate the reaction chain. As a result, lipid peroxidation occurs in cascades, and hydroperoxides can be converted, among other products, to JA by allene oxide synthase and other enzymes found in three different compartments – chloroplasts, cytoplasm and peroxisomes – to finally modulate gene expression in the nucleus (Schaller 2001; Munné-Bosch and Alegre 2002). It has been shown that elevated levels of JA are needed to promote leaf senescence in *A. thaliana* (He et al. 2001, 2002). As it occurs with ABA and SA, JA is a growth inhibitor and therefore can contribute to growth arrest, a pre-requisite for leaf senescence to occur. Furthermore, gene expression analysis in senescing leaves of plant lines defective in signaling pathways involving SA, JA and ethylene have revealed that these three pathways are also all required for expression of many genes during developmental senescence (Buchanan-Wollaston et al. 2005). Therefore, signals derived from plastids, such as ABA, SA and JA, in cooperation with other hormones, such as the senescence promoting ethylene and the senescence-inhibitor cytokinins, regulate the progression of senescence in leaves and coordinate the concomitant changes in gene expression.

V. Conclusions

Plastids are found in all plant cells and develop into specific forms corresponding to the tissue context of their host cell. By this means, the developmental stage of plastids represents a reporter for the developmental stage of the tissue and, subsequently, of the plant. During the life cycle of a plant, plastids will change their structural and functional properties. The latter are a comprehensive source of information since plastid function is tightly connected to the surrounding cell as well as to the environment. Numerous plastid functions are influenced by environmental variations and report on these changes to the nucleus which in turn initiates appropriate acclimation responses. Therefore, the mutual communication between nucleus and plastids integrates import information which is essential for development and growth in all stages of the life cycle of a plant.

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Chapter 23

Chloroplast Control of Leaf Senescence

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Summary

Chloroplasts have an active role in the induction and course of senescence through signals, derived from reactive oxygen species (ROS), exiting to the cytosol. In this way, leaf chloroplasts mimic the role of mitochondria in the apoptosis of non-photosynthetic organs. Oxylipins, magnesium-protoporphyrin IX (Mg-ProtoIX) and proteins are the best studied signal candidates that exit from chloroplasts that, through a poorly-understood network of transduction intermediates, inhibit the expression of genes encoding components of the photosynthetic machinery and activate the expression of senescence-associated genes (*SAG*) in the nucleus, ultimately resulting in activation of programmed cell death (PCD). There are many similarities in the mechanisms of production and scavenging of ROS in chloroplasts during stress defense and in senescence. However, there are also differences and one significant difference is that the feed-back effect of chloroplast signals on the ROS level in

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defense responses changes to a feed-forward effect that favors further increases of ROS in the PCD associated with leaf senescence or the hypersensitive response (HR). Therefore, a key question related to the induction of leaf senescence resides in the molecular basis of the shift from a feed-back to a feed-forward suicide response of the network that regulates the chloroplast ROS level. Up-regulation of nuclear genes encoding components of the thylakoid Ndh complex and of lipoxygenase (LX) and down-regulation of the nuclear genes for chloroplastic superoxide dismutase (SOD) seem to be among the changes responsible for the feed-forward increase of ROS associated with (and probably determining) PCD, but further investigations are needed to identify the intermediates and their cause-effect relations in the signal transduction network. These investigations must include hormones, H_2O_2, proteins and microRNAs, whose levels and interactions are also affected by environmental factors acting inside and outside the chloroplast.

I. Introduction

The decay of chloroplast structure and function is the most prominent feature of leaf senescence, which is visibly associated with the loss of chlorophyll. Chloroplasts usually contain, mainly as protein, more than 50% of the leaf nitrogen (Dalling 1985), which is remobilized during senescence to young and developing structures after conversion to exportable forms such as asparagine and glutamine (Morita 1980; Feller and Fischer 1994). Proteases, RNAses, chlorophyllases, amino acid reconversion enzymes and, in general, proteins involved in the disassembly of chloroplasts have been implicated in leaf senescence and the encoding genes have been identified. Most of these genes are located in the nucleus and the encoded proteins are targeted to chloroplasts or to the vacuole, where the degradation process is somehow completed, which suggested that leaf senescence is primarily controlled at the nuclear level. An extensive body of scientific literature describes the advances during the last quarter of the twentieth century of the knowledge of the molecular and ultrastructural changes in senescent chloroplasts and the hormonal and environmental factors that control them (Beevers 1976; Thimann 1978; Thomas and Stoddart 1980; Woolhouse 1984; Sabater 1985; Leshem et al. 1986; Thompson et al. 1987; Noodén et al. 1990; Smart 1994; Matile 2001; Noodén 2004).

At the end of the twentieth century, increasing evidence indicated that the role of chloroplasts in leaf senescence is not completely passive and that a complex interplay between nucleus, chloroplast and cytoplasm is involved in the induction and course of leaf senescence (Chen and Dickman 2004; Zapata et al. 2005; Yao and Greenberg 2006; Wright et al. 2009; Zuppini et al. 2009; Chen et al. 2010; Doyle et al. 2010; Mohapatra et al. 2010; Van Doorn and Yoshimoto 2010). Similarities between animal cell apoptosis and plant cell senescence became increasingly evident, and the chloroplast has emerged as the organelle in mesophyll cells performing a similar role as mitochondria in the programmed cell death (PCD) of heterotrophic cells. Reactive oxygen species (ROS) and other signals generated in chloroplasts

Abbreviations: ABA – Abscisic acid; ^{3}Chl* – Triplet excited chlorophyll; *Csd2* – Gene encoding the chloroplastic Cu/ZnSOD; HR – Hypersensible response; JA – Jasmonic acid and derivatives; LX – Lipoxygenase; Mg-ProtoIX – Magnesium-protoporphyrin IX; Ndh – Thylakoid NADH dehydrogenase complex; *ndh* – Genes for components of the thylakoid Ndh complex; NPQ – Non-photochemical quenching; $^1O_2$ – Singlet oxygen; $O_2^{\bullet -}$ – Superoxide anion radical; PCD – Programmed cell death; PET – Photosynthetic electron transport; POS – Photooxidative stress; PPR – Pentatricopeptide-repeat; PRDX – Peroxiredoxin; PSI (II) – Photosystem I (II); PX – Peroxidase; ROS – Reactive oxygen species; *SAG* – Senescence-associated genes; SAR – Systemic acquired resistance; SOD – Superoxide dismutase; TO – Terminal oxidase

seem to play key roles in leaf senescence, the discussion of which will be the focus of this chapter.

II. Reactive Oxygen Species (ROS), Stress and Leaf Senescence

A. ROS in Leaf Senescence and Stress Responses

Increased levels of ROS and of related free radicals during leaf senescence were recognized many years ago (Dhinsa et al. 1981; Leshem et al. 1981; Leshem 1984; Kar and Feierabend 1984) and are generally assumed to facilitate the associated degradation processes (Mehta et al. 1992). Later, evidence accumulated indicating an additional inductive role of ROS in senescence (Burgess and Taylor 1987) and, in general, as cell signals regulating the expression of genes involved in senescence and in the response to different stresses (Martín et al. 1996; Potikha et al. 1999; Karpinski et al. 2001; Van Breusegem et al. 2001; Apel and Hirt 2004; Zimmermann and Zentgraf 2005; Gechev et al. 2006; Zentgraf 2007). ROS scavenging enzymes were found to decrease during senescence (Casano et al. 1994, 1999), which suggested a similarity between leaf cell senescence and the mitochondrial- and ROS-mediated mechanisms of PCD in animal systems (Kane et al. 1993; Orr and Sohal 1994; Green and Reed 1998). Subsequently, a number of nuclear and chloroplast genes for enzymes and transcription-related factors were found to be expressed during senescence and stress responses (Lim et al. 2007). Nowadays, it is generally accepted that mechanisms determining the initiation and the development of leaf senescence are linked to the control of ROS generation and scavenging.

1. ROS Production and Scavenging in Chloroplasts

As in mitochondria, the superoxide anion radical ($O_2^{\bullet -}$) as a major ROS is produced in the chloroplast, mainly by the transfer of one electron from reduced iron-sulfur proteins to O_2 (Mehler reaction). Subsequent enzymic and nonenzymic reactions produce other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO•) and different peroxi-organic derivatives. In addition, the chloroplast produces singlet oxygen ($^1O_2$) by excitation transfer from triplet excited chlorophyll (^{3}Chl*) to O_2 (Fig. 23.1). These processes, linked to reduced components of the photosynthetic electron transport (PET) chain and to excited pigments, make chloroplasts the main source of ROS in the leaf (Karpinski et al. 2001). Excess light, above the capacity to consume the produced NADPH (e. g., due to low temperature, CO_2 supply or high light intensity), increases the steady-state level of reduced electron transporters and of excited pigments, thereby favoring the production of ROS that destroy different cell components (photooxidative stress: POS). Chloroplasts have evolved both enzymic and nonenzymic mechanisms that scavenge ROS and mitigate this situation. In chloroplasts, ROS are produced, albeit at a low rate, even under non-stressing conditions, but scavenging of $O_2^{\bullet -}$ and H_2O_2 by superoxide dismutase (SOD) and peroxidase (PX) maintains a low steady-state level of ROS.

Figure 23.1 shows the main pathways of ROS generation and scavenging in chloroplasts (a) and their primary deleterious effects (b). $^1O_2$, $O_2^{\bullet -}$ and HO• are short-lived and mainly damage chloroplast components. H_2O_2 is more stable and may penetrate membranes; therefore it can damage extrachloroplastic components. The breakdown of lipids, nucleic acids and proteins increases during senescence due to an increase of the steady-state levels of ROS, due to changes in the rates of production and scavenging.

Probably, the drainage of electrons from PET by photorespiration decreases the production of ROS (Douce and Heldt 2000); however, the photorespiratory oxidation of glycolate to glyoxalate produces H_2O_2 in peroxisomes (Levine 1999). The mobilization of phosphorylated light-harvesting complex II from PSII to PSI at high light intensities decreases the entry of electrons into PET by

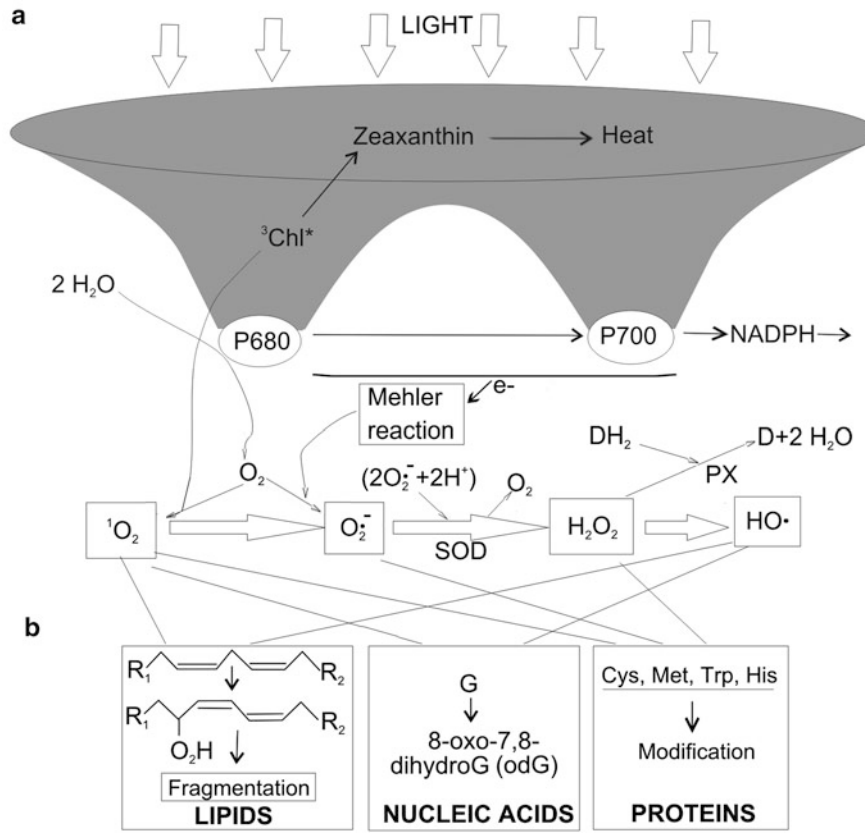

Fig. 23.1. Reactive Oxygen Species (ROS) generation, scavenging and damage effects in chloroplasts. (**a**) Main ROS generating and scavenging processes in chloroplasts. Triplet excited chlorophyll ($^3Chl^*$) can transfer excitation to oxygen to produce singlet oxygen ($^1O_2$) which, by interaction with diverse molecules, can be transformed to the anion radical superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ can also be formed in the Mehler reaction by the transfer of one electron to O_2, mainly from reduced iron-sulfur proteins. In a reaction catalyzed by superoxide dismutase (SOD), two $O_2^{\bullet-}$ ions plus two protons produce H_2O_2 and O_2. In reactions catalyzed mainly by divalent cations, H_2O_2 decomposes to two hydroxyl radicals (HO•). (**b**) Main damage targets of ROS. $^1O_2$, directly, and HO• with oxygen transform 1,4-unsatured fatty acids to hydroperoxi-derivatives ($-O_2H$) which further undergo different transformations, including fragmentations that disassemble membranes. Again, mainly $^1O_2$ and HO• modify bases in DNA, RNA and free bases, especially guanine (G) which is transformed to 8-oxo-7,8-dihydro guanine (odG), which can be paired with C or A, producing erroneous proteins and mRNA and DNA mutations. Most of the amino acids, whether free or in polypeptide chains, can be modified by ROS; cysteine is especially sensitive to $O_2^{\bullet-}$ and H_2O_2; cysteine, methionine, tryptophan and histidine are especially sensitive to $^1O_2$.

lowering the exciton transfer to PSII (Ohad et al. 2001) and could also decrease the production of ROS. Moreover, photooxidative stress (POS) triggers (Karpinski et al. 2001) the photoinhibition of PSII (Osmond 1994), which, in a feed-back action decreases the entry of electrons in PET and the production of ROS. As indicated, the protection against POS by ROS scavengers is mainly provided by SOD and PX activities (Fig. 23.1). At least one PX activity uses reduced plastoquinone as an electron donor (Casano et al. 1999, 2000) and, hence, in addition to scavenging H_2O_2, it also drains excess electrons from PET. At relative high light intensities, the xanthophyll violaxanthin is converted to zeaxanthin, which drains excitons from chlorophylls and dissipates them as heat (Fig. 23.1), decreasing the formation of $^1O_2$ and constituting the main factor responsible

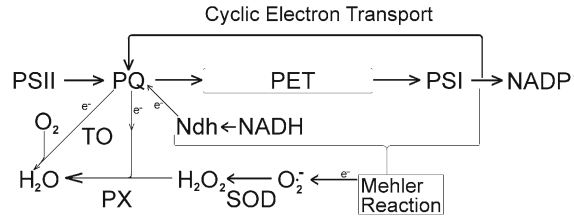

Fig. 23.2. Chlororespiratory electron transport. Proposed chlororespiratory electron transport that poises the redox level of cyclic electron transporters by feeding NADH electrons by the Ndh complex and draining electrons by Mehler reaction, TO and plastoquinol PX; the latter uses the H_2O_2 formed by SOD from $O_2^{\bullet -}$ generated by Mehler reaction. Electron feeding and draining reactions are not simultaneous; they take place under relatively low activities of PSII and PSI, respectively.

for the non-photochemical quenching (NPQ) of the chlorophyll fluorescence. When low light intensities return, zeaxanthin is again converted to violaxanthin (that cannot dissipate energy), thus completing the xanthophyll cycle (Eskling et al. 2001). Therefore, the xanthophyll cycle has a key role in terrestrial plants (Külheim et al. 2002) subjected to rapid fluctuations of light intensity (Pearcy 1994; Martín et al. 2009). Obviously, all or some of the critical factors that adjust the ROS in chloroplasts to a low level collapse during leaf senescence.

The heat dissipation by zeaxanthin requires a proton gradient (ΔpH) across the thylakoid membrane (Eskling et al. 2001), which is also required for photophosphorylation. Plausibly, the cyclic PET sustains the necessary ΔpH with the lack of CO_2 (by stomata closing) or a strong photoinhibition of PSII drastically decreases the acyclic PET. However, as pointed out by Heber and Walker (1992), the functioning of cyclic PET requires the balancing of the intermediary transporters. When the electron transporters are over-reduced, the generation of $O_2^{\bullet -}$ by the Mehler reaction seems to be the main valve that drains the excess of electrons (Biehler and Fock 1996). The scavenging of H_2O_2 by plastoquinol PX could also drain excess electrons (Casano et al. 2000). Therefore, the generation of $O_2^{\bullet -}$ and H_2O_2 would be a less harmful alternative in POS, due to the fact that by maintaining an active poised cyclic PET, it favors ΔpH and NPQ, decreasing the formation of $^1O_2$. The collapse of ΔpH by a low rate of cyclic PET would also occur when transporters become over-oxidized by a sudden transition to low light after PSII photoinhibition by high light intensities. To prevent this situation, the redox poising of transporters seems to be restored by feeding electrons from NADH by the thylakoid Ndh complex (EC 1.6.5.3) (Casano et al. 2000), a product of the plastid *ndh* genes. Accordingly, the NPQ is impaired in *ndh*-defective mutants (Burrows et al. 1998; Martín et al. 2004) and the balancing of the redox state of cyclic electron transporters is achieved by a chlororespiratory electron transport chain (Casano et al. 2000; Joët et al. 2002; Nixon and Rich 2007; Martín et al. 2009; Martín and Sabater 2010) that provides electrons from NADH to plastoquinone through the Ndh complex, and, operating discontinuously in time, drains electrons through the concerted actions of the Mehler reaction, SOD, plastoquinol PX (Casano et al. 2000) and the plastid terminal oxidase (TO) (Rumeau et al. 2007) as shown in Fig. 23.2.

Under field conditions, high light intensities and fluctuations, extreme temperatures, water deficit, contaminants (such as ozone, O_3) rapidly change the rates of electron supply (from PSII) and consumption (by Calvin-Benson cycle), therefore requiring rapid redox balancing to maintain the cyclic PET functional and minimize photooxidative damage. Accordingly, the level of the components of the chlororespiratory chain (Ndh complex, SOD, PX and TO) increases in chloroplasts

when plants are exposed to stressing agents (Casano et al. 1994, 1999; Martín et al. 1996, 2004; Endo et al. 1999; Prochazkova et al. 2001; Rumeau et al. 2007; Serrot et al. 2008). Catalase is not present in chloroplasts, but cytoplasmic catalase activities could contribute to scavenge H_2O_2 released from chloroplasts. Although chloroplast ascorbate peroxidase could also be involved in ROS control (Asada 1999), it is rapidly inactivated by H_2O_2 (Mano et al. 2001). The 2-cysteine peroxiredoxin (PRDX), an abundant thiol peroxidase of chloroplasts, could also be involved in the protection of photosynthesis as an H_2O_2 scavenger and a redox signal (Dietz 2003; Muthuramalingam et al. 2009).

2. ROS Levels During Biotic Stress and Senescence

During the last decade of the twentieth century, significant evidence accumulated for the involvement of ROS in the responses of plants to abiotic and biotic stresses. Frequently, the plant-pathogen interaction results in a burst of ROS that destroys the invading microorganism and, sometimes, the surrounding plant tissue in a hypersensitive response (HR) where damage by H_2O_2 and, especially, $O_2^{\bullet -}$ is linked to the activation of PCD (Lam et al. 2001; Epple et al. 2003). ROS, especially H_2O_2, and derived products act as signals that induce PCD in the immediate tissue and, frequently, the systemic acquired resistance (SAR), by which distant tissues develop defenses against pathogens and predators. The similarities between HR, PCD and leaf senescence were highlighted (see, e.g.: Levine 1999; Van Breusegem et al. 2001; Overmyer et al. 2003) and prompted investigations on the mechanisms that trigger the production of ROS by chloroplasts during leaf senescence and on the mechanisms through which ROS and ROS-derived signals activate PCD (Casano et al. 1994, 1999, 2001; Abarca et al. 2001a, b; Zapata et al. 2005; Zimmermann and Zentgraf 2005; Gechev et al. 2006).

ROS are also probably involved in dark-induced leaf senescence (Becker and Apel 1993; Fischer-Kilbienski et al. 2010) in mesophyll cell necrosis induced by the phytotoxin tenuazonic acid (Chen et al. 2010) and in the PCD of non-photosynthetic tissues, such as those of tracheary element differentiation (Potikha et al. 1999), root hypoxia (He et al. 1996), UV-induced plant death (Gao et al. 2008) and cell cultures (Doyle et al. 2010). We shall refer here to the natural senescence of leaves in light and will discuss the chloroplast control of leaf senescence and the molecular basis of the increase of ROS and the induction of leaf senescence syndrome by ROS.

Nuclear and plastid genes encoding components of the photosynthetic machinery are repressed in leaf senescence, whereas those related to macromolecular breakdown and re-mobilization (senescence-associated genes: *SAG*) are induced (Thomas and Stoddart 1980; García et al. 1983; Guéra et al. 1989; Vera et al. 1990; Jayabaskaran et al. 1990; Bate et al. 1991; Tomás et al. 1992; Becker and Apel 1993; Biswal and Biswal 1999; He and Gan 2002; Chen et al. 2002a; Hayden and Christopher 2004; Jones 2004; Lim et al. 2007; Liu et al. 2008; Thomas et al. 2009). Thus, *SAG* became excellent tools to investigate the role of ROS in gene expression, their interactions with hormones and the chloroplast and nucleus interactions in the gene-controlled PCD process of senescence (Yen and Yang 1998; Overmyer et al. 2003; Morgan and Drew 2004).

3. Increased Production and Decreased Scavenging of ROS in Chloroplasts as Related to Leaf Senescence

The level of $O_2^{\bullet -}$ increases in illuminated chloroplasts during senescence (McRae and Thompson 1983), and both increased production and decreased scavenging of $O_2^{\bullet -}$ seem to be the cause. In contrast to peroxisomal SOD, which increases (Del Rio et al. 1998), chloroplastic SODs are impaired during senescence. Several nuclear-encoded SODs (containing Fe, Cu, Zn or Mn ions) are present in cell compartments while Fe-SOD and Cu/Zn-SOD seem more frequently identified in chloroplasts. Transcripts and activities of chloroplastic SOD increase

when the leaves are subjected to different POSs (Bowler et al. 1992). However, the induction of SOD in response to stress, in contrast to chloroplastic and extrachloroplastic PX activities, becomes progressively impaired when leaves enter senescence (Casano et al. 1994, 1999; Kurepa et al. 1997; Abarca et al. 2001a; Prochazkova et al. 2001; Ohe et al. 2005). In animal systems, transgenic Drosophila over-expressing SOD and catalase show a significant life-span extension (Orr and Sohal 1994). Similar assays have not yet been reported on plant senescence, although transgenics over-expressing SOD targeted to chloroplasts show increased tolerance to POS (Bowler et al. 1992). The responses of transgenic plants overexpressing SOD, PX and/or catalase are sometimes difficult to interpret because a high SOD activity not accompanied by PX and catalase would result in an increase of H_2O_2, itself harmful and involved in defense or necrosis responses (Levine 1999; Quirino et al. 2000). Although the chloroplast is also the main compartment of H_2O_2 production in photosynthetic cells (Darehshouri and Lutz-Meindl 2010), its free diffusion suggests that different cell compartments contribute to scavenge H_2O_2. Evidences strongly suggest that in leaves (as in animal PCD), certain stages in the signal transduction chains that increase chloroplastic SOD under POS fail at the beginning of senescence, resulting in an increase of $O_2^{\bullet -}$, probably not accompanied by H_2O_2 (therefore avoiding further PET electron drain and defense responses). In this regard, the *Arabidopsis thaliana* ecotype Cvi, that has a different gene (*Csd2-2*) for chloroplastic Cu/ZnSOD from the Ler ecotype (*Csd2-1*), shows higher levels of mRNA and activity of Cu/Zn-SOD (Abarca et al. 2001b) and longer post-bolting rosette longevity (45 days) than Ler (24 days) (Luquez et al. 2006).

Related to ROS production, the ROS damage of Calvin-Benson enzymes (Mehta et al. 1992) increases the reduction level of electron transporters, favoring further production of ROS and induction of leaf senescence. Moreover, the increase of the thylakoid Ndh complex seems to be an earlier occurring event triggering ROS production and leaf senescence.

As pointed out in Sect. II.A.1, the thylakoid Ndh complex regulates the redox level of cyclic electron transporters by providing electrons that are removed by the Mehler reaction and the action of SOD, PX and TO to prevent the over-reduction of the transporters (Fig. 23.2). The level and the activity of the Ndh complex increases early during leaf senescence (Martín et al. 1996; Casano et al. 1999, 2000; Lascano et al. 2003), which strikingly parallels the increase of the mitochondrial complex I during human ageing (Pich et al. 2004). Both complexes increase the reduction level of electron transporters and, hence, the generation of ROS in the respective organelle, which, aggravated by the poor response of SOD in aged tissues, further enhances PCD processes.

Probably, the mitochondrial complex I increases in aged tissues to restore the rates of ATP production, impaired by the damage of components of the respiratory chain (Pich et al. 2004), not, as in young tissues, to cope with an increased ATP demand. Thus, a high level of complex I in aged tissues increases the reduction of electron transporters and the production of ROS but does not restore high ATP production, feeding a "vicious cycle" leading the cell to death. In chloroplasts, by feeding electrons, the Ndh complex contributes to optimize the cyclic PET (Fig. 23.2) to produce ATP and to decrease the production of $^1O_2$ (Sect. II.A.1). In young leaves, the components (Ndh, SOD and PX) of the chlororespiratory electron transport chain (Casano et al. 1999, 2000) increase to alleviate POS and/or low ATP levels. However, when the leaf ages and the induction of SOD fails, the low production of H_2O_2 impairs the electron drain by plastoquinol PX (Fig. 23.2) while the increase of the Ndh complex further over-reduces electron transporters, activating an autocatalytic cycle of production of $^1O_2$ and $O_2^{\bullet -}$ in chloroplasts. The consequences are a further decrease of ATP production, increased oxidative damage and increased levels of ROS-derived signals required for the expression of *SAG*. Accordingly, transgenic tobacco plants defective in the *ndhF* gene and Ndh

complex show an approximate 30-day-delay in leaf senescence with respect to the wild-type (Zapata et al. 2005). Significantly, all tested photosynthetic angiosperms showing developmentally-regulated leaf senescence contain plastid *ndh* genes that are, however, absent or accompanied by pseudogenes in several long-lived-leafy gymnosperms (Martín and Sabater 2010), as *Pinus longaeva* whose 35-year-old needles remain functional. The Ndh complex was found to be also essential for the senescence-like tomato fruit ripening (Nashilevitz et al. 2010). The persistence in aged leaves of the mechanisms that regulate the levels of the Ndh complex but not of SOD in chloroplasts leads the cell to a growing spiral of ROS production and death. Reactions that feed and remove electrons from the cyclic electron transport chain must be finely tuned, and so they are in young healthy cells (Casano et al. 1999). Sooner or later, a break of the balance favors electron-feeding reactions that trigger the autocatalytic generation of ROS, the loss of induction of chloroplastic SODs and PCD in leaves.

III. Nucleus-Chloroplast Interaction in the Control of Leaf Senescence

As explained above, levels of both SOD and the Ndh complex increase under oxidative stress and some clues can be deduced from the mechanisms controlling their responses to biotic and abiotic stresses. Although most Ndh subunits are encoded in the plastid genome SOD and a still unknown number of subunits of the Ndh complex are encoded in the nucleus (Martín and Sabater 2010). Therefore, the increases of SOD and Ndh complex in young leaves in response to the POS generated in chloroplasts must involve signals travelling from chloroplast to nucleus (Pfannschmidt and Munné-Bosch, Chap. 22).

A. Feed-Forward Control of ndh Gene Expression During Leaf Senescence

External application of 10 mM H_2O_2 increases the levels of *ndh* gene transcripts, protein and activity of the Ndh complex in leaf segments (Casano et al. 2001; Lascano et al. 2003). The activation of pre-existing complexes is achieved by Ca^{2+}-dependent phosphorylation of the threonine-181 of the Ndh-F subunit (Lascano et al. 2003; Martín et al. 2009). The effects of H_2O_2 mimic the stimulating effects of POS and senescence (Martin et al. 1996, 2009; Catalá et al. 1997; Casano et al. 2001). In young leaves, the stimulating effect of POS on the Ndh complex does not further increase ROS because it is accompanied by parallel increases of SOD and PX (Casano et al. 1999, 2000; Zapata et al. 2005) that scavenge $O_2^{\bullet -}$ and H_2O_2 and balance the redox level of cyclic electron transporters.

Favory et al. (2005) reported that the chloroplastic RNA polymerase requires the nuclear encoded sigma4 factor for the transcription of the plastid *ndhF* gene, suggesting that some *ndhF* product could in turn stimulate the expression of the other plastid *ndh* genes which, in the last instance, would be under nuclear control. Nuclear control could also act through the nuclear-encoded polypeptides of the Ndh complex. Further identification of intermediates (for example; hormones, ROS, trans- and cis-factor) of the stress transduction chain are required to clarify the nuclear control of *ndh* gene expression. Within chloroplasts, extensive evidence indicates that the expression of the *ndh* genes, as is the case of many other plastid DNA encoded genes (Bailey-Serres et al. 1999), is predominantly under post-transcriptional control (Del Campo et al. 2000, 2002; Serrot et al. 2008), through which the processing of the primary polycistronic transcripts increases the levels of translatable monocistronic *ndh* transcripts under stress conditions (Del Campo et al. 2006; Serrot et al. 2008).

The plastid DNA of most plants contains 11 *ndh* genes (Maier et al. 1995; Martín and Sabater 2010). The *ndhB* (repeated) and the *ndhF* genes are probably transcribed as monocistronic mRNAs. The other nine genes are aligned in two transcriptional units: the *ndhC-J* (for the *ndhC, ndhK* and *ndhJ* genes) and the *ndhH-D* (for the *ndhH, ndhA, ndhI,*

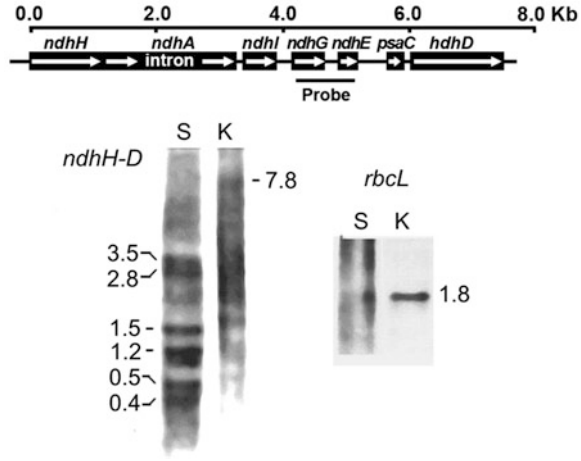

Fig. 23.3. Changes in the *ndhH-D* transcript profile associated to the delaying of leaf senescence by kinetin. RNA was isolated from senescing 14-day-old (S) and senescence-delayed (K) (by 20 h treatment with 45 μM kinetin) leaves of barley. Northern blots with probes of the *ndhG–ndhE* gene region of the *ndhH-D* operon (see map above) and of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*, used as control) were performed as described (Martínez et al. 1997). Comparison of S and K lanes shows a low RNA maturation processing of the primary *ndhH-D* transcripts in kinetin treated leaves. Numbers sided to lanes indicate transcript sizes in kilo-bases.

ndhG, ndhE and *ndhD* genes) which also includes the gene *psaC* (encoding a polypeptide of the PSI) between genes *ndhE* and *ndhD*. The primary polycistronic transcripts of the *ndhC-J* and *ndhH-D* operons mature to translatable monocistronic transcripts by posttranscriptional processes that include C to U editing of certain bases, intergenic cleavage and intron splicing in the case of the *ndhA* (Del Campo et al. 2000, 2002). Primary monocistronic transcripts may also require editing *(ndhB* and *ndhF*) and intron splicing (*ndhB*). In fact, the chloroplast contains a large variety of transcripts containing *ndh* messages at different stages of processing. Alternative splicing of transcripts derived from the *ndhH-D* operon leads to a preferential accumulation of the *psaC* translatable mRNA or of *ndh* translatable mRNAs (Del Campo et al. 2002). Larger-sized, low-processed transcripts of the *ndh* operons accumulate in young leaves, whereas monocistronic translatable transcripts accumulate in senescent leaves and in leaves under POS (Martínez et al. 1997; Casano et al. 2001; Del Campo et al. 2006; Serrot et al. 2008). The treatment of barley leaves with the cytokinin kinetin, which delays senescence, in addition to increasing the level of the mRNA of the *rbcL* gene (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase), decreases the levels of translatable low-sized transcripts of the *ndhH-D* operon and accumulates large-sized, low-processed transcripts (Fig. 23.3). Therefore, the control of the *ndh* transcript processing must provide clues on the intrachloroplast events leading to leaf senescence. Nuclear-encoded proteins, some of which contain a pentatricopeptide-repeat (PPR), are involved in the processing of plastid primary transcripts (Barkan and Goldschmidt-Clermont 2000; Monde et al. 2000; Felder et al. 2001; Kotera et al. 2005; Okuda et al. 2009), which make their encoding genes additional targets to investigate the molecular mechanisms controlling leaf senescence.

In addition to the enhanced Cu/Zn-SOD expression (Sect. II.A.3), deficient *ndh* transcript processing could contribute to the delayed senescence of the Cvi ecotype of Arabidopsis with respect to the Ler ecotype.

Codon 17 (UCC for serine) of the *ndhG* gene is edited (to UUC for phenylalanine) in all Arabidopsis Ler *ndhG* transcripts. However, the corresponding codon in Arabidopsis Cvi (GCC for alanine) is edited (to GUC for valine) in only a fraction of transcripts (Martínez et al. 1997; Tillich et al. 2005). Conceivably, defective transcript processing and amino acid differences among the Ndh-G subunits of Ler and Cvi ecotypes could also contribute to the delayed senescence of Cvi providing additional evidences for the role of *ndh* gene expression in the induction of leaf senescence.

B. Signals Exiting from Chloroplasts

The mechanism controlling the expression of the plastid-encoded *ndh* genes and the activation of the Ndh complex may include the synthesis of proteins (sigma4 factor, plastid transcriptional factors, nuclear-encoded subunits of the Ndh complex and RNA processing factors), their incorporation into chloroplasts and their possible activation. The direct action of growth substances, such as cytokinins, jasmonates (JAs) and abscisic acid (ABA), in the chloroplast cannot be excluded, but direct evidence is sparse. As in young leaves, the expression of the *ndh* genes during leaf senescence is stimulated by ROS signals, with the specificity that the expression in aged leaves results in the autocatalytic increase of ROS production. As discussed, the expression of the nuclear-encoded chloroplastic SOD is also stimulated by ROS. As these are mainly produced in the chloroplast, the immediate question is what are the ROS or ROS-derived signals that exit chloroplasts and stimulate the nuclear expression of *ndh*-related genes, of genes for chloroplastic SODs (only in young leaves) and, in general, of genes related to ROS production such as the lipoxygenase (LX), which will be discussed later.

In contrast to the short-lived $^1O_2$, $O_2^{\bullet -}$ and HO•, H_2O_2 could be considered a signal that exits the chloroplast and regulates nuclear gene expression (Piñas and Strand 2008). Increase of H_2O_2 levels in the cytoplasm frequently occurs in young and adult leaves. H_2O_2 is involved in the signal transduction of the SAR and probably, at high levels, in HR (Levine 1999; Van Breusegem et al. 2001; Overmyer et al. 2003). In animal systems, growth factors inhibit membrane-bound PRDX by phosphorylation of a tyrosine, increasing the level of H_2O_2 that acts as a downstream signal (Woo et al. 2010). The involvement of cytosolic and chloroplast PRDXs in plant development and in the response to stress (Dietz 2003) could also modulate the level of the H_2O_2 signal. H_2O_2, reportedly produced by plasmalemma NADPH oxidase, increases in the JA-induced senescence of detached rice leaves (Hung et al. 2006). However, there is no evidence of the increase of H_2O_2 production in chloroplasts during natural leaf senescence under normal light conditions, although it could increase in peroxisomes (Del Rio et al. 1998). On the other hand, the increase of PX activities combined with low SOD activities suggests that H_2O_2 is not the signal that exits chloroplasts in leaf senescence. Accordingly, the PCD of bundle sheaths in the maize *camouflage1* (*cf1*) mutant, in contrast to HR, does not accumulate H_2O_2 (Huang and Braun 2010). Thus, although H_2O_2 increases *ndh* gene expression and Ndh levels (Casano et al. 2001; Lascano et al. 2003), the increase of the Ndh complex during senescence does not seem to be due to the increase of H_2O_2 production in chloroplasts and, probably, other ROS-derived signals must exit chloroplasts to induce nuclear PCD events.

The synthesis of ABA, presumably in chloroplasts, increases during leaf senescence. However, ABA synthesis in response to water stress is higher in young than in senescent leaves (Eze et al. 1981; Quarrie and Henson 1981). The increase of ABA occurs late in senescence (Samet and Sinclair 1980) and the effect of ABA stimulating leaf senescence varies among different plants. Therefore, we will discuss other possible senescence signals departing from chloroplasts.

1. Jasmonates

Oxylipins, further transformed to jasmonic acid (JA) and derivatives, could be ROS-derived

signals leaving chloroplasts that modify nuclear gene expression. The biosynthesis of JA initiates from free linolenic acid (Schaller et al. 2005) which, in a reaction with O_2 catalyzed by LX, is transformed into 13-hydroperoxy linolenic acid. Further reactions in chloroplasts lead to the oxylipin (9S,13S)-12-oxo-phytodienoic acid which is transformed into jasmonic acid in peroxisomes and to other JA in cytosol (Creelman and Mulpuri 2002; Wasternack 2007). The possibility cannot be excluded that LX could also attack esterified linolenic acid in lipid membranes resulting in the 13-hydroperoxy linolenic acid later released by a lipase. On the other hand, $^1O_2$ in the absence of LX produces 13-hydroperoxy derivative acids from polyunsaturated fatty acids of membrane lipids (Fig. 23.1b) generating JA that mediates a rapid stress response toward the production of $^1O_2$ (Wagner et al. 2004) and inhibits the synthesis of proteins for the photosynthetic machinery (Reinbothe et al. 1993). It stimulates the expression of genes related to stress defense, *SAG*s (Creelman and Mulpuri 2002) and chloroplast LX (Bachmann et al. 2002) and increases the production of $^1O_2$ (Guo et al. 2010) and the level of the thylakoid Ndh complex (Cuello et al. 1995). JA increases under a variety of stress conditions and externally applied JA accelerates senescence (Wasternack 2007). Although assays with plants generated with LX2-RNA interference introduce doubts on the involvement of JA (Seltmann et al. 2010), the suggestive picture emerges for the chloroplast control of leaf senescence through an autocatalytic cross-communication between the chloroplast and nucleus by which, when a critical level of $^1O_2$ is reached, the JA produced induces nuclear genes for LX and the thylakoid Ndh complex (and, in general, *SAGs*) that further increase $^1O_2$ and JA leading to the leaf senescence syndrome.

2. Mg-ProtoChl

Tetrapyrrole intermediates of the biosynthesis of chlorophyll, especially Mg-Protoporphyrin IX (Mg-ProtoIX), were proposed as intermediates of the retro-inhibition of nuclear and plastid genes encoding proteins of the photosynthetic machinery (Rodermel 2001; Fernández and Strand 2008). Mg-ProtoIX accumulates in the chloroplast and cytoplasm under stress conditions (Ankele et al. 2007) and negatively affects the expression of photosynthesis genes. At the same time, the increase of Mg-ProtoIX favors the expression of defense genes (Von Gromoff et al. 2006). In the defense response, genetic evidences in Chlamydomonas for the induction of the HSP70 identifies the (G/C)CGA(C/T)N(A/G)N15 (T/C/A)(A/T/G) motif as that conferring responsiveness to Mg-ProtoIX (Von Gromoff et al. 2006). In the repression of photosynthetic genes, evidences from Arabidopsis mutants suggest that Mg-ProtoIX and other photosynthesis associated stress signals converge in chloroplasts through GUN1, a PPR protein located in chloroplasts, to a common signal that exits chloroplasts and inhibits the expression of activation factors in the nucleus, such as GLK1 (Kakizaki et al. 2009), and/or facilitates the binding of the repressor ABI4 to its target CCAC motif upstream of several photosynthesis genes (Koussevitzky et al. 2007), hence avoiding the binding of light-induced G-box-mediated transcription activation factors.

Whatever its role, the cytosolic increase of Mg-ProtoIX under stress probably is derived from chloroplasts (Ankele et al. 2007; Piñas and Strand 2008). ProtoIX, a biosynthetic precursor and probable degradation product of Mg-ProtoIX, when supplied externally, induces a HR-like PCD, probably dependent on the production of $^1O_2$ from photo-activated ProtoIX and on H_2O_2 over-production in mitochondria (Yao and Greenberg 2006). Therefore, possibly, ProtoIX could feed to the vicious cycle of ROS production triggering leaf PCD within a complex network of factors and cell compartments.

3. Proteins

PPRs are a large family of proteins encoded in the nucleus, most of which bind to RNA and/or DNA and are targeted to chloroplast and/or mitochondria where they are required for pre-mRNA splicing and editing

(Kotera et al. 2005; Beick et al. 2008; Okuda et al. 2009). Until now, only one PPR protein has been demonstrated to be located in the nucleus where it interacts with RNA polymerase (Ding et al. 2006). It cannot be excluded that the same GUN1 PPR protein could be a signal that exits chloroplasts and activates the network repressing photosynthetic genes.

In fact, a large number of proteins have been found to be targeted to both chloroplast and mitochondria and even, some of them, also to nucleus. Some of these proteins are translocated from one compartment to another (Krause and Krupinska 2009). Among them, Whirly proteins bind in vivo to chloroplast DNA, contributing to its stability (Maréchal et al. 2009) and RNA (Prikryl et al. 2008). In the nucleus, they are transcriptional factors of defense genes (Desveaux et al. 2005). If, as proposed by Krause and Krupinska (2009), Whirly1 (a member of the group) could be released from chloroplast to act as transcriptional regulator in nucleus, an investigation of its distribution in the different organelles during leaf development could provide clues on the chloroplast-nucleus interaction during senescence.

C. Nuclear Gene Expression that Feed-Forwards Chloroplast ROS Accumulation in Senescence

Due to the complex interaction of developmental and environmental factors that influence the production and scavenging of ROS in chloroplasts, extrapolations of the mechanisms controlling gene responses to stress to gene expression that initiates leaf senescence must be made with caution. We have discussed evidence suggesting that, above a critical chloroplast level of $^1O_2$ plus $O_2\bullet^-$, an autocatalytic increase of ROS makes the path to death irreversible. Above that critical level, gene expression addressed to decrease ROS levels is substituted by gene expression addressed to further increase ROS. Obviously, genetic, environmental and developmental factors modulate the critical $^1O_2$ plus $O_2\bullet^-$ level. By focusing this chapter on the chloroplast control of leaf senescence, we do not discuss the control of the many *SAG* genes expressed during senescence (see useful information in Buchanan-Wollaston 1997; Guo and Gan 2006; Lim et al. 2007; Thomas et al. 2009). In addition, as we have discussed the expression of the plastid *ndh* genes, we will focus below on the nuclear gene expression presumably responsible for the suicide feed-forward increase of ROS, after $^1O_2$ plus $O_2\bullet^-$ reach the critical levels. Between the signals exiting chloroplasts up to nuclear gene expression and, again, to the further increase of $^1O_2$ plus $O_2\bullet^-$, complex transduction networks (summarized in Fig. 23.4) operate that are only fragmentarily known. In fact, by identifying a few chloroplast signals and nuclear genes whose expression is affected, we only know two ends of that transduction network whose internal functioning must be modulated by a variety of hormonal and environmental factors.

Most investigations so far done on the control of nuclear-encoded genes by chloroplasts are related to the inhibition of photosynthesis genes by signals leaving the chloroplast under stress conditions, which we have briefly discussed. In contrast, positive effects of those signals in the nucleus could be expected on conventional *SAG* and on genes for LX, Ndh complex subunits and the plastid transcript processing machinery. The genes for chloroplast SODs, that fail to express under the POS in senescence, deserves specific attention.

1. Control of Super oxide Dismutase (SOD) Gene Expression

In many plant systems, the increase of chloroplast SOD under stress becomes impaired when leaves age and senesce (Sect. II.A.3). Differences of upstream sequences of Cu/Zn-SOD genes in *Arabidopsis thaliana* Ler and Cvi ecotypes (GenBank Accession Nr. AP000423 and DQ136312, respectively) suggest the existence of mechanisms responsible for the low expression in the Ler ecotype (Abarca et al. 2001b) and the higher SOD activity and, consequently, the delayed senescence of the Cvi ecotype (Luquez

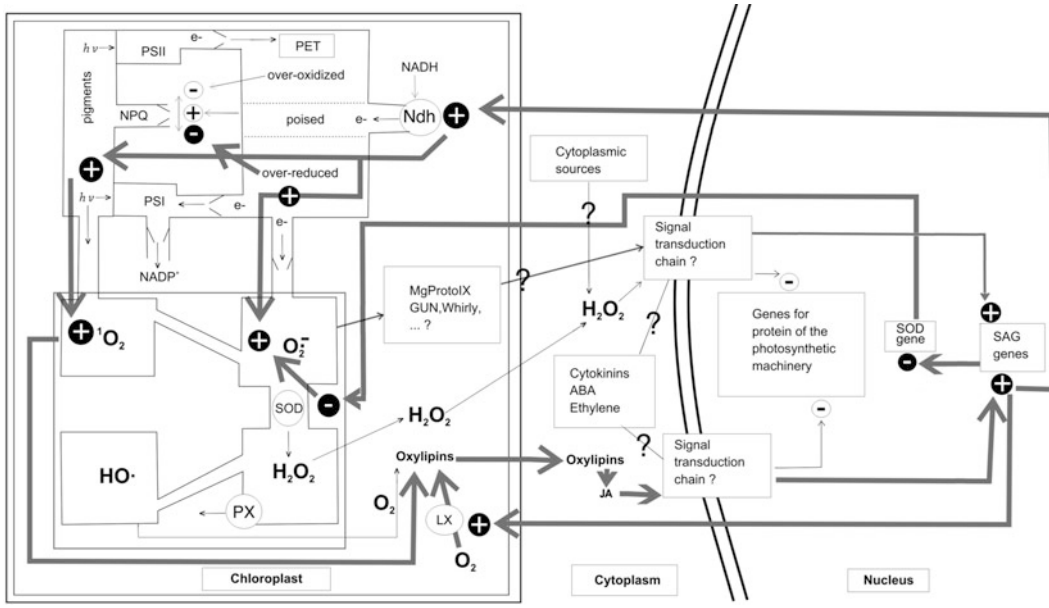

Fig. 23.4. Chloroplast signals and autocatalytic ROS production in leaf senescence. The figure shows the main production, pool and scavenging of $^1O_2$, $O_2^{\bullet -}$, H_2O_2 and HO• in chloroplasts and the derived signals that exit to the cytoplasm/nucleus transduction chains. The general main control of pools and processes are indicated by *arrows* pointing to ⊕ or ⊖ for stimulation or inhibition, respectively. Symbols ⊕ and ⊖ and thick *gray arrows* mark the proposed control mechanisms that trigger the autocatalytic ROS/JA production and chloroplastic SOD down-regulation characteristic of leaf senescence.

et al. 2006). The sequence of the 700 bases upstream of the ATG initiation codon of the *Cu/Zn-SOD* gene (*Csd2*) is very similar in the two ecotypes except for a 20-base deletion in Cvi (the −257 to −238 sequence of Ler) (Fig. 23.5). The sequence between, approximately, −700 to −257 (positions referred to the Ler gene) includes several motifs usually associated to recognition by the transcription machinery, among them:

- two W-boxes (TGAC(T)) recognized by WRKY transcription factors (Eulgem et al. 2000), some of them induced during leaf senescence (Singh et al. 2002),
- three I-boxes (AGATAA) recognized by type IV zinc finger factors (Reyes et al. 2004),
- eight TATA boxes involved in the binding of the transcriptional complex,
- two GTCAAAAT boxes recognized by Whirly transcriptional factors involved in SAR response (Eulgem 2005),
- one GT-element (GGTTAA) involved in the expression of genes for the photosynthetic machinery (Zhou 1999).

Significantly, one AGATAA and two TATA box motifs are immediately upstream of the region deleted in Cvi. In addition, the 20-base sequence present in Ler but not in Cvi contains the AACTAA motif recognized by some MYB factors (Fig. 23.5). MYBs are factors represented by many genes in Arabidopsis that, upon binding to the respective target motifs, inhibit the transcription of the downstream gene (Jin et al. 2000). As the expression of several MYB factor genes increases in HR (Daniel et al. 1999; Vailleau et al. 2002) and, in leaf senescence (Chen et al. 2002b; Buchanan-Wollaston et al. 2005), it seems plausible that MYB factors over-expressed in senescence bind to the AACTAA motif inhibiting the downstream progression of the transcription complex to express the *Csd2* gene, thus favouring ROS accumulation and senescence in the Ler ecotype. By preventing MYB factor binding, the 20-base deletion in Cvi allows the transcription of *Csd2* and contributes to delaying leaf senescence.

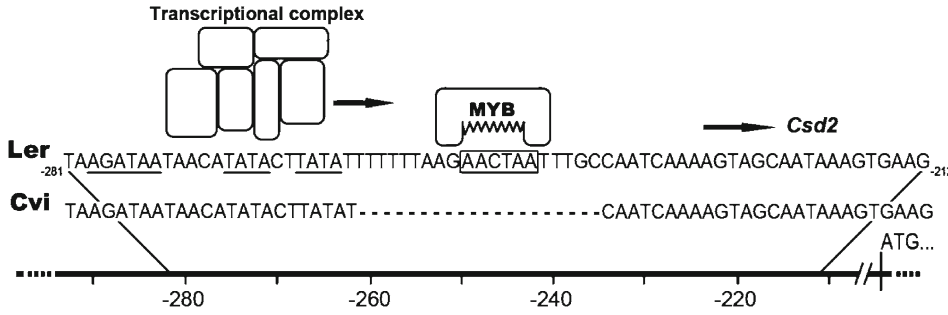

Fig. 23.5. Proposed down-regulation of the *Csd2* gene in *Arabidopsis thaliana* Ler during senescence. The MYB transcriptional factor increases during senescence and binds to the AACTAA motif located −248 to −243 bases upstream of the first codon of the nuclear *Csd2* gene for the chloroplastic Cu/ZnSOD. Bound MYB factor should prevent the advance of the transcriptional complex in the Ler ecotype. Due to a 20-base deletion around the −240 position (sequence below), the Cvi ecotype lacks the AACTAA motif, thus avoiding the MYB factor inhibition and allowing the transcription of *Csd2*, higher levels of Cu/ZnSOD and delayed senescence. The bar scale corresponds to the base number upstream of the ATG start codon of the *Csd2* gene.

As the MYB factor that binds to the AACTAA motif of *Csd2* is the product of an *SAG* presumably induced by ROS, the down-regulation of the chloroplast Cu/ZnSOD feed-forwards the suicidal increase of ROS generation during leaf senescence as marked by thick gray arrows in Fig. 23.4.

It is noteworthy that the Arabidopsis Cvi ecotype shows, at least, two genetic features that contribute to delayed rosette senescence, namely the one described above associated to Cu/ZnSOD and that described in Sect. III.A associated to the partial editing of the *ndhG* gene transcript. Probably, a long life span of Arabidopsis is advantageous in the bioclimatic conditions of the Cape Verde Islands. Hence, several genetic changes delaying senescence were fixed in the ancestry of the actual Cvi ecotype characteristic of these islands.

2. The SOD-LX-Ndh-JA Irreversible Journey to Death

In addition to the connection (through MYB factors) of SOD-repression with *SAG*-induction, the effects of JA induced genes for chloroplast LX (Bachmann et al. 2002) and increasing the levels of the Ndh complex (Cuello et al. 1995) and $^1O_2$ production (Guo et al. 2010) feed-forward the levels of ROS and lead to leaf senescence. The biosynthesis of JA is under the positive control, among others, of a TCP transcriptional factor (Schommer et al. 2008), which is in turn under control of a microRNA, that recognizes several motifs (..GGACCAC, ..AGACCAC.., ..GGACCAA.. and ..GTGGTCC..) upstream of the gene (*LOX2*) of the chloroplast LX. Therefore, it should be interesting to investigate the level of the TCP factor during leaf senescence.

The transduction chain of signals exiting chloroplasts and affecting nuclear gene expression is far from being known and its characterization is an exciting field of investigation for the coming years. In addition to the few factors described, the cause-effect order and the interactions with many other factors, hormones, H_2O_2, Ca^{+2}, protein kinase cascades and, more recently identified, microRNAs (Sunkar et al. 2006; Schommer et al. 2008; Kim et al. 2009) deserve intense research in the future.

IV. Concluding Remarks

Leaf senescence is the result of changes in the multiple regulatory processes controlling the functioning of the cell, and finally leads to death. Extensive evidence shows that the ROS produced in chloroplasts are active

players in the organelle's degradation and in the network of processes that regulate leaf senescence. In focusing on chloroplast control, we have not discussed other aspects of leaf senescence that must not be underestimated. In addition, the robustness of the genetic approaches have relegated certain essential investigations, such as enzyme and substrate modifications, sub-cellular trafficking and membrane changes, to a second plane that is only briefly mentioned here. Nevertheless, data on the role of the chloroplast provides new insights and promising avenues for further investigations of leaf senescence.

Feed-forward relations between the decrease of chloroplast SOD and the increase of the level of the Ndh complex, LX and ROS suggest a plausible explanation for PCD induction in leaf senescence. Evidences indicate that, in leaf senescence, the production of ROS in chloroplasts feed-forwards the expression of the nuclear genes favoring the increase of the Ndh complex and LX and the decrease of SOD in chloroplast, which further increases ROS production as indicated by thick gray arrows in Fig. 23.4. Only a few of the regulatory factors of the feed-forward system are known. Among them, JA (that also feed-forward increases) is especially relevant in leaf senescence. Most components, if not all, of the cell regulatory network leading to senescence are functional in processes that do not lead to PCD. Especially, as evidenced by the cross interactions between stress and senescence responses (Behera et al. 2003; Deo et al. 2006), most of the network components involved in the PCD of leaf senescence are also activated in defense responses against different stresses that the cell perceives as oxidative stress due to the increase of ROS (Martín et al. 2004). In addition to their damaging effects, ROS act as early signals in the transduction network leading to the defense (feed-back) or the suicide (feed-forward) response of the cell. Therefore, the questions in the induction of PCD would refer to the molecular basis of the shift from feed-back regulation to feed-forward regulation of the level of ROS. Except for the HR, which shows many similarities with natural PCD, usual responses to stress are addressed to the surveillance of the cell. With respect to pure defense responses, the distinctive trait of PCD in leaf senescence and HR is that, due to a too strong or too weak response of certain network components, the production of the ROS increases out of control in a suicide response.

Uncertainties remain regarding the identity of the ROS-derived signals exiting chloroplasts (to date, oxylipins are the only ones for which there are compelling evidences) and whether they are different in defense and PCD responses. Many *SAGs* encode proteins involved in the macromolecular degradation and nutrient mobilization in senescing leaves, while others encode transcription factors, such as the MYB factors proposed to down-regulate the expression of SOD (Fig. 23.5). However, the high number of genes presumably encoding transcription factors possess the formidable task of functionally integrating them in the cascades directing the regulation of the genome. Available evidences for leaf senescence and comparison with other animal and plant cell processes suggest that, instead of independent cause-effect chains, gene regulation is controlled by a network of signal transduction chains that share a number of components (Hu et al. 2010) and that are affected, through a poorly-understood pathway, by hormonal and environmental factors (Fig. 23.4).

Far from straightforward independent cause-effect chains, lateral interactions among the components of the network add a complexity that probably requires new approaches such as those related to the network stability, sometimes identified with entropy maximization (Demetrius and Manke 2005; Lezon et al. 2006). From the evidence discussed here, one could hypothesize that a stable high-entropy network, designed for defense responses, collapses and prompts PCD when critical feed-back regulation of ROS production changes to feed-forward regulation. In this regard, relevant questions concern the molecular basis of this change

and its effects on the network entropy. The decrease of SOD and the increase of the levels of the Ndh complex and LX in chloroplasts seem promising tools for a thorough characterization of the molecular basis of leaf senescence.

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Chapter 24

The Role of Trehalose Metabolism in Chloroplast Development and Leaf Senescence

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Summary

Trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside) is a non-reducing disaccharide that acts as a storage carbohydrate, transport sugar and stress protectant in microorganisms and invertebrate animals. In plants, trehalose synthesis is catalyzed by trehalose 6-phosphate synthase (TPS) followed by trehalose 6-phosphate phosphatase (TPP). Despite activity of this biosynthetic pathway, trehalose does not usually accumulate to large amounts in plant tissues. The low content of trehalose indicates that the conventional role as carbon source or compatible solute is unlikely in plants. Instead, sucrose has assumed this role, while trehalose metabolism mainly appears to fulfill a signaling function. Work with transgenic plants has revealed a role of trehalose 6-phosphate (T6P), the precursor of trehalose in the biosynthetic pathway, as signal for high availability of carbon, in particular in the form of sucrose. While T6P is likely to be synthesized in the cytosol, it stimulates starch synthesis in the chloroplasts. In seedlings, T6P activates pathways for the synthesis of amino acids, proteins and nucleotides, but also represses photosynthesis genes. These changes in gene expression can be explained by inhibition of the activity of sucrose non-fermenting-1-related protein kinase 1 (SnRK1) by T6P in growing tissues. Since SnRK1 serves as an integrator of transcriptional networks in starvation signaling, the inhibitory effect of T6P is consistent with its role as a high carbon signal. High T6P also improves sucrose utilization and growth of seedlings. In mature leaves, however, T6P does not inhibit SnRK1. Although photosynthesis on a leaf area basis is enhanced in response to high T6P content, altered leaf shape restricts overall plant carbon gain and growth. Transgenic plants expressing genes for trehalose synthesis generally show improved photosynthetic function during stress. More recently, enhanced stress tolerance, while avoiding negative effects of T6P on leaf development, has been achieved by expressing TPS/TPP fusion constructs or by targeting trehalose synthesis to the chloroplast. This suggests a protective role of T6P and/or trehalose in the chloroplast, although trehalose content was probably too low to act as a compatible solute. Trehalose metabolism also affects reproductive plant development, including a role of T6P in floral initiation as well as in the regulation of leaf senescence. Development of photosynthesis is therefore regulated by trehalose metabolism in many different ways, with T6P typically acting as a signal for high carbon availability.

I. Trehalose Metabolism in Plants

Trehalose is a non-reducing sugar formed from two glucose molecules that are linked by an α-1,1 bond. It is widely distributed in nature, occurring in bacteria, archaea and invertebrate animals. Owing to its chemical properties, trehalose can protect cellular components, including proteins and membranes, and can therefore act as a stress protectant (Wiemken 1990; Crowe et al. 1998).

Abbreviations: Φ PSII – PSII operating efficiency; AGPase – ADP-glucose pyrophosphorylase; G6P – Glucose 6-phosphate; SnRK1 – Sucrose non-fermenting-1-related protein kinase 1; T6P – Trehalose 6-phosphate; TPP – Trehalose 6-phosphate phosphatase; TPS – Trehalose 6-phosphate synthase

With the exception of so called "resurrection plants" that can survive complete dehydration, such as *Selaginella lepidophylla* (Adams et al. 1990) and *Myrothamnus flabellifolia* (Drennan et al. 1993), trehalose does not accumulate to high amounts in plants. However, small contents of trehalose were found in, e.g., Arabidopsis (Vogel et al. 2001), potato (Roessner et al. 2000) and sugarcane (Glassop et al. 2007), suggesting that trehalose synthesis is widespread in plants.

A. Genes Involved in Trehalose Synthesis and Breakdown

Several pathways for trehalose synthesis exist in nature. The most common pathway, which is also the one used by eukaryotes, involves

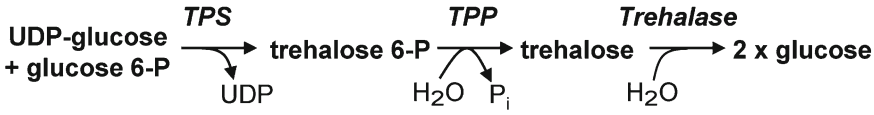

Fig. 24.1. Pathway of trehalose synthesis and breakdown in plants. The formation of trehalose 6-phosphate (T6P) is catalyzed by trehalose 6-phosphate synthase (TPS). T6P is then hydrolyzed to trehalose by trehalose 6-phosphate phosphatase (TPP). Trehalose can be hydrolyzed to glucose by trehalase.

formation of T6P (trehalose 6-phosphate) by trehalose 6-phosphate synthase (TPS), followed by hydrolysis of T6P to trehalose by trehalose 6-phosphate phosphatase (TPP) (Fig. 24.1). Complementation of yeast mutants in trehalose synthesis by transformation with Arabidopsis cDNA libraries identified one functional TPS gene (*AtTPS1*; Blázquez et al. 1998) and two functional TPP genes (*AtTPPA* and *AtTPPB*; Vogel et al. 1998). Since then, genome sequencing has resulted in the annotation of a total of 11 TPS-like and of 10 TPP-like genes. The exact functions of the additional genes, however, still remain unresolved and none of them has so far been demonstrated to encode proteins with TPS or TPP activity (Vogel et al. 2001; Harthill et al. 2006; Ramon et al. 2009). Instead, differential expression of these genes dependent on cell and tissue type, throughout development and in response to carbon supply, light, hormones and stress suggests that they may have a regulatory function, for example by monitoring T6P or through regulating T6P synthesis (Lunn 2007; Paul et al. 2008; Ramon et al. 2009). The vital importance of *AtTPS1* in embryo development and throughout vegetative growth (Eastmond et al. 2002; Gómez et al. 2010) demonstrates that other TPS isoforms cannot compensate for the lack of *AtTPS1* and thus indicates that they may not be catalytically active. The evolution and the presence/absence of functional domains of TPS and TPP genes are discussed in detail by Lunn (2007).

The observation that trehalose rarely accumulates to high amounts in plants suggests an effective mechanism for its breakdown. Trehalose is hydrolyzed into two glucose molecules by trehalase (Fig. 24.1). Only one trehalase gene, *AtTRE1*, has been identified in Arabidopsis (Müller et al. 2001). In contrast, a large number of genes are involved in the breakdown of sucrose; 17 invertase and 6 sucrose synthase genes in Arabidopsis. Sucrose degradation may therefore be tightly regulated, whereas regulation of the synthesis and breakdown of T6P rather than trehalose itself may be of major importance in plants.

B. Localization of Trehalose Metabolism

T6P is synthesized from glucose 6-phosphate (G6P) and UDP-glucose (Fig. 24.1) and the content of T6P is closely related to sucrose content (Lunn et al. 2006; Paul et al. 2010). UDP-glucose, G6P and sucrose have a predominantly cytosolic localization, although G6P is also present in chloroplasts and sucrose in vacuoles (Gerhardt et al. 1987) and with evidence that it can be present in chloroplasts too (Gerrits et al. 2001). As synthesis of T6P cannot proceed without UDP-glucose, all evidence would predict a cytosolic site of T6P synthesis. In support of this, *AtTPS1* has been confirmed as cytosolic by immunogold labeling and translational fusions with GFP (Geelen et al. 2007). Despite the probably non-plastidic localization of T6P synthesis, T6P affects starch synthesis in the chloroplast (Sect. II.A), chloroplast function during stress (Sect. III), development of photosynthesis (Sect. IV) and the decline of photosynthetic function during leaf senescence (Sect. V.B). Some TPPs (At5g51460 = AtTPPA, At4g22590 and At5g10100) have predicted subcellular localization in the chloroplast (Emanuelsson et al. 2007) and presence of a T6P transporter across the chloroplast envelope has been inferred (Smith and Stitt 2007), which could explain the effects of T6P in chloroplasts.

However, trehalose, the product of TPP activity, does not appear to be degraded in the chloroplast. Frison et al. (2007) showed that the Arabidopsis trehalase is bound to the plasma membrane and has extracellular activity. This would require export of trehalose out of the cell before it can be hydrolyzed. Trehalose transporters have been identified in a range of organisms, but not in plants. One possibility would be that trehalose is translocated by one of the several sucrose transporters described for plants (Kühn 2003). Extracellular effects of trehalose are consistent with a role of trehalase in the defense against trehalose-producing pathogens, such as *Plasmodiophora brassicae* (Brodmann et al. 2002).

II. The Role of Trehalose Metabolism in Sugar Signaling

Observations of the effects of modifying the trehalose pathway in transgenic plants led to the postulation that trehalose metabolism is somehow involved in sugar signaling (Goddijn et al. 1997; Goddijn and Smeekens 1998). Such an involvement was unexpected and came before the publication of the Arabidopsis genome sequence which indicated an extensive genetic basis. However, Blázquez et al. (1998) and Vogel et al. (1998) had already shown functional enzymes for the pathway in Arabidopsis which had begun to challenge the view that trehalose was only of importance in specialized resurrection plants. Main features of transgenic plants with altered trehalose metabolism include effects on leaf growth and development (Sect. IV.B) rather than accumulation of trehalose. A hypothesis was proposed that low-abundance signals emanating from the pathway were interacting with a sugar signaling mechanism. Significantly, it became clear that such signals could override determinants of growth such as the supply of sucrose and the light environment (Sects. II.B and IV.B). The signaling mechanism appears critical for the utilization of sugars. For example, expression of the *E. coli* gene *otsA*, encoding a TPS, enhanced the ability of seedlings to utilize sucrose for growth, whereas expression of the *E. coli* gene *otsB*, encoding a TPP, diminished this ability, such that seedling growth could be modulated dependent on the amount of T6P (Schluepmann et al. 2003; Paul et al. 2010). The embryo lethality of Arabidopsis *tps1* knockouts (Eastmond et al. 2002) also showed that this signaling system is absolutely required for cell growth.

A. Enhancement of Starch Synthesis in Chloroplasts by Trehalose 6-Phosphate

Chloroplasts were shown to be an important site of interaction of T6P through its effect on starch synthesis (Kolbe et al. 2005; Lunn et al. 2006). The key enzyme of starch synthesis, ADP-glucose pyrophosphorylase (AGPase) is subject to redox regulation which involves reversible disulfide bridge formation between the two smaller subunits of the AGPase heterotetramer. In leaves, AGPase is redox activated by thioredoxin-dependent reduction, which results in monomerization. The mechanism resembles the light activation of enzymes of the Calvin-Benson cycle where electrons are transferred from photosystem I to ferredoxin and via ferredoxin:thioredoxin reductase to thioredoxins f and m, which activate target enzymes by reduction of regulatory disulfides (Buchanan and Balmer 2005). Transgenic plants with altered amounts of T6P displayed increased activation of AGPase by formation of the reduced monomer (Kolbe et al. 2005). Feeding of T6P to isolated chloroplasts showed that T6P specifically increased redox activation of AGPase. The precise molecular interactions of this mechanism are not yet known. However, given that T6P responds to the sucrose content (Lunn et al. 2006; Paul et al. 2010), it represents a means through which chloroplast starch synthesis can respond to the availability of sucrose in the cytosol. Since T6P is likely to be synthesized in the cytosol and transporters of T6P have not been characterized (Sect. I.B), it is not known whether T6P enters the chloroplast or is perceived at the chloroplast envelope.

Seedling growth in response to sucrose is enhanced in plants with high T6P levels (Schluepmann et al. 2003; Paul et al. 2010). Starch has been described as an integrator of growth (Sulpice et al. 2009) and at first sight the impact of T6P on starch synthesis and starch content could be seen as a means whereby T6P could affect growth rate. However, accumulation of starch is inversely related to growth rate, and rather than being a driving force for growth, it is likely that starch content responds to other factors that determine growth rate and use starch in growth processes. The impact of T6P on starch synthesis can therefore not adequately explain the impact of T6P on seedling growth.

B. Inhibition of the Sucrose Non-fermenting-1-Related Protein Kinase 1 by Trehalose 6-Phosphate

A model that can explain the effect of T6P on the growth of seedlings was recently published (Zhang et al. 2009). T6P inhibits the catalytic activity of sucrose non-fermenting-1-related protein kinase 1 (SnRK1). SnRK1 is a member of the SNF1-related/AMPK group of protein kinases that are universal in eukaryotes. These protein kinases are activated by starvation of energy and carbon source. Using a protoplast transient expression system it was shown that SnRK1 activates processes at the level of gene expression that enable survival under limiting conditions, including mobilization of reserves (catabolism) and cessation of growth (Baena-González et al. 2007). Inhibition of SnRK1 by T6P promotes the opposite response – biosynthetic processes (anabolism) and seedling growth (Zhang et al. 2009; Paul et al. 2010). As T6P responds to sucrose supply, this provides a framework whereby T6P relates sucrose availability with growth processes.

SnRK1 is involved in the direct inactivation of cytosolic enzymes such as sucrose phosphate synthase, nitrate reductase and 3-hydroxy-3-methylglutaryl-CoA reductase and was thought to be located in the cytosol. However, a recent report provided evidence of an association of SnRK1 with chloroplasts (Fragoso et al. 2009). The regulatory βγ subunit of SnRK1 has a chloroplast transit peptide and the β subunits of SnRK1 are known to regulate the subcellular localization of SnRK1 (Polge and Thomas 2007). It is possible that SnRK1s of specific subunit composition may be chloroplast. This could also explain the strong association of SnRK1 with starch metabolism, which paradoxically includes activation of the starch mobilization pathway at the transcriptional level (Baena-González et al. 2007) as well as redox activation of the starch biosynthetic enzyme AGPase in response to sucrose in potato tubers (Tiessen et al. 2003). β subunits contain a putative glycogen-binding domain which may facilitate interaction between SnRK1 and starch granules (Gissot et al. 2006). Interestingly, in mammals AMPK has been reported to be inhibited by glycogen directly through interaction with the β subunit (McBride et al. 2009).

While T6P inhibits SnRK1 in seedlings and young leaves, mature leaves lack an additional protein factor that is required for this inhibitory effect (Zhang et al. 2009). The observation that T6P inhibits SnRK1 in leaves that are fully green but not yet fully expanded, but not in leaves that have stopped growing indicates that the effect of T6P on SnRK1 is more closely related to growth than the sink-source transition during leaf development. T6P effects on the function of mature leaves and during senescence may therefore not be mediated by SnRK1, unless they are consequences of earlier developmental changes mediated through SnRK1.

III. The Role of Trehalose Metabolism During Stress

In agreement with the role of trehalose in resurrection plants and as stress protectant in other organisms, there are many accounts of improved stress tolerance in plants transformed with yeast, *E.coli* or plant genes for trehalose synthesis. Often, photosynthetic function under stress conditions

was improved (e.g. Pilon-Smits et al. 1998; Garg et al. 2002), suggesting a role of either T6P or trehalose in the protection of chloroplast components.

A. Improved Stress Tolerance Through Genetic Engineering of Trehalose Synthesis

Initial attempts to improve drought tolerance of tobacco plants by expressing the yeast (Holström et al. 1996) or *E. coli* (Romero et al. 1997) TPS genes succeeded, but also resulted in growth abnormalities, such as stunted growth and altered leaf shape (Sect. IV.B). When both the *E. coli* TPS gene, *otsA*, and the TPP gene, *otsB*, were expressed together in tobacco, the pleiotropic growth effects were reduced, while drought tolerance was still increased, as indicated by improved photosynthetic function during drought stress (Pilon-Smits et al. 1998). This suggests that pleiotropic growth effects in plants that were transformed with TPS are caused by T6P accumulation, whereas the protective function of the trehalose pathway may depend on trehalose formation. However, trehalose contents were considered too low by Pilon-Smits et al. (1998) to play a role in osmotic adjustment. Other sugar contents were found to be increased in the *otA*- and *otsB*-expressing plants, which could also contribute to the improved stress tolerance.

Sophisticated methods were developed to improve stress tolerance while avoiding detrimental developmental effects of increased T6P synthesis. The *otsA* and *otsB* genes were fused to reduce T6P accumulation. Rice plants expressing such fusion genes showed improved stress tolerance and accumulated trehalose without having a growth phenotype under non-stress conditions (Garg et al. 2002; Jang et al. 2003). The increased trehalose content was, however, probably still too low to act as compatible solute. Tolerance of drought, freezing, salt and heat stress was also improved by expression of a bifunctional fusion of the yeast TPS (*ScTPS1*) and TPP (*ScTPS2*) genes in Arabidopsis (Miranda et al. 2007) and by transformation with an *ScTPS1*/*ScTPS2* double construct in tobacco (Karim et al. 2007). A role for trehalose synthesis in protecting the chloroplast during drought stress was shown by expressing the yeast *ScTPS1* gene in the chloroplast instead of the nuclear genome (Lee et al. 2003). In contrast to nuclear transformants, pleiotropic growth effects were avoided in the chloroplast transformants despite enhanced trehalose accumulation. T6P probably plays signaling roles in the cytosol as well as in the chloroplast (Sect. II). It appears that cytosolic signaling interactions are responsible for abnormal growth, whereas T6P or trehalose in the chloroplast may fulfill a function in stress protection.

B. Mechanisms of Increased Stress Tolerance

A way in which stress tolerance could be increased in transgenic plants with enhanced trehalose synthesis is activation of stress response pathways. Bae et al. (2005) showed that treatment with trehalose resulted in altered expression of stress response genes. However, both increased and reduced expression of these genes was found and no analysis was performed to determine if stress response genes were over-represented among the differentially expressed genes. Veyres et al. (2008) found increased expression of genes associated with stress in the *sweetie* mutant of Arabidopsis, which has increased T6P and trehalose contents. It is not clear whether this effect indicates that T6P or trehalose enhance the stress response or whether the *sweetie* mutant suffers from stress. The latter is supported by the early senescence phenotype of this mutant (Sect. V.B). Evidence that increased trehalose synthesis may activate other stress response pathways was also obtained in rice plants over-expressing the TPP gene, *OsTPP1* (Ge et al. 2008). Conversely, stress could activate trehalose synthesis by increased expression of endogenous genes. Some of the Arabidopsis *AtTPP* genes (Iordachescu and Imai 2008; Li et al. 2008) and the rice *OsTPP1* gene (Ge et al. 2008) were regulated by stress, suggesting that trehalose formation in response to stress

is caused by increased TPP expression. Together with the activation of stress response pathways by trehalose, this could result in a feed-forward loop that allows plants to rapidly respond to stress conditions.

Overall, recent research suggests that trehalose does not necessarily improve stress tolerance by stabilizing cellular components, such as the photosynthetic apparatus, but by activating other stress response pathways. Downstream pathways could involve ABA signaling (Avonce et al. 2004). In this context, it is interesting that *AtTPS1* expression is very high in the guard cells after ABA treatment (Leonhardt et al. 2004). It is therefore possible that trehalose or T6P accumulates to high concentrations in the guard cells, although the possible impact on guard cell function is not known.

In addition to the beneficial effects of trehalose, T6P could be involved in stress responses through its signaling function, e.g. by affecting SnRK1-dependent signaling (Sect. II.B). SnRK1 has been demonstrated to act as an integrator of stress and energy signaling (Baena-González et al. 2007; Baena-González and Sheen 2008). In young, growing tissues where T6P inhibits SnRK1, it would be expected that high T6P could interfere with stress signaling. However, SnRK1 is likely to be involved in stress pathways that result in starvation, which is not always the case during stress. For example, during drought stress, carbon utilization for growth is more strongly affected than photosynthetic activity. Drought stress can therefore result in increased rather than decreased carbon availability (Hummel et al. 2010). Similarly, sugars accumulate under other stress conditions, such as cold stress. T6P could thus signal high carbon availability under stress conditions where growth is more severely reduced than photosynthesis. On the other hand, artificially high T6P in transgenic plants over-expressing TPS genes could have a negative impact under other stress conditions that result in starvation, such as extended nights or severe shading. In this case, inhibition of SnRK1 could impair metabolic adjustment in response to starvation.

IV. Regulation of Chloroplast Development by Trehalose Metabolism

Several studies have shown that trehalose metabolism plays an important role in the development of photosynthesis including effects on gene expression, photosynthetic function and leaf growth and shape. Research with transgenic plants suggests that these effects are mainly caused by T6P rather than by trehalose.

A. Effects on the Photosynthetic Apparatus

In *otsA*-expressing Arabidopsis seedlings grown in liquid culture, expression of photosynthetic genes, including genes involved in light reactions as well as in the Calvin-Benson cycle, were down-regulated (Zhang et al. 2009). In contrast, genes for other biosynthetic pathways, including amino acid, protein and nucleotide synthesis, were up-regulated, together with genes for glycolysis, the TCA cycle and mitochondrial electron transport. This regulation was opposite to the effects found in protoplasts over-expressing the SnRK1 gene *KIN10* and supports the inhibitory effect of T6P on SnRK1 activity (Zhang et al. 2009) and the function of SnRK1 during starvation (Baena-González et al. 2007). As outlined above (Sect. II), T6P content increases in response to carbon supply and is likely to act as a signal for high carbon availability. The observed changes in gene expression, including down-regulation of photosynthesis, are consistent with this proposed role of T6P in carbon signaling.

However, no negative impact on photosynthetic function was seen in mature leaves of plants with increased T6P. *otsA*-expressing Arabidopsis plants even had higher protein and chlorophyll contents than wild-type plants on an area basis, while chlorophyll and Rubisco contents were reduced in *otsB*-expressing plants (Fig. 24.2). These differences are also reflected in the operating efficiency of PSII (Φ PSII), which was higher in *otsA*- than in *otsB*-expressing plants, indicating higher electron transport rates. Similar

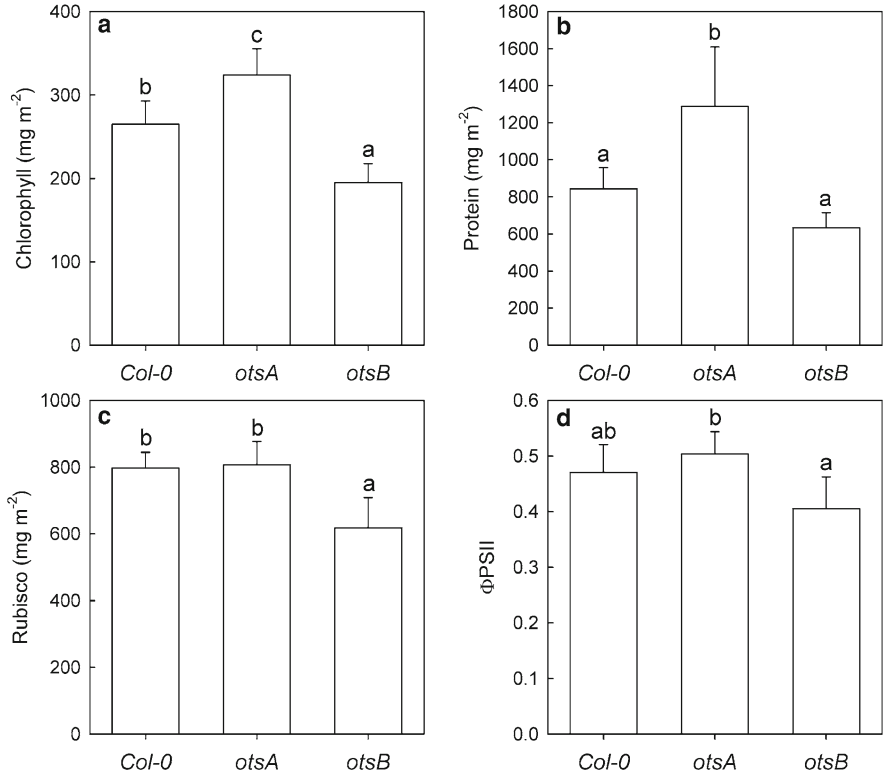

Fig. 24.2. Chlorophyll content (**a**), protein content (**b**), Rubisco content (**c**) and PSII operating efficiency (Φ PSII) (**d**) in mature rosette leaves of wild-type Col-0 and transgenic plants expressing the TPS gene *otsA* or the TPP gene *otsB*. Data are means of 5–8 plants ± SD. Different letters indicate statistically significant differences (ANOVA followed by Tukey's pairwise comparison; $p < 0.05$.).

to these results for Arabidopsis, components of the photosynthetic apparatus were increased in *otsA*-expressing and decreased in *otsB*-expressing tobacco plants, resulting in higher (*otsA*) or lower (*otsB*) rates of photosynthetic CO_2 assimilation on a leaf area basis (Pellny et al. 2004). Reduced photosynthesis associated gene expression due to high T6P in seedlings is therefore not reflected in photosynthetic function in mature plants, which is not surprising considering that mature, fully expanded leaves lack a factor that is required for inhibition of SnRK1 by T6P (Zhang et al. 2009).

B. Effects on Leaf Shape and Plant Growth

In addition to its effect on photosynthetic function, T6P also affects leaf shape. Overall, the leaf phenotype suggests that T6P acts in light signaling, with high T6P resulting in leaf characteristics that are typical for sun leaves (thick, dark green leaves with reduced area), whereas leaves with low T6P resemble shade leaves (thin, pale green leaves with increased area). These changes in leaf area can counteract the increased photosynthetic rates in plants with high T6P. Tobacco plants with increased T6P due to expression of *otsA* had lancet-shaped leaves (Goddijn et al. 1997). Similarly, lancet-shaped leaves were described for tobacco plants overexpressing the yeast *ScTPS1* (Romero et al. 1997), showing that this effect is not caused by the presence of the bacterial otsA protein but by altered trehalose metabolism. The altered leaf shape resulted in decreased leaf area in *otsA*-expressing tobacco plants and, as a consequence, increased photosynthetic rates in

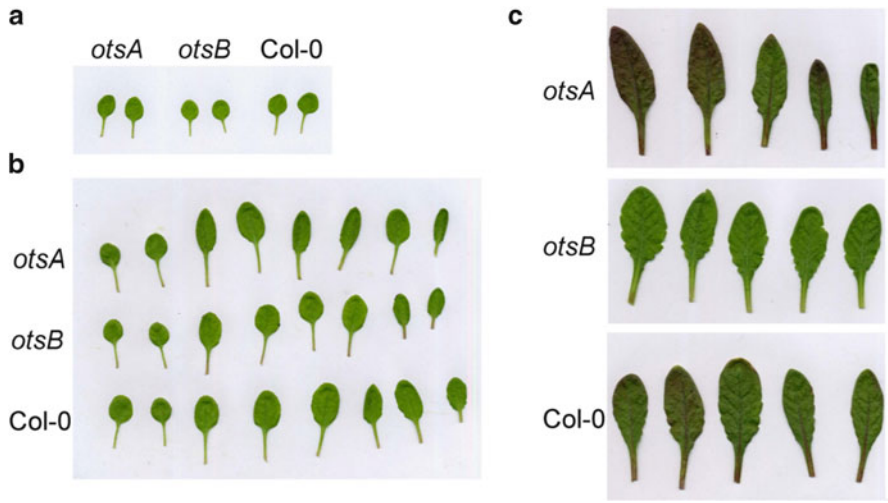

Fig. 24.3. Comparison of leaf growth of wild-type Col-0 and transgenic plants expressing the TPS gene *otsA* or the TPP gene *otsB*. (**a**) Leaves 1 and 2 on day 18. (**b**) Leaves 1–8 on day 25. (**c**) Leaves 16–20 on day 46.

Table 24.1. Leaf length, width, length/width ratio and mass per area of mature leaves of wild-type Col-0 and transgenic plants expressing the TPS gene *otsA* or the TPP gene *otsB*. Data are means of five plants (length, width and length/width ratio) or of eight plants (leaf mass per area) ± SD. Different letters in *brackets* indicate statistically significant differences (ANOVA followed by Tukey's pairwise comparison; $p < 0.05$.).

Plant	Length (mm)	Width (mm)	Length/width	Mass per area (g m^{-2})
Col-0	59.0 ± 1.0 [b]	19.5 ± 1.0 [a]	3.04 ± 0.11 [b]	27.9 ± 0.6 [a]
otsA	60.6 ± 1.6 [b]	18.7 ± 0.7 [a]	3.25 ± 0.13 [c]	35.0 ± 4.7 [b]
otsB	55.0 ± 1.8 [a]	19.7 ± 0.8 [a]	2.80 ± 0.13 [a]	25.2 ± 2.7 [a]

plants with high T6P did not lead to increased plant growth (Pellny et al. 2004). In contrast, leaf area of *otsB*-expressing plants was increased and growth enhanced, confirming that T6P is responsible for these effects (Pellny et al. 2004). Larger, paler green leaves in plants with low T6P could result in improved light harvesting with beneficial effects for plant growth. In contrast, improved seedling growth in plants with high T6P (Schluepmann et al. 2003; Paul et al. 2010) may not translate into stronger growth during later stages of development.

Leaf shape was also affected by expression of *otsA* or *otsB* in Arabidopsis (Fig. 24.3). Leaf development was delayed in *otsB*-expressing plants and leaves had a rounder appearance. Mature leaves of these plants were shorter. In addition, differences were found in the length/width ratio, which was higher in *otsA*- and lower in *otsB*-expressing plants than in wild-type (Table 24.1). As in tobacco (Pellny et al. 2004), *otsA*-expressing plants also had higher leaf mass per area.

Altered anthocyanin accumulation – reduced in *otsB*-expressing plants and increased in *otsA*-expressing plants (Fig. 24.3) – is also consistent with altered light and/or sugar signaling (Teng et al. 2005; Shi and Xie 2010). The seedling phenotype of *otsA*-expressing plants, including shorter and thicker hypocotyls and anthocyanin accumulation, indicates that altered light signaling also affects the development of seedlings (Paul et al. 2010). Differential expression of genes involved in light signaling in these seedlings supports the view that altered light signaling is responsible for changes in the development of the photosynthetic apparatus in response to T6P.

V. Regulation of Flowering and Leaf Senescence by Trehalose Metabolism

In monocarpic plants, i.e. plants that flower and set seed once and then die, leaf senescence and reproduction are often linked. Signals that coordinate leaf senescence and reproduction could regulate early stages of reproductive development (i.e. floral initiation) or act later when fruit formation affects the source-sink relationship. QTL analysis has revealed a genetic basis for interactions between floral initiation and whole-rosette senescence in Arabidopsis (Wingler et al. 2010). However, a locus that affects senescence without an impact on flowering was also identified, suggesting that senescence is also regulated independently of floral initiation. How senescence is influenced by fruit development varies widely among species. Removal of the fruits can, e.g., delay senescence in soybean and sunflower, but accelerate it in maize (Noodén and Guiamét 1989; Sadras et al. 2000). In contrast, removal of the inflorescence does not affect senescence of individual Arabidopsis leaves, but plant longevity is increased through formation of new leaves (Noodén and Penney 2001). The signals linking reproductive development and senescence are still not well characterized, but it is likely that sugars as well as phytohormones are involved in this interaction. Recent evidence indicates that T6P synthesis in response to carbon availability plays a role in flowering and senescence regulation.

A. Regulation of Flowering

Floral initiation depends on carbon availability. Sucrose can promote flowering of Arabidopsis plants grown in the dark (Roldán et al. 1999), but no single sugar or hormonal signal is responsible for floral initiation in all species (Corbesier and Coupland 2006). Instead, the protein product of *Flowering Locus T* (*FT*) has been identified as a floral stimulus transported from the leaves to the shoot apex. This does, however, not preclude an important function of the carbon balance of the leaves in promoting flowering. In the shoot apical meristem itself, changes in the expression of genes involved in carbohydrate metabolism (Wong et al. 2009) could modulate the flowering response to FT.

In agreement with the function of T6P as a signal for high carbon availability (Sect. II), a role in floral initiation has also been discovered. In the absence of *AtTPS1* expression, Arabidopsis plants do not flower (van Dijken et al. 2004; Gómez et al. 2010). Flowering was also delayed in mutants with point mutations in the *AtTPS1* gene that were induced using the TILLING (targeted induced local lesions in genomes) approach (Gómez et al. 2010). However, constitutive over-expression of *AtTPS1* can result in delayed flowering too (Avonce et al. 2004), suggesting that T6P formation has to be tightly balanced to allow plants to flower. While *AtTPS1* is expressed throughout the plant, expression is higher in the shoot apex than e.g. in leaves, where expression is concentrated within the guard cells (Leonhardt et al. 2004). In soybean, expression of a TPS gene was increased in the shoot apical meristem during floral induction and the transcript was present in the floral as well as in axillary meristems (Wong et al. 2009). This supports the proposed role of T6P in determining meristem identity of maize, where the *ramosa3* mutation in TPP results in irregular inflorescence branching (Satoh-Nagasawa et al. 2006). In *otsB*-expressing Arabidopsis plants flowering was delayed, which also suggests a function of T6P in floral initiation (Schluepmann et al. 2003). Expression of the Arabidopsis trehalase gene, *AtTRE1*, is highest in flowers and seeds (Müller et al. 2001; data from Schmid et al. 2005), indicating that any trehalose synthesized is rapidly degraded in flowers. Overall, these findings show that T6P and not trehalose is likely to be required to initiate flowering.

B. Regulation of Leaf Senescence

Leaf senescence can be regulated by a range of environmental factors, including biotic and abiotic stress. Senescence can, e.g., be induced by shading of individual leaves allowing nutrients, such as nitrogen, to be mobilized from the shaded leaves to sun-exposed leaves

and thus maximizing a plant's carbon gain (Boonman et al. 2006). However, while shaded leaves may be carbon starved, sugars accumulate in unshaded leaves during senescence. The senescence response to stress is also often associated with high leaf sugar contents (Wingler and Roitsch 2008). Importantly, treatment of nitrogen-starved plants with glucose results in changes in gene expression that are in agreement with a role of sugar signaling during natural senescence, thus suggesting that high rather than low carbon availability accelerates senescence (Pourtau et al. 2006; Wingler and Roitsch 2008; Wingler et al. 2009).

There is evidence that T6P acts as a signal for high carbon availability in senescing leaves. For example, the *sweetie* mutant, which contains increased amounts of T6P, shows accelerated senescence (Veyres et al. 2008). This could imply a role of T6P formation in senescence regulation. Since the contents of trehalose, glucose, fructose and sucrose were also increased in this mutant, it is, however, not clear if T6P, trehalose or other sugars are responsible for the senescence response. In addition, *SAG12*, a marker gene for developmental senescence, was not induced in *sweetie*. The senescence phenotype of *sweetie* may therefore be stress-related rather than indicative of altered development.

Senescence was delayed in transgenic plants expressing the *E. coli* TPP gene, *otsB* (Wingler et al. 2012). This effect could either be caused by decreased T6P or by increased trehalose contents in these plants. However, it is unlikely that trehalose is involved in senescence regulation since plants expressing the functional *E. coli* trehalase gene, *TreF*, do not show a senescence phenotype (Schluepmann et al. 2003). Furthermore, as pointed out by Fernandez et al. (2010), expression of the trehalase gene *AtTRE1* is upregulated during senescence, which could result in rapid degradation of trehalose. In contrast, T6P may signal carbon availability in senescing leaves. In parallel with the contents of other sugars, T6P strongly accumulates during leaf senescence (Wingler et al. 2012).

A role of T6P in promoting senescence would be in agreement with its inhibitory effect on SnRK1 activity. Over-expression of the Arabidopsis SnRK1 gene *KIN10* results in delayed seedling senescence in response to nutrient deprivation as well as in delayed flowering and senescence under long-day conditions (Baena-González et al. 2007). Based on the proposed role of SnRK1 as an integrator of starvation signaling, delayed senescence in *KIN10* over-expressors supports a function of starvation signals in the extension of life span, as e.g. found in heterotrophic organisms (Wingler et al. 2009). However, T6P does not inhibit SnRK1 of fully expanded leaves, as these leaves are lacking a factor that is required for the interaction of T6P with SnRK1 (Zhang et al. 2009). If inhibition of SnRK1 by T6P is responsible for senescence regulation, delayed senescence in *KIN10* over-expressors and *otsB* transgenics would therefore likely be a consequence of earlier developmental changes.

Senescence was not analyzed in detail in Arabidopsis TILLING lines with weak *AtTPS1* alleles or in an embryo-rescued *tps1* mutant, but it was noted that these plants do senesce even if they remained in the vegetative state due to severely delayed flowering (Gómez et al. 2010). This is not surprising, since, so far, no Arabidopsis mutant or transgenic plant has been identified that does not senesce eventually. The reason why senescence cannot be completely inhibited is probably that it is regulated by a plethora of environmental and age-related factors. Even though several of the environmental conditions that result in early senescence may be integrated by sugar signals (Wingler et al. 2006), there may be more than one sugar signaling pathway involved in senescence regulation. In addition to the function of T6P, acting upstream or independent of SnRK1, hexokinase-dependent sugar signaling has been shown to play a role (Moore et al. 2003; Pourtau et al. 2006).

VI. Conclusions

The ancient lineage of trehalose metabolism from the prokaryotic ancestor of chloroplasts has persisted and developed during

the evolution of plants. Genes for the pathway are all nuclear-encoded and serve to regulate chloroplast function through the regulation of starch synthesis by T6P-mediated redox activation of AGPase. In seedlings, expression of photosynthesis genes is affected by T6P through inhibition of the protein kinase SnRK1. SnRK1 is thought to be mainly cytosolic, yet chloroplast forms of SnRK1 are also possible. A major function of the pathway is to relay information on the availability of sucrose through the signaling function of T6P to processes that use sucrose in biosynthetic and growth processes. Trehalose metabolism also affects development and photosynthetic function in mature leaves, for example during stress and senescence. In contrast to seedlings, these effects are, however, unlikely to be directly caused by inhibition of SnRK1 by T6P and the exact pathways involved in T6P signaling in mature leaves remain largely unresolved.

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Part VI

Environmental Signals and Chloroplast Development

Chapter 25

Photoregulation of Chloroplast Development: Retrograde Signaling

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Summary

Chloroplasts are the major organelles of the plant cell, which perform photosynthesis and thereby support life on Earth. Light not only provides energy for photosynthesis, it also regulates several proteins which are involved in chloroplast biogenesis and functioning. This strategy helps plants to conserve their energy and resources for an appropriate time when the surrounding environment is favourable for their growth and development. This direct signaling pathway is called anterograde signaling. Apart from this direct signaling pathway, chloroplasts also have a retrograde route through which, based on their developmental status, they can modulate the expression of genes regulated by light. Chloroplasts emit some signals, called plastid signals, when they are under some kind of stress, to keep the nucleus updated

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on their developmental and metabolic status. These plastid signals use different components of the light signaling pathway such as phytochrome B (PHYB), cryptochrome 1 (CRY1), elongated hypocotyl 5 (HY5) and constitutive photomorphogenic 1 (COP1) and invariably convert them from a positive regulator of light-regulated genes into a negative regulator or vice-versa, depending upon the functional state of the chloroplast. Apart from this, also a light-independent pathway mediated by GUN1 (Genomes Uncoupled 1) exists. Thus, interplay of anterograde and retrograde signaling, crossing each other at different junctions, helps plants to respond appropriately to the ambient light environment and to regulate chloroplast development.

I. Introduction

A plant cell has three genetic compartments. Most of the genetic information is present in the nucleus, but chloroplasts and mitochondria also have their own genomes. According to the endosymbiotic theory, plastids evolved as a result of an endosymbiosis of a unicellular free living photosynthetic cyanobacteria and an ancient eukaryotic cell. During the course of evolution, the plastid genome, which may have contained all the necessary information to support an independent photoautotrophic lifestyle, underwent a series of progressive and drastic reduction in its coding capacity. The chloroplast genome of a higher plant encodes for less than 100 proteins most of which are involved in photosynthesis and regulation of plastid gene expression. The nuclear genome, on the other hand, is predicted to encode more than 3,000 chloroplast proteins in Arabidopsis and rice (Richly and Leister 2004; Larkin and Ruckle 2008). The genes associated with photosynthesis can therefore be classified into photosynthesis-associated nuclear genes (PhANGs) and photosynthesis-associated plastid genes. The proteins involved in photosynthesis are large multisubunit complexes which are composed of both nuclear-encoded and plastid-encoded proteins. Therefore, a well-coordinated expression of PhANGs and photosynthesis-associated plastid genes is required for proper development and maintenance of the chloroplast machinery (Larkin and Ruckle 2008). There are two signaling pathways by which exchange of information between nucleus and plastid takes place.

Anterograde signaling; it refers to the forward flow of information from the nucleus to the plastids, which largely controls plastid development and gene expression.

Abbreviations: 2CPA – 2-CYS Peroxiredoxin-A; ABI4 – ABA-insensitive 4; APX2 – Cytosolic ascorbate peroxidases; AtGLK1 – Golden 2 like 1; AtMYC2 – MYC-related transcriptional activator; CAB – Chlorophyll a/b binding protein; CCA1 – Circadian clock associated 1; CHLD – Mg-chelatase D subunit; CHLH – Mg-chelatase H subunit; CHLI –Mg-chelatase I subunit; COP1 – Constitutive photomorphogenic 1; CRY1 – Cryptochrome 1; CRY2 – Cryptochrome 2; DET – De-etiolated 1; EID1 – Empfindlicher im dunkelroten licht 1; ELIPS – Early light-inducible proteins; END – Enhanced de-etiolation; EX1 – Executor 1; EX2 – Executor 2; FHY3 – Far-red elongated hypocotyl 3; FLU – Fluorescent; GBF – G-box binding factor; GO – Gene ontology; GUN – Genomes uncoupled; HFR – Long hypocotyl in far-red; HRB1 – Hypersensitive to red and blue; HSP – Heat shock protein; HY1 – Long hypocotyl mutant 1; HY2 – Long hypocotyl mutant 2; HY5 – Elongated hypocotyl 5; JA – Jasmonic acid; LHCB – Light harvesting complex of photosystem II subunit; LHCII – Light-harvesting complex II; LREs – Light responsive elements; PGE – Plastid gene expression; PhANGs – Photosynthesis-associated nuclear genes; PIF3 – Phytochrome interacting factor 3; PIF4 – Phytochrome interacting factor 4; PPR – Pentatricopeptide-repeat;PQ – Plastoquinone pool; PRIN2 – Plastid redox insensitive 2; PRR5 – Pseudo-response regulator 5; PTAC2 – Plastid transcriptionally active chromosome protein 2; RBCS – Rubisco (small subunit); Rimb – Redox-imbalanced; SMR – Small mutS-related; SPA1 – Suppressor of phytochrome A-1; STN7 – STT7 homolog; TIC – Translocon of the inner envelope of the chloroplast; TOC – Translocon of the outer envelope of the chloroplast; ZAT10 – Zinc finger transcription factor

Retrograde signaling; it refers to the backward flow of information from chloroplast to nucleus by which PhANGs are controlled depending on the functional and developmental status of the chloroplast (Nott et al. 2006; Pfannschmidt 2010).

A. Anterograde Control

Chloroplast development on exposure to light is a part of a larger process called photomorphogenesis (Khurana et al. 1998, 2009). The process of photomorphogenesis is controlled by a vast array of components which can be broadly classified into photoreceptors like phytochromes, cryptochromes, phototropins, early signaling components like phytochrome interacting factor 4 (PIF4), suppressor of phytochrome A-1 (SPA1), empfindlicher im dunkelroten Licht 1 (EID1), central integrators like de-etiolated 1 (DET1) and constitutive photomorphogenic 1 (COP1) and downstream components like HY5 and phytochrome interacting factor 3 (PIF3) (Chory 2010) (Fig. 25.1).

The light signal perceived by photoreceptors ultimately reaches the downstream effectors which then alter the expression pattern of *LHCB* (light harvesting complex of photosystem II subunit), *RBCS* (rubisco small subunit) and other PhANGs involved in chloroplast development by binding to the light responsive elements (LREs) like G-box, GT-elements, I-box or GATA motifs and AT-rich motifs present in their promoters (Tyagi and Gaur 2003; Larkin and Ruckle 2008). The translated products of these light regulated genes are then transported to the chloroplast and targeted to the appropriate compartment to help assemble the functional chloroplast.

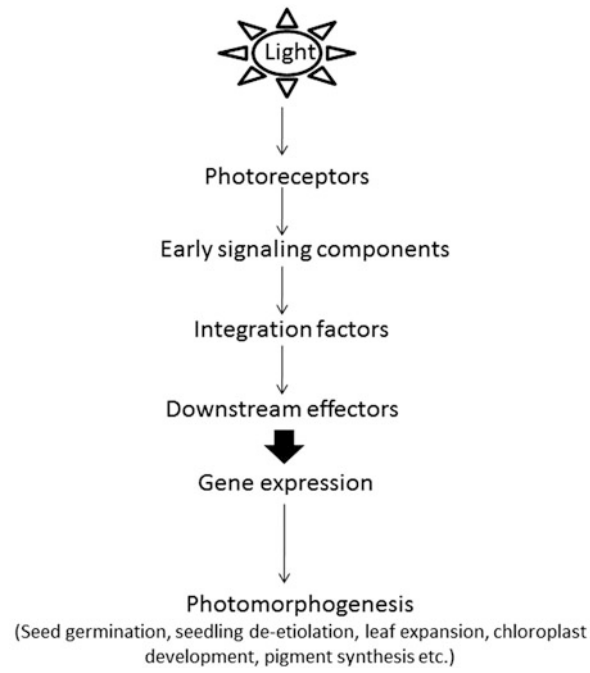

Fig. 25.1. **Diagram depicting the pathway of anterograde light signaling.** On perception of light by photoreceptors, a broad array of components like early signaling components, integration factors and downstream effectors acting at different levels of the pathway relay the signals to bring changes in gene expression which ultimately lead to photomorphogenesis.

B. Retrograde Control

The retrograde signaling evolved as a feedback loop to control the proper development and functioning of chloroplasts and mitochondria since their genomes lack the genes for regulatory proteins or RNAs (Pogson et al. 2008). This feedback control can act and bring things in order during adverse conditions. Also, the fact that the genomes of these organelles do not harbour genes for all their constituent proteins and instead a sizeable number is encoded by the nucleus raises the issue of the supply of requisite proteins at the appropriate time to these organelles. As a result, a system has been evolved through which plastids and mitochondria may send signals to the nucleus indicating the status of their development or function, and in turn the nucleus expresses the genes for the proteins required by them at that time. In general, these proteins represent the subunits of different enzymes or regulatory proteins. Different approaches have been used to study various aspects of cellular development and functioning. The approach mainly used to study the components involved in retrograde signaling has either been forward genetics, i.e. study of mutants defective in retrograde signaling, or using chemicals which inhibit this signaling pathway at different junctions. These can be antibiotics like chloramphenicol and lincomycin which inhibit plastid protein synthesis, or herbicides like norflurazon (which inhibits biosynthesis of photo-protective carotenoid pigments) and amitrole (which inhibits lycopene cyclization). Retrograde signaling has been largely classified based on different types of signals: retrograde signaling involving chlorophyll biosynthetic precursors, plastid gene expression dependent signaling pathway and retrograde signaling involving chloroplast redox signals.

Several articles reviewing the status of light induced changes in PhANGs and some with emphasis on anterograde signaling have been written in recent years (Woodson and Chory 2008; Waters and Langdale 2009). The focus of this article is thus on the significance of retrograde signaling in light control of chloroplast development which is much more complex and requires in-depth analysis.

II. Types of Retrograde Signaling

A. Retrograde Signaling Involving Chlorophyll Biosynthetic Precursors or Tetrapyrrole Signaling

Retrograde signaling plays an important role in synchronizing the biosynthesis of chlorophyll in the chloroplast with the expression of nuclear-encoded chlorophyll binding proteins (Nott et al. 2006). One of the intermediates of chlorophyll biosynthesis, Mg-protoporphyrin, a tetrapyrrole, was shown to be involved in retrograde signaling (Pesaresi et al. 2007). Higher plants contain four classes of tetrapyrroles, i.e. chlorophyll, heme, siroheme and phytochromobilin. Chlorophyll is a central component of the phytosynthetic apparatus and is a tetrapyrrole with a characteristic fifth ring, a Mg^{+2} ion and a phytol chain (Tanaka and Tanaka 2007). Heme and siroheme are closed macrocycles containing iron. Heme plays an important role in respiration and photosynthesis while siroheme is a prosthetic group of nitrite and sulphite reductases. Phytochromobilin is a linear tetrapyrrole which serves as a chromophore of phytochromes, the red/far-red sensing photoreceptor (Tanaka and Tanaka 2007).

The role of tetrapyrroles in retrograde signaling was first deduced from the studies involving *Chlamydomonas reinhardtii* cells which failed to accumulate *LHCB* transcripts when they were treated with compounds which inhibit the late steps of tetrapyrrole biosynthesis, but not when treated with inhibitors of early steps of tetrapyrrole biosynthesis. This indicated that some intermediates of the late steps of tetrapyrrole biosynthesis trigger inhibition of nuclear gene expression (Pesaresi et al. 2007). Heat shock protein 70A and 70B are located in the cytosol and in plastids, respectively. In addition to induction by heat stress, the genes encoding these proteins are also induced by light. Later, it was found that induction of these *heat shock protein 70* genes

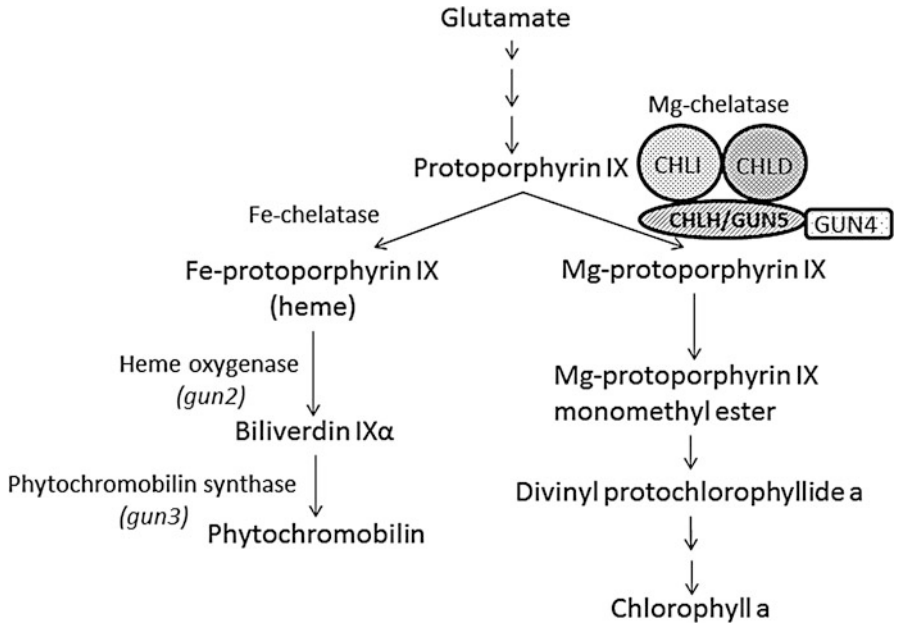

Fig. 25.2. **Tetrapyrrole biosynthetic pathway showing roles of different genomes uncoupled (GUN) proteins at different steps of the pathway.** *Genomes uncoupled* (*gun*) mutants are shown in *grey colour*. There are four *gun* mutants which function at different steps of the tetrapyrrole biosynthetic pathway. *GUN2* encodes heme oxygenase while *GUN3* encodes phytochromobilin synthase. They are involved in the formation of phytochromobilin. GUN4 is a Mg-chelatase cofactor while *GUN5* encodes the H-subunit of Mg-chelatase (CHLH). They are involved in Mg-protoporphyrin IX biosynthesis (Adapted from Nott et al. 2006.).

(*HSP70A* and *HSP70B*) by light is mediated by Mg-protoporphyrins, which served as a plastid derived signal in this process (Kropat et al. 1997, 2000). More evidence for the role of Mg-protoporphyrin IX in retrograde signaling came from reports in which direct feeding of Mg-protoporphyrin IX to Chlamydomonas cell cultures lead to the induction of *HSP70* genes. The conditions which abolished the light induction of the porphyrin pool also abolished the light induction of *HSP70* genes (Kropat et al. 2000; Beck 2005).

Recent reports have indicated that Mg-protoporphyrin IX acts as a coordinator of organelle and nuclear DNA replication in red algae and tobacco BY-2 cells (Kobayashi et al. 2009). Thus, Mg-protoporphyrin IX also affects nuclear gene expression through coordinating the cell cycle (Inaba 2010). Exogenous application of Mg-protoporphyrin IX leads to inhibition of *LHCB* expression in Arabidopsis protoplasts (Strand et al. 2003). There were reports, however, which challenged the role of Mg-protoporphyrin IX in retrograde signaling as it was found that the Mg-protoporphyrin IX levels were observed to be dramatically reduced in norflurazon treated plants (Inaba 2010). Zhang et al. (2011) showed that the levels of Mg-protoporphyrin IX are different in short-term and long-term norflurazon treated plants. There is an accumulation of Mg-protoporphyrin IX and *LHCB* repression when short-term norflurazon treatment is given. However, long-term norflurazon treatment causes marked changes in the tetrapyrrole pool accompanied by continuous repression of *LHCB* expression. How Mg-protoporphyrin IX is able to transfer plastid signals to the nucleus is still not very well understood. One view is that the steady-state level of Mg-protoporphyrin IX convey the status of tetrapyrrole biosynthesis either directly or via the H subunit of Mg-chelatase (CHLH) to the nucleus (Pesaresi et al. 2007) (Fig. 25.2).

Genetic studies carried out in *Arabidopsis thaliana* gave further evidence for the role of tetrapyrrole biosynthesis intermediates in retrograde signaling. Genetic screening for

identification of Arabidopsis mutants with defects in retrograde signaling lead to the identification of a number of mutants including five independent *genomes uncoupled* (*gun*) mutants (Susek et al. 1993). These mutants did not show repression of *LHCB* transcript in presence of norflurazon (Jarvis 2007). Later work revealed that *GUN2* encodes heme oxygenase and *GUN3* encodes phytochromobilin synthase. Both of these enzymes are involved in biosynthesis of phytochromobilin that is covalently attached to phytochromes at the conserved cysteine residue located towards N-terminus. Their mutants accumulate high levels of heme, which leads to negative feedback regulation of chlorophyll biosynthesis including Mg-protoporphyrin IX (Nott et al. 2006). GUN4 is a Mg-chelatase cofactor (Verdecia et al. 2005). *GUN5* encodes the H-subunit of Mg-chelatase (CHLH) involved in Mg-protoporphyrin IX biosynthesis (Fig. 25.2) (Mochizuki et al. 2001). GUN1 is a chloroplast-localized pentatricopeptide-repeat (PPR) protein which acts downstream of all the three pathways. GUN1 has an additional small *m*utS-*r*elated (SMR) domain at its carboxyl terminus. A GUN1 fragment containing both PPR and SMR domains was able to bind to DNA. Thus, GUN1 integrates information from multiple plastid signals (Jarvis 2007; Koussevitzky et al. 2007). GUN1 has been found to transmit the plastid signal independent of tetrapyrrole export from plastids (Zhang et al. 2011).

ABA-insensitive 4 or ABI4 is an apetala 2 (AP2)-type nuclear transcription factor which has an important role in ABA mediated seed development and germination (Finkelstein et al. 1998). The analysis of promoters of genes down-regulated in *gun1gun5* mutants revealed that ABRE elements were present more frequently in these genes. By analysing ABA signaling mutants it was found that *abi4* shows a *gun1* phenotype. Further investigations showed that ABI4 acts downstream of GUN1 as overexpression of ABI4 suppresses the *gun1* phenotype. ABI4 has also been shown to repress photosynthesis related genes in response to high levels of soluble sugar, like glucose, indicating the presence of a plastid signal generated by an increasing sugar level in plastids (Rook et al. 2006; Jarvis 2007; Koussevitzky et al. 2007).

B. Signaling Pathway Involving Plastid Gene Expression

The transcriptional and translational activity of plastids has been found to affect the expression of nuclear genes. On treating 6–7 day old dark grown wheat seedlings with tagetitoxin, which is an inhibitor of plastid RNA polymerase, the light induction of *rbcS* mRNA is blocked (Mathews and Durbin 1990). The plastid transcription rate also affects the initial increase in *rbcS* mRNA which occurs during early leaf development. Tagetitoxin also has an inhibitory effect on the accumulation of *cab* mRNA while no effect was seen on the *ACTIN* expression. This suggests that tagetitoxin mediated inhibition of plastid transcription inhibits only those nuclear genes which encode for plastid localized proteins (Rapp and Mullet 1991). The characterization of *prin 2* (Plastid Redox Insensitive 2) mutants also suggested that the plastid transcriptional status affects nuclear gene expression. PRIN2 is a plastid transcription machinery component which is localized to chloroplast nucleoids and is involved in redox-mediated retrograde signaling. *prin2* mutant alleles showed low expression of the plastid-encoded RNA polymerase (PEP) dependent genes as well as of the nuclear encoded *LHCB* gene (Kindgren et al. 2012). It has been found that plastid-specific translational inhibitors, like chloramphenicol and lincomycin, cause repression of nuclear photosynthesis gene expression. This lead to the discovery of plastid gene expression (PGE) pathway associated with retrograde signaling (Inaba 2010). The inhibitory effect of these treatments could be observed only during the first 3 days of seedling development (Sullivan and Gray 1999; Pogson et al. 2008). However, the *prors1* mutant, which is defective in prolyl-tRNA synthetase present in plastids and mitochondria, was found to induce light independent down-regulation of PhANGs in adult plants, indicating that

the PGE signaling pathway is not restricted to the first three days of seedling development but is active in adult plants also. Only the *prp11mrp11* double mutant and not *prp11* (defective in plastid ribosome function) and *mrp11* (defective in mitochondrial ribosome function) mutants were found to have specific down-regulation of photosynthesis related genes indicating that PhANGs are regulated by translational rates synergistically contributed by both chloroplasts and mitochondria (Pesaresi et al. 2006, 2007). GUN1 acts in the PGE pathway as the *gun1* mutant shows de-repression of nuclear genes in plants treated with plastid translation inhibitors. GUN1 is a pentatricopeptide repeat (PPR) protein which is structurally similar to the plastid protein pTAC2, which is thought to be a part of a large DNA-multiprotein complex called "Transcriptionally Active Chromosome" (TAC) involved in plastid transcription (Pogson et al. 2008). Like pTAC2, GUN1 possesses a small MutS-related (SMR) domain (Pfalz et al. 2006; Pesaresi et al. 2007). *pTAC2* mutants are defective in plastid-encoded RNA polymerase mediated transcription, and it is proposed that pTAC2 is part of a multi protein complex which is associated with plastid transcription. Based on these similarities, GUN1 is also presumed to mediate expression of plastid genes (Pogson et al. 2008). A PGE signal generated due to an abnormal plastid translational activity could be sensed or mediated by GUN1. Thus, it has been hypothesized that under normal conditions when the nuclear encoded plastid genes are being expressed, GUN1 is in a state in which it is associated with RNA and/or plastid-encoded transcriptional complexes in plastids and unable to generate any PGE signal. Repression of plastid transcription or translation leads to a change in the GUN1 state which in turn initiates a PGE signal for repression of nuclear genes like *LHCB*. In the *gun1* mutant initiation of this plastid signal irrespective of PGE is impaired (Pogson et al. 2008).

The plastid protein import process also generates a plastid signal for retrograde signaling. The plastid proteins encoded by the nucleus usually contain a transit peptide sequence at their N-terminus. This transit peptide is eventually cleaved off within the plastid. The transit peptide which is essential and sufficient for import of proteins into the plastids is recognized by the TOC-TIC machinery of plastids (See Ling et al., Chap. 12). This machinery is a super-protein complex which consists of two parts – TOC (*t*ranslocon of the *o*uter envelope of *c*hloroplasts) and the TIC (translocon of the inner envelope of chloroplasts). The core of the TOC machinery consists of receptors and channel protein components. The recognition of plastid proteins is mediated by two GTPases, Toc34 and Toc159 (Inaba 2010). Any defect in the import of plastid proteins due to defects in the plastid import machinery generates a plastid signal which in turn suppresses the expression of nuclear genes encoding plastid proteins. Toc159 is involved in chloroplast biogenesis. Toc 159 mutation (*ppi* mutant) causes repression of nuclear encoded chloroplast genes like *LHCB* and small subunit of *RUBISCO*, which are normally abundant in the plastid, but does not repress expression or import of less abundant non-photosynthetic plastid proteins (Bauer et al. 2000). The primary signaling molecules involved in retrograde plastid signaling mediated by defects in plastid protein import machinery have not yet been identified and appear to be different from Mg-protoporphyrin IX (Inaba 2010). Kakizaki and his co-workers (2009) found that the route this signal takes to suppress gene expression is mediated by GUN1 and AtGLK1 (Golden 2 like 1) and does not involve AtABI4 (Fig. 25.3). AtGLK1 belongs to the GARP superfamily of proteins. The Golden 2 like 1 (*GLK1*) gene transcript accumulates exclusively in leaf tissue and its level of expression is light-regulated. GLK1 is also known to be involved in chloroplast development and to increase the photosynthetic capacity of tissues with high photosynthetic workload like cauline and rosette leaves (Fitter et al. 2002). AtGLK1, a transcriptional activator, is known to interact with GBF1 and

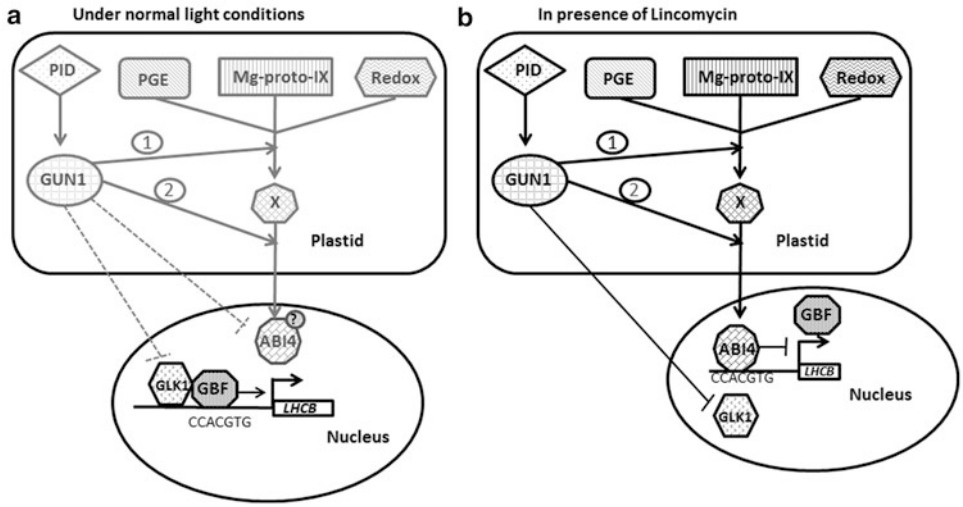

Fig. 25.3. **Operating model for retrograde signaling pathways.** (**a**) In normally developed, non-stressed plastids, the genomes uncoupled 1 (GUN1) derived signal is not generated. Thus, in the nucleus, ABA-insensitive 4 (ABI4) does not bind to *LHCB* (light harvesting complex of photosystem II subunit) promoters, and GBF (G-Box binding factor) mediated expression of *LHCB* occurs. Also, since there is no PID (Protein Import defect) induced signal, there is no repression of AtGLK1 (Golden 2 like 1) by GUN1, and normal expression of *LHCB* by AtGLK1 takes place. (**b**) Under stress conditions or when plastid development is impaired, plastid gene expression is inhibited, Mg-protoporphyrin IX accumulates and/or there is a change in the redox status of plastoquinone pool. Also these changes generate a common downstream signal 'X'. The role of GUN1 could either be to generate this signal X (pathway 1) or to perceive this signal (pathway 2). In either of the cases, in response to this signal, ABI4 binds to the *LHCB* promoter and thus prevents GBF (G-box binding factor) required for light induced expression of *LHCB* from binding to *LHCB* promoter. In case of PID (Protein Import defect), the signal generated is perceived by GUN1 which then represses the activity of AtGLK1 (adapted from Jarvis 2007; Kakizaki et al. 2009).

GBF3 (Tamai et al. 2002). The plastid signal generated by defects in the protein import machinery down-regulates the expression of the *AtGLK1* and this repression is mediated by GUN1 (Kakizaki et al. 2009).

C. Retrograde Signaling Involving Chloroplast Redox Signals

The redox states of photosynthetic electron transport components have been known to regulate expression of plastid encoded genes. But the redox states of photosynthetic electron transport components have also been proposed to influence expression of nuclear genes via retrograde pathways (Nott et al. 2006). It is proposed that two different sources are present which initiate redox signals for retrograde signaling (Pesaresi et al. 2007). Besides the intersystem electron transport including the plastoquinone pool (PQ), the acceptor side of photosystem I including a variety of redox-active components (e.g. NADPH, thioredoxin, glutathione and glutaredoxin) are sources of redox signals.

The redox state of the plastoquinone pool acts as a photon sensing system and affects the expression of the *LHCB* gene in the green alga *Dunaliella tertiolecta* (Escoubas et al. 1995). In *Chlamydomonas reinhardtii*, integrity of the plastoquinol-oxidizing site of the cytochrome b_6/f complex controls the expression of light-induced expression of nuclear genes encoding plastid proteins (Shao et al. 2006). In winter rye plants, however, the expression of *LHCB* is controlled by phosphorylation of light-harvesting complex II (LHCII) and not by the redox state of plastoquinone (Pursiheimo et al. 2001). In flowering plants, the regulation of nuclear

photosynthesis gene expression would involve different redox signals depending upon whether it is a short-term change or a long-term change. The short-term changes in illumination will depend mainly on the redox state of the PSI acceptor side while the long-term changes depend on the redox state of the plastoquinone pool and might not involve GUN signaling or cytosolic photoreceptors.

Reactive oxygen species (ROS) are produced when plants are exposed to excess light and these plastid derived ROS are known to act as a plastid signal. The main reactive oxygen species generated in chloroplasts are $^1O_2$, O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radicals. Chloroplast-derived $^1O_2$ has a short half-life and limited diffusion. Therefore, oxylipins generated by $^1O_2$-responsive lipoxygenases may be involved in $^1O_2$ mediated gene induction as stable secondary messengers (Op den Camp et al. 2003; Krieger-Liszkay 2005). $^1O_2$ mediated retrograde signaling has been studied using *flu*, *ex1* and *ex2* mutants. *flu* or *fluorescent* mutants are conditional mutants which were identified to elucidate the biological activity of $^1O_2$. Analysis of *flu* mutants revealed that the primary effect of $^1O_2$ generation and the resulting downstream $^1O_2$ mediated signaling has a role in regulating genes involved in biotic and abiotic stress responses rather than in chloroplast biogenesis. $^1O_2$ is a positive regulator of genes involved in biotic and abiotic stress response (Op den Camp et al. 2003; Lee et al. 2007). FLU is a negative regulator of tetrapyrrole metabolism, as a result of which, the *flu* mutants in the dark accumulate excess of the photosensitizer protochlorophyllide (Pchlide), a chlorophyll precursor, which upon illumination generates excess of $^1O_2$ causing cell death and growth arrest (Danon et al. 2006; Lee et al. 2007; Pogson et al. 2008). Given the short span of $^1O_2$, secondary messengers would be involved in $^1O_2$ mediated changes in nuclear gene expression. A second-site-mutant screening of *flu* mutants lead to the identification of two nuclear encoded chloroplast proteins, EXECUTOR 1 (EX1) and its closest homolog, EXECUTOR 2 (EX2). These are novel thylakoid–localized proteins. Mutant analysis revealed that EX1 and EX2 are involved in $^1O_2$ mediated induction of nuclear genes. Therefore, these two proteins are downstream components of $^1O_2$–mediated signaling which jointly control the expression of $^1O_2$–responsive nuclear genes (Lee et al. 2007). H_2O_2 is a more stable and lesser toxic ROS as compared to others whose production is stimulated under stress. On exogenous application of H_2O_2, expression of genes encoding APX2, ZAT10 and ZAT12 (cytosolic ascorbate peroxidases and zinc finger transcription factors, respectively) is induced. Changes in the redox state of the plastoquinone pool might lead to an interaction between H_2O_2 and $^1O_2$ directed signals. H_2O_2 movement from chloroplasts to the cytosol may be mediated by aquaporin channels (Pogson et al. 2008).

STN7 (STT7 homolog), a dual function thylakoid protein kinase and five *redox-imbalanced* RIMB proteins might be additional components involved in redox and ROS signaling. STN7 is required for state transitions and photosynthetic acclimation while *rimb* mutants show decreased *2CPA* (2-CYS peroxiredoxin-A) transcription and are defective in transcription of genes encoding other nuclear encoded chloroplast antioxidant enzymes (Pesaresi et al. 2007).

III. Authenticity of Plastid Factors

Although a number of components have been suggested to act as signaling molecules in retrograde signaling, the absence of experimental evidence to support them has raised a number of questions which challenge the authentication of these components as true plastid signaling factors. Critical evaluation of some of these tentative signals is discussed below.

A. Tetrapyrroles

Recent reports have suggested that changes in Mg-protoporphyrin IX accumulation do not correlate with changes in *LHCB* expression. When the steady-state levels of Mg-protoporphyrin IX were quantified along

with its neighbouring intermediates in Arabidopsis plants with altered plastid signaling responses, the gene expression of *LHCB1* and *RBCS* was not found to be correlated with steady-state levels of Mg-protoporphyrin IX (Mochizuki et al. 2008). When liquid chromatography-mass spectrometry (LC/MS) method was applied to measure the levels of tetrapyrrole intermediates, no Mg-protoporphyrin IX or any other chlorophyll biosynthesis intermediate could be detected when the plants are treated with norflurazon which leads to suppression of PhANGs expression. On the contrary, when endogenous levels of Mg-protoporphyrin IX were artificially increased, the expression of PhANGs were induced (Moulin et al. 2008) Therefore, it is possible that Mg-protoporphyrin is not acting as a direct signaling molecule, and in *gun* mutants a perturbation of tetrapyrrole synthesis might lead to changes in the redox state of the plastid or to localized production of ROS which in turn may transmit the signal to the nucleus (Moulin et al. 2008; Kleine et al. 2009).

B. Redox State and Reactive Oxygen Species (ROS)

The redox state of chloroplasts or their ROS pools are considered to be involved in retrograde signaling. The fact that most of the ROS species have a short lifetime and dissociate before they can cross the chloroplast envelope reduces their possibility of acting as a direct signal. For example, $^1O_2$ mainly acts close to its site of production and has a high reactivity, short half-life and limited diffusion. In cells, the half-life of $^1O_2$ is about 200 ns (Gorman and Rodgers 1992) and the possible diffusion distance in physiologically relevant situations is up to 10 mm (Sies and Menck 1992). Thus, the possibility of $^1O_2$ crossing both the inner and outer envelope of chloroplasts and acting as a long distance signal is rather unlikely (Kleine et al. 2009). The other view is that because of its short lifespan $^1O_2$ directly oxidizes a component of the signal transduction chain (Krieger-Liszkay 2005). ROS are also involved in a number of other stress-related processes like pathogen defence or wound response. Therefore, they may be unspecific signaling molecules. H_2O_2 because of its longer half-life and less toxic nature is suggested to diffuse freely across the chloroplast envelope to activate a cytosolic mitogen-activated protein kinase (MAPK) cascade, which in turn modulates nuclear gene expression (Apel and Hirt 2004; Kleine et al. 2009). The problem with this model is that H_2O_2 is polar in nature and therefore its mobility across the membrane might be restricted. The other possibility is that it might be transported through aquaporins but this movement has yet to be demonstrated *in planta*. H_2O_2 is produced at different sites in the cell and under various stresses and stimuli. How H_2O_2 specifically communicates the information on the functionality of chloroplasts to the nucleus needs to be thoroughly investigated (Kleine et al. 2009). The exact process through which photosynthetic redox signals from plastoquinone pool transfer the signal to the nucleus is not clear. It is thought that the signal generated by the redox state is converted into a phosphorylation cascade but no substrates have been identified yet (Pesaresi et al. 2009; Steiner et al. 2009).

IV. Mechanisms of Retrograde Signaling Pathways

The impairment of plastid biogenesis or stress in plastids results in the generation of a common plastid signal due to accumulation of Mg-protoporphyrin IX, inhibition of plastid gene expression and/or altered redox status. This signal is either generated or perceived by GUN1. The signal thus generated is detected in the nucleus by ABI4 which binds to the promoter of *LHCB* gene and represses its expression. The promoter of *LHCB* has a G-box element (ACGT) called CUF1. This CUF1 is preceded by two cytosines resulting in two overlapping elements – CCAC and ACGT. ABI4 binds to CCAC while the G-box-binding factor or GBF binds to ACGT. Therefore, ABI4 and GBF

might compete for binding at the promoter of *LHCB*. GBF promotes expression of *LHCB* while ABI4 represses its expression. Therefore, under normal conditions, GBF binds to the *LHCB* promoter while under plastid dysfunction, ABI4 binds to the *LHCB* promoter. Most of the PhANGs but not all, contain CCAC and ACGT elements in close proximity suggesting that the above mentioned mechanism might be applicable to regulation of other PhANGs (Jarvis 2007) (Fig. 25.3).

The signal derived from inhibition of the import of nuclear encoded plastid proteins due to defects in the protein import machinery follows a different route. The signal is perceived by GUN1 which then suppresses the activity of AtGLK1 which is a positive regulator of *LHCB* gene expression (Kakizaki et al. 2009) (Fig. 25.3).

V. Retrograde Signaling and Light

The early evidence for the existence of photoregulation of chloroplast development through retrograde signaling came in 1995 when it was found that in addition to red and blue light, the level of early light-inducible proteins (ELIPS) is also dependent on positive signaling factors originating from plastids (Adamska 1995).

The original screening of *gun* mutants yielded no light signaling mutants apart from *gun2* and *gun3*, which were allelic to *hy1* (*long hypocotyl mutant 1*) and *hy2* (*long hypocotyl mutant 2*), respectively, and were defective in biosynthesis of phytochromobilins, the chromophore for phytochromes. The *gun2* and *gun5* mutants showed similar cluster analysis of genes which show more than three-fold expression difference in *gun* mutants as compared to wild type. Also inhibitors of the tetrapyrrole biosynthetic pathway like dipyridyl were used which lead to more accumulation of Mg-Proto porphyrin IX in *gun2* and *gun5* mutants. When these dipyridyl treated mutants were grown on norflurazon, the *gun* phenotype was lost (Strand et al. 2003). So the *gun* phenotype displayed by *gun2* and *gun3* mutants was a result of reduced levels of Mg-protoporphyrin IX instead of a direct defect in phytochrome signaling (Larkin and Ruckle 2008).

It has long been known that the blue light sensing photoreceptor CRY1 induces *LHCB* expression but identification of *gun* mutants harbouring a defective *cry1* allele suggests that CRY1 represses *LHCB* when chloroplast biogenesis is blocked (Ruckle et al. 2007). Since GUN1 is also involved in plastid dysfunction mediated *LHCB* repression, analysis of *cry1gun1* double mutant was done in blue and white light. This analysis showed that the *LHCB* expression in the double mutants was only 1.5–2.7 fold more than single mutants indicating that partial redundancy exists in the pathways used by GUN1 and CRY1 for repressing *LHCB* gene expression in the presence of chloroplast biogenesis inhibitors (Ruckle et al. 2007). The *gun1cry1* double mutant analysis showed that the accumulation of *LHCB* mRNA was always greater in blue light than in white light when the chloroplast biogenesis is blocked. The level of *LHCB* mRNA accumulation is the same in untreated wild-type seedlings and the *gun1cry1* double mutants treated with lincomycin, suggesting that, in blue light, most of the *LHCB* repression is mediated by both GUN1 and CRY1. The full repression of *LHCB* also requires an additional light quality other than blue light as *gun1cry1* double mutants showed 30 % less *LHCB* accumulation than the untreated wild-type control under white light and, the de-repression of *LHCB* in *cry1* mutants is not seen under red light (Ruckle et al. 2007). This indicates that an additional component probably from some other photoreceptor pathway is also involved in *LHCB* repression in addition to GUN1 and CRY1. Analysis of *cry2* and a *cry1cry2* double mutant suggests that cryptochrome 2 (CRY2) can partially compensate for CRY1 in *cry1* background under blue light. Further analysis of other light photoreceptor mutants like *phot1, phot2, phyA, phyB* and *cry3* showed that these mutants do not act as *gun* mutants under light. Transcription of the *RBCS* and *LHCB* genes is known to be repressed in wild-type plants when chloroplast biogenesis

is blocked. De-repression of *RBCS* was seen in *gun1* mutants but not in *cry1* or *cry1gun1* double mutants when chloroplast biogenesis is blocked. In fact, the expression of *RBCS* was less in *cry1* or *cry1gun1* double mutants indicating that regardless of the presence or absence of chloroplast biogenesis inhibitors, CRY1 always acts as an inducer of *RBCS* (Ruckle et al. 2007). Initially, it was thought that CRY1 regulates photomorphogenesis by binding and inhibiting COP1, an E3 ubiquitin ligase which targets positive regulators of photomorphogenesis like HY5 (Wang et al. 2001; Yang et al. 2001; Khurana et al. 2009). Co-immunoprecipitation analyses showed that this interaction is independent of light as CRY1-COP1 interaction was found in both dark grown and light grown seedlings (Yang et al. 2001). But recently, Liu and co-workers (2011) showed that the interaction of CRY1 modulates the activity of COP1 by interacting with SPA1 (Suppressor of Phytochrome A) in a blue light dependent manner. SPA1 interacts with COP1 through its coiled-coil domain, and thus activates COP1-dependent activation of light regulated transcription factors like HY5. In response to blue light, photoactivated CRY1 interacts with SPA1 via the CCE domain of CRY1 and the WD repeat domain of SPA1. As a result, SPA1 is not able to interact with COP1 because of which COP1 cannot target HY5 for degradation. But previous studies of the light independent CRY1-COP1 interaction suggests that photoactivated CRY1 may directly suppress COP1 (Liu et al. 2011). Analysis of *LHCB* gene expression in *cop1*, *gun1*, *cop1gun1, cry1* and *cry1cop1* mutants revealed that while in *gun1* mutant, the presence or absence of lincomycin had no effect on its expression, in *cop1* and *cry1cop1* double mutants, the expression of *LHCB* was inhibited in the presence of lincomycin. This suggests that CRY1 utilises a COP1 mediated mechanism to regulate *LHCB* expression in presence of lincomycin and COP1 is epistatic over CRY1. The slight inhibition of *LHCB* expression in lincomycin treated seedlings of *gun1cry1* double mutants as compared to *gun1* single mutants suggests that largely GUN1 does not employ COP1 for *LHCB* regulation (Ruckle et al. 2007). However, other factors downstream of COP1 might be also involved in the pathway.

It is well-known that COP1 regulates photomorphogenesis by targeting positive regulators of photomorphogenesis like HY5 for degradation. The analysis of the *hy5* mutant, impaired in expression of PhANGs (Lee et al. 2007), showed that de-repression of *LHCB* expression takes place when chloroplast biogenesis is blocked. The results obtained with the *cry1hy5* and *gun1hy5* double mutants indicated that HY5 functions in a pathway involving CRY1 and is distinct from the pathway which utilizes GUN1 to repress transcription of *LHCB* (Ruckle et al. 2007). The *RBCS* expression in *gun1hy5* and *gun1cop1* double mutants was higher than in *gun1* and *gun1cry1* double mutants (Ruckle et al. 2007) indicating that HY5 and COP1 are not involved in CRY1 mediated induction of *RBCS* in the presence of lincomycin. COP1 and HY5 may repress *RBCS* expression in the presence of lincomycin but these effects are only visible when GUN1 is not active. Therefore, the pathway utilized by CRY1 to induce *RBCS* transcription in the presence of chloroplast biogenesis inhibitors needs a more detailed analysis.

Based on the evidences so far presented, it can be suggested that plastid signals can change CRY1 dependent regulation of *LHCB* transcription by converting HY5 from a positive to negative regulator. Since it is already known that HY5 can bind to the promoter of *LHCB* and that binding to the promoter is necessary but not sufficient for HY5 to regulate transcription, it has been suggested that, in addition to HY5, other factors are also required to induce and repress *LHCB* transcription (Ruckle et al. 2007) (Fig. 25.4).

Therefore, it is likely that under normal growth conditions CRY1 inhibits COP1 mediated degradation of HY5 by interacting with SPA1. The HY5 transcription factor is, therefore, free and can bind to the promoter of the *LHCB* gene. Since HY5 binding is necessary but not sufficient for regulation of *LHCB* gene expression, another factor 'W' associates with *LHCB* promoter using a HY5

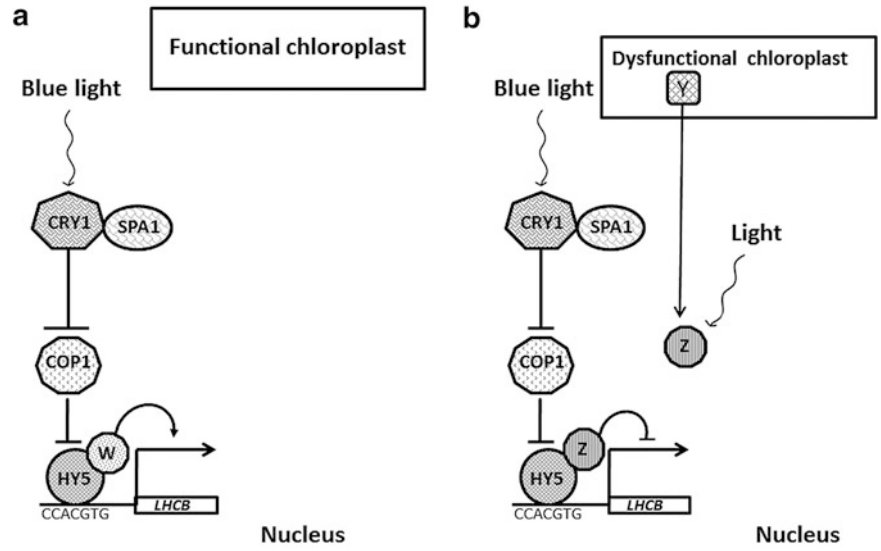

Fig. 25.4. **Working model showing how light signaling mediated by CRY1 is rewired by plastid signals.** (**a**) When the chloroplast is functioning properly, long hypocotyl 5 (HY5) is associated with a factor 'W' in the nucleus. This association makes HY5 a positive regulator of *LHCB* (light harvesting complex of photosystem II subunit) and induces its expression. The role of cryptochrome1 (CRY1) is to prevent constitutive photomorphogenesis 1 (COP1) from degrading HY5. (**b**) When the chloroplast is dysfunctional, the chloroplast generates a signal 'Y'. This 'Y' converts CRY1 into repressor of *LHCB* expression by converting HY5 into a repressor. It is proposed that signal 'Y' and light activate a factor 'Z' which binds to HY5, thereby, making HY5 a repressor of *LHCB* expression (adapted from Larkin and Ruckle 2008.).

dependent mechanism. When however, the cells contain dysfunctional chloroplasts, e.g., upon treatment with inhibitors of chloroplast biogenesis, another plastid signal 'Y' is generated which activates a factor 'Z' replacing 'W' in binding to HY5 and inhibiting the transcription of the *LHCB* gene. Under all these conditions, the role of CRY1 is to prevent HY5 from degradation (Larkin and Ruckle 2008) (Fig. 25.4).

The mechanism for regulating *RBCS* gene expression in the presence of chloroplast biogenesis inhibitors seems to differ from the one proposed for the *LHCB* gene. A hypothetical model can be proposed in which CRY1 ultilizes COP1 and HY5 for inducing *RBCS* transcription in the presence of light and in the absence of chloroplast biogenesis inhibitors. Upon treatment with inhibitors like lincomycin, CRY1 continues to induce *RBCS* expression but does not utilize COP1 and HY5 for its induction. Another factor 'B' which has the ability to bind to *RBCS* promoter and induces its expression seems to be used by CRY1. The GUN1 mediated pathway, however, does repress *RBCS* transcription (Fig. 25.5). Whether COP1 and HY5 are somewhere involved in this pathway is an open question.

Although CRY1 and GUN1 might play a dominant role in repression of *LHCB* when chloroplast biogenesis is blocked in blue light, the repression of *LHCB* in white light may be more complex and may involve additional photoreceptors such as phytochrome B. Since maximum PhANG repression also requires red light, analysis of *phy* mutants was done. The data suggested that *phyA* or *phyB* do not behave like *gun* mutants. In the *phyB* mutant *LHCB* gene transcription is repressed in the presence of chloroplast biogenesis inhibitors but only when GUN1 is absent, and phyA induces *LHCB* expression in the absence of GUN1. There was no effect of PHYA and PHYB seen on the expression of *RBCS* as the expression of *RBCS* was same

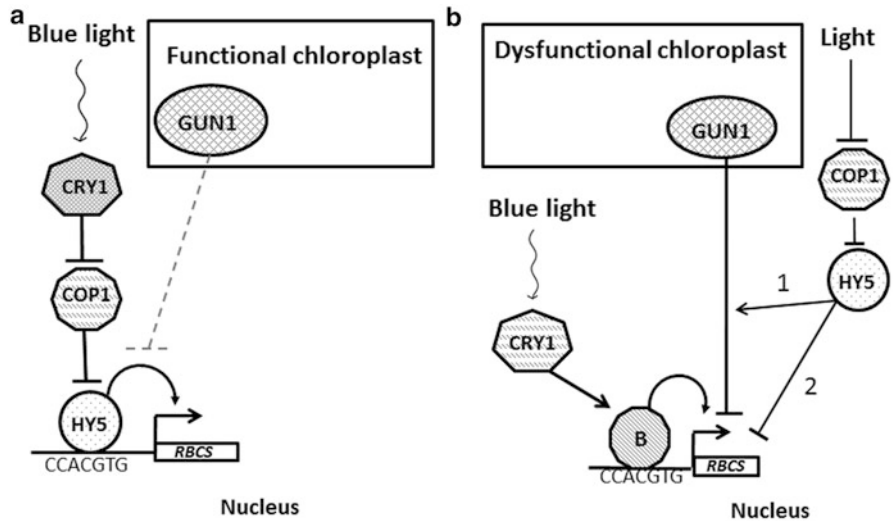

Fig. 25.5. **Working model showing *RBCS* regulation in presence of chloroplast biogenesis inhibitors.** (**a**) When chloroplast is functioning properly, long hypocotyl 5 (HY5) is active in the nucleus as the activity of constitutive photomorphogenesis 1 (COP1) is inhibited by cryptochrome1 (CRY1) and *RBCS* (Rubisco small subunit) transcription takes place. Genomes uncoupled 1 (GUN1) mediated inhibition of *RBCS* does not takes place in a functional chloroplast. (**b**) When the chloroplast is dysfunctional, the GUN1 mediated pathway becomes active and it represses *RBCS* expression. CRY1 no longer utilizes HY5 mediated *RBCS* activation but utilizes another factor 'B' for induction of *RBCS* expression. HY5 and COP1 may be involved in *RBCS* repression either through GUN1 mediated pathway (1) or light pathway independent of GUN1 (2).

in the *phyA* single mutant as well as the *phyAgun1* double mutant. The same was true for *phyB* single and double mutant analysis. Thus, PHYA and PHYB unlike CRY1, are not able to induce or repress *RBCS* expression by themselves and need the help of some additional factors (Ruckle et al. 2007).

VI. Reciprocal Regulation of Photomorphogenesis by Plastid Signals

Ruckle and Larkin (2009) reported on an antagonistic pathway through which plastid signals are able to regulate photomorphogenesis of seedlings when chloroplast biogenesis is blocked. Analysis of *gun1* and *cry1* mutants revealed that GUN1 can inhibit cotyledon expansion in the presence of lincomycin in low-fluence blue light. Also CRY1, which under high-fluence blue light induces cotyledon expansion, acts as an inhibitor of cotyledon expansion in the presence of lincomycin.

It has been observed that anthocyanin biosynthesis is induced during photomorphogenesis and stress. One of the major roles played by anthocyanins is to protect chloroplasts from photo-oxidative stress (Gould 2004). GUN1 plays a major role in induction of anthocyanin when chloroplast biogenesis is blocked and this pathway does not include CRY1 and HY5. Analysis of *cry1*, *hy5* and *gun1* mutants revealed that plastid signals produced during chloroplast dysfunction stimulate hypocotyl elongation while CRY1 and HY5 always inhibit hypocotyl elongation whether chloroplast biogenesis inhibitors are present or not (Ruckle and Larkin 2009).

A hypothetical model for regulation of photomorphogenesis by plastid signals has been proposed in which GUN1 mediated plastid signaling inhibits cotyledon expansion and morphogenesis and another signal 'X' turns CRY1 from a positive regulator to a negative regulator of cotyledon expansion and morphogenesis in the presence of lincomycin. Plastid stress, generated by high light

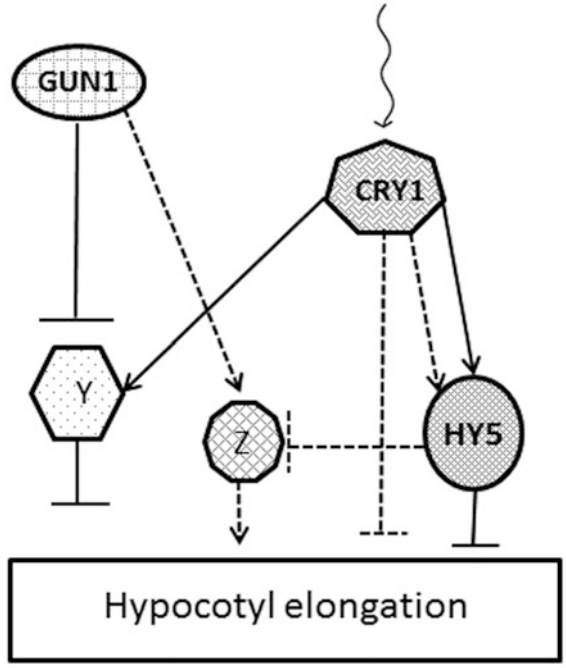

Fig. 25.6. **Hypothetical models showing two different possible mechanism of regulation of hypocotyl elongation by plastid signals.** (1) In the first model (depicted by *normal arrows*), there are two negative regulators of hypocotyl elongation – HY5 and another unknown factor 'Y'. CRY1 induces both these negative regulators while GUN1 inhibits 'Y' when chloroplast biogenesis is blocked. (2) In the second model (depicted by *dashed arrows*), a positive regulator of hypocotyl elongation, factor 'Z', is present which is downstream of HY5. In presence of chloroplast biogenesis inhibitors like lincomycin, CRY1 inhibits hypocotyl elongation either by inducing HY5 which, in turn, inhibits factor 'Z' or in a HY5 independent mechanism. GUN1 here plays an inducer of this factor 'Z'.

intensity or inhibitors of chloroplast biogenesis causes GUN1 and CRY1 to induce the biosynthesis of anthocyanins thereby, protecting the plastids from damage caused by excessive light and scavenging reactive oxygen species (Ruckle and Larkin 2009).

The mechanism for controlling hypocotyl elongation has been explained by two models (Ruckle and Larkin 2009). In the first model, there may be a negative regulator of hypocotyl elongation 'Y' apart from HY5, which is inhibited by GUN1 but induced by CRY1 when chloroplast biogenesis is blocked. In the second model, a factor 'Z' is present which is downstream of HY5 and is a positive regulator of hypocotyl elongation. In the presence of lincomycin, CRY1 inhibits hypocotyl elongation in HY5 independent and dependent mechanisms. HY5 inhibits 'Z' but GUN1 induces 'Z' (Fig. 25.6) (Ruckle and Larkin 2009).

VII. Effects of Light and Retrograde Plastid Signaling on the Transcriptome of Arabidopsis

A. Effects of Plastid Signals on Light-Regulated Genes and Vice-Versa

Microarray studies were carried out to unravel the impact of plastid signals on the expression of light-regulated genes. Seedlings grown on medium with or without lincomycin were kept for 6 days in 0.5 μmol/m^2/s of 40 % blue and 60 % red (BR) light. They were then shifted to 60 μmol/m^2/s of BR light. Samples for microarray analysis were harvested at 0, 1, 4 and 24 h. This analysis showed that approximately 6,424 genes of Arabidopsis were regulated by light and half of these genes were also regulated by plastids. In comparison, only 680 genes are exclusively controlled by plastids and are

unaffected by the fluence-rate shift (Ruckle et al. 2012). Agglomerative hierarchical clustering analysis was done to find more genes which follow *LHCB1* expression pattern in the presence or absence of lincomycin. This analysis revealed that there are at least 100 genes in Arabidopsis whose expression can be shifted from upregulation to downregulation and vice-versa in presence of lincomycin (Ruckle et al. 2012).

B. Effects of Plastid Signals on Different Pathways Regulated by Light

Plastid signals not only change the nature of regulation of expression of plastid-associated genes by light but can also convert light from a positive to a negative regulator of genes annotated to be involved in cell cycle, DNA replication and in the response to jasmonic acid (JA) indicating that the targets of these plastid signals are not restricted to plastids (Ruckle et al. 2012).

Plastid signals were found to repress the light-regulated genes involved in the JA response and can change the nature of light from an inducer to the repressor of genes involved in JA biosynthesis. This suggests that plastid signals can affect the integration of light and JA signaling (Ruckle et al. 2012). One of the factors known to be involved in light signaling as well as JA signaling is AtMYC2 (MYC-related transcriptional activator). AtMYC2 is a negative regulator of blue light signaling (Yadav et al. 2005) but a positive regulator of JA signaling (Lorenzo et al. 2004). So, probably, plastid signals are affecting AtMYC2 activity which in turn is bringing the changes in light-regulated genes involved in the JA response.

Plastid retrograde signaling also affects expression of light-regulated circadian genes like *CCA1* (Circadian clock associated 1) and *PRR5* (Pseudo-response regulator 5). This suggests that natural stress perceived by chloroplasts induce plastid signals which change the expression of genes involved in circadian rhythm by changing the nature of components involved in light signaling (Ruckle et al. 2012).

Changes in the expression pattern of genes involved in abiotic stress were also observed in seedlings grown in the presence of lincomycin and shifted from low BR fluence to high BR fluence. The down-regulation of these genes following BR fluence-rate shift was abolished in the presence of lincomycin, thereby, converting a negative regulator of these genes to a positive regulator. The general observation seen from the microarray analysis suggests that when chloroplasts function well, light induces the expression of genes involved in growth and development and down-regulates genes involved in stress tolerance. When the chloroplast becomes dysfunctional, the plastid signals emitted by it use the same light signaling components to divert the plant resources from growth and development to stress tolerance (Ruckle et al. 2012).

C. END Genes and the Integration of Light and Plastid Signaling

Genes that were found to have a higher expression level in lincomycin treated seedlings than in untreated seedlings on exposure to light were selected for functional analyses in mutants. These genes should also have a GO annotation as genes involved in signaling, transcription or of unknown function. This analysis resulted in isolation of *enhanced de-etiolation* (*end*) mutants. These mutants showed twofold more chlorophyll accumulation than the wild-type plants during de-etiolation. The enhanced de-etiolation phenotype was also analysed in mutants of known light signaling components. It has been found that mutants of genes like *AtMYC2*, *HFR* (long hypocotyl in far-red), *GBF1* (G-box binding factor 1), *HRB1* (hypersensitive to red and blue) and *SPA1* also overaccumulate chlorophyll on de-etiolation while mutants of genes like *COP1*, *DET1*, *PIF1* (phytochrome interacting factor 1), *PIF3* (phytochrome interacting factor 3) and *FHY3* (far-red elongated hypocotyl 3) attenuate chlorophyll accumulation during de-etiolation (Ruckle et al. 2012).

Analysis of *end* mutants revealed that they induce chloroplast biogenesis without affecting photo-oxidative stress and some of

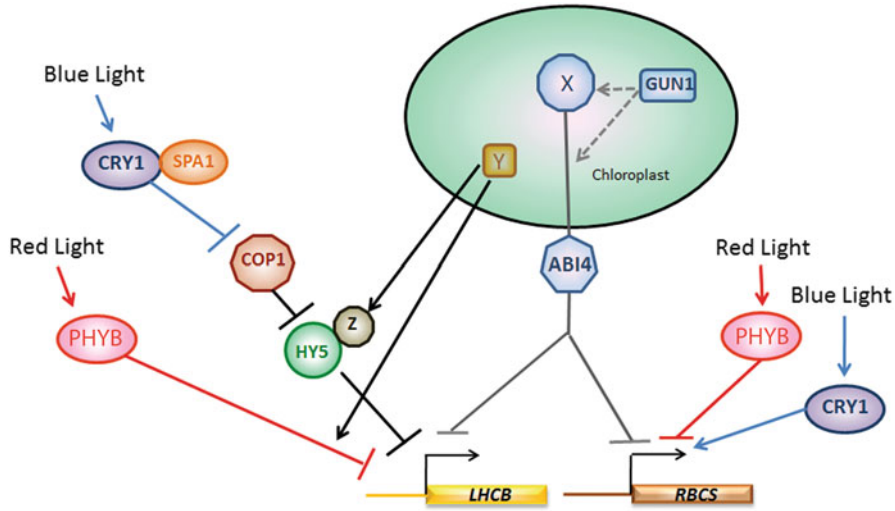

Fig. 25.7. **Summary of the different pathways known to be involved in retrograde plastid signaling**. Two major pathways exist which are involved in retrograde signaling. One is light dependent pathway which involves components like cryptochrome1 (CRY1), phytochrome B (PHYB), long hypocotyl 5 (HY5) and constitutive photomorphogenic 1 (COP1) and the other is a light independent pathway and involves genomes uncoupled 1 (GUN1). When chloroplasts become dysfunctional, GUN1 either emits or perceives the signal X, consequently, ABI4 binds to the promoters of *LHCB* (light harvesting complex of photosystem II subunit) and *RBCS* (rubisco small subunit) and prevents other factors like G-box binding factors (GBF) from binding to them. As a result *LHCB* and *RBCS* are not expressed. In the light dependent pathway, photoreceptors like CRY1 and PHYB, which under normal conditions are inducers of *LHCB* and *RBCS*, repress the expression of *LHCB* upon perceiving signal 'Y' generated from dysfunctional chloroplasts. The pathway of CRY1 mediated repression involves SPA1 (suppressor of phytochrome A-1), COP1 and HY5. The components involved in PHYB mediated repression are still to be elucidated. CRY1, however, induces *RBCS* expression even when the chloroplast is dysfunctional.

these *END* genes also regulate photosynthesis-related gene expression. Therefore, it can be concluded that integration of light and retrograde signaling induced by plastid signals induce a number of *END* genes, which have diverse biological functions promoting the functionality of chloroplast (Ruckle et al. 2012).

VIII. Conclusions

The signaling cascade involved in regulation of chloroplast development by light is quite complex and many of its components are still to be elucidated. The light signals, through anterograde signaling, control the expression of chloroplast genes such as *LHCB* and *RBCS*. But this control of light at the gene expression level also requires feedback information from plastids in the form of plastid signals which keep the plant updated on the status of chloroplast functioning. If the chloroplast dysfunction takes place, the plastid signals released from it have the capacity to convert a positive regulator of light-regulated genes into a negative one and vice-versa. The data available so far suggest that retrograde signaling has at least two different pathways involved which are triggered when chloroplast biogenesis is blocked (Fig. 25.7). One pathway includes rewiring of the light signal pathway involved in expression of PhANGs like *LHCB* from a positive to a negative regulator. The other pathway gets activated in a light independent manner and involves GUN1. Both pathways act to suppress the expression of PhANGs. Microarray analysis has revealed that approximately 3,212 genes are jointly regulated by light and plastids. Plastid signals also affect the expression of light-regulated genes involved in different

processes such as the cell cycle, JA signaling, biotic and abiotic stress responses. Screening of mutants to isolate more components involved in this integration of light and plastid signaling pathways resulted in the isolation of *END* genes which have diverse functions but show a common phenotype of enhanced de-etiolation. Thus, the process of retrograde signaling involves components from diverse pathways and their interlinking enables the plant to respond to the ambient environment and modulate its development.

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Chapter 26

Regreening of Yellow Leaves

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Summary

Yellowing of leaves is the most widely observed manifestation of senescence in plants. Various aspects of this type of programmed cell death are extensively covered elsewhere in this book. One largely overlooked, and perhaps peculiar, feature of plant senescence is regreening of leaves which are already in advanced stages of yellowing. This rejuvenation of main energetic and nutrition tissues is governed by molecular processes and signals which are mostly uncharacterized and poorly understood. Most of the data describing regreening of leaves comes from ultrastructural studies of plastids found in various stages of this process. Reversal of gerontoplast formation has been studied at the level of plastoglobuli formation and disappearance, reassembly of thylakoid structures, lipid remobilization, photosynthetic pigment retention and synthesis. Several investigations have been also focused on naturally occurring regreening models, either induced by insect habitation on leaves, or by physical damage or removal of meristematic tissues. It is now clear that plant hormones, mostly cytokinins, play very important roles in promoting reassembly of the photosynthetic apparatus and the consequent re-establishment of energy supply to the plant. Furthermore, a special kind of regreening was observed in some *aurea* mutants of deciduous woody species. *Aurea* mutants are able to regreen their bleached leaves several times during the vegetation season. It seems that remnants of the thylakoid membrane system are necessary for re-assembly of functional thylakoids in plants.

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I. Introduction

Greening of young and developing plant tissues is the most important series of events that lead to the development of autotrophic, photosynthetic organs, such as leaves. Leaves are the primary sources of energy for the growth of the plant, they are also necessary for the progression of plants into reproductive phase. Damage or defoliation has dramatic effects on the metabolism of the entire plant and can even lead to its death; however, it can also lead to delayed senescence or even regreening (Li et al. 2005). Age-dependent senescence is a genetically programmed series of molecular events that lead to cell death and leaf abscission (Lim et al. 2007). Regulated senescence and controlled breakdown of proteins and nutrients needed for re-mobilization to other organs is crucial for winter survival of deciduous woody species (Krupinska 2007). The loss of soluble proteins, chlorophylls, RNA and starch is generally considered as a criterion for leaf yellowing and senescence (Kirk and Tilney-Bassett 1978). Greening and senescence are normally on the opposite poles of the life span of a plant tissue. Perhaps the best studied molecular processes which occur during greening are those involved in protochlorophyllide photoconversion. Already in 1950 it has been established that the conversion of protochlorophyllide to chlorophyll *a* is very rapid when dark-grown corn seedlings are exposed to white light (Koski 1950). Most of the protochlorophyllide was converted to chlorophyll *a* within 60 s. The rate of conversion is directly proportional to the light intensity (Smith and Benitez 1954). This conversion is carried out by the NADPH-dependent enzyme protochlorophyllide oxidoreductase (POR). Whereas angiosperms make use of the light-dependent enzymes POR A, B, and C, cyanobacteria, algae, bryophytes, pteridophytes and gymnosperms contain an additional, light-independent enzyme dubbed dark-operative protochlorophyllide oxidoreductase (DPOR) (Reinbothe et al. 2010). Illumination initiates the rapid disappearance of POR, so that by the time chlorophyll accumulation reaches its maximum rate, only trace amounts of the enzyme remain (Reinbothe et al. 2010) In the light, excited porphyrin molecules can interact with oxygen to give rise to highly reactive singlet oxygen (Triantaphylidès and Havaux 2010). Like other types of reactive oxygen species (ROS), singlet oxygen has damaging effects on plants, including growth inhibition and cell death (Triantaphylidès and Havaux 2010). To avoid such damage, chlorophylls do not occur as free pigment molecules, instead they are bound together with carotenoids to certain proteins of the photosystems and arranged in the lipid bilayers of the thylakoid membranes, thereby reducing the risk of uncontrolled interaction of excited pigments with oxygen (Nelson and Yocum 2006). In angiosperms, carotenoids unlike the chlorophylls, are formed in the dark, although to a lesser extent than in the light (Lu and Li 2008). Furthermore, carotenoids can be stored within chloroplasts either in plastoglobuli (Austin et al. 2006) or in fibrillar structures (Prebeg et al. 2006). To avoid formation of unbound photodynamic chlorophylls during greening of plant tissues chlorophyll biosynthesis and protein biosynthesis need to be tightly coordinated. After illumination, in some instances, the protein content of the isolated plastid fraction increased by 210% in 2 days, while the protein content of the whole leaf can rise by about 60% in this time (De Deken-Grenson 1954). Additional to photosynthetic pigments and proteins, lipids are essential building blocks for the assembly of photosynthetic macromolecular complexes. During greening

Abbreviations: DCMU – 3-(3,4-diclorophenyl)-1,1-dimethylurea; DGDG – Digalactosyl diacylglyceride; F_m – Maximal fluorescence; F_v – Variable fluorescence or F_m-F_o; F_v/F_m – Quantum efficiency or yield; LHCP – Light harvesting chlorophyll protein; MAPK – Mitogen activated protein kinase; MDA – Malonyldialdehyde; MGDG – Monogalactosyl diacylglyceride; NADPH – Nicotinamide adenine dinucleotide phosphate; PI – Performance index; POR – Protochlorophyllide oxidoreductase; PS – Photosystem; SOD – Superoxide dismutase

of etiolated leaves of *Vicia faba* the amount of fatty acids per leaf doubled (Crombie 1958). Linolenic acid, which is the major fatty acid of the vascular plant chloroplast, makes up 72% of the new fatty acids. Early works on greening of etiolated *Phaseolus vulgaris* leaves (Wallace and Newman 1965) indicate that during greening the relative amounts of palmitic, stearic, oleic, and linoleic acids in the plastids decreased, while the level of linolenic acid increased. The ratios unsaturated/saturated, and C_{18}/C_{16} for the fatty acids increased as well during greening (Wallace and Newman 1965). Galactolipids are specific for chloroplasts and play important roles in the biogenesis of photosynthetic membranes (see Block et al., Chap 7). It appears that during early stages of greening the galactolipid content of plastids decreases, while the chlorophyll content of plastids reaches one-third of its final value. However, at later stages of leaf development the galactolipid content of chloroplasts was about 8.5 times as high as in the plastids of the dark-grown leaves (Wallace and Newman 1965). The phospholipid content of these plastids increased by 63% after 36 h of illumination and by 94% after 11 days illumination.

It is evident that the greening process requires substantial amounts of endogenous energy. The cells gain such energy by oxidizing carbohydrates or lipids, or both. Already in 1865, Böhm has demonstrated the necessity for oxidative metabolism in greening (Böhm 1865). He showed that etiolated plants would not form chlorophyll if illuminated in an atmosphere of pure nitrogen, hydrogen, or carbon dioxide. Clearly, greening of leaves in spring uses different sources of stored energy than germinating seeds. Also, regreening most likely utilizes reserve material stored in other plant tissues and/or relies on the remaining photosynthetic capacity in senescing leaves.

At the ultrastructural level, plastids found in greening etiolated tissues undergo massive restructuring accompanied by the light-induced pigment biosynthesis and thylakoid biosynthesis steps from prolamellar bodies and plastoglobuli (Wellburn 1982). The exact series of assembly events is still not resolved and the controversy whether thylakoid formation requires material from the inner plastid membrane remains. Some authors have reported that during the development of the etioplast, there was an accumulation of vesicles which were produced by invagination of the inner plastid membrane (Vothknecht and Westhoff 2001). It seems that the crystalline center of the prolamellar body was formed by fusion of these vesicles. Other studies do not dismiss this assumption; however no accumulation of vesicles could be observed (Gunning and Jagoe 1967; Kesselmeier and Laudenbach 1986). Unlike in etiolated tissues, plastids found in regreening leaves resemble gerontoplasts which are filled with large (magnoglobuli) and smaller plastoglobuli. Plastoglobuli in mature or senescent plastids contain residual hydrophobic components of degraded thylakoid membranes (Biswal and Biswal 1988; Matile 1992; Lichtenthaler, Chap. 15). It has been suggested that chloroplasts can arise only from an early gerontoplast stage, whereas later stages are no longer retransformable to chloroplasts. In addition to plastoglobuli, these gerontoplasts contain substantial amounts of thylakoid membrane remnants which are often found in concentric or cup-shaped structures (Fulgosi et al. 2008). The residual membranes seem to be important for the reassembly of functional thylakoid membrane system.

II. Leaf Regreening After Removal of the Growing Shoot Apex

More than 50 years ago Mothes and Baudisch (1958) have demonstrated that when all buds and leaves except one yellow leaf are removed from a *Nicotiana rustica* plant, then the regreening of that leaf can occur. Their observations have been repeated in a study which aimed at a more thorough ultrastructural analyses of the changes taking place during yellowing and regreening of *Nicotiana* leaves (Ljubešić 1968). Regreening could be divided into three stages; recovery stage, regreening stage, and starch accumulation stage (Fig. 26.1).

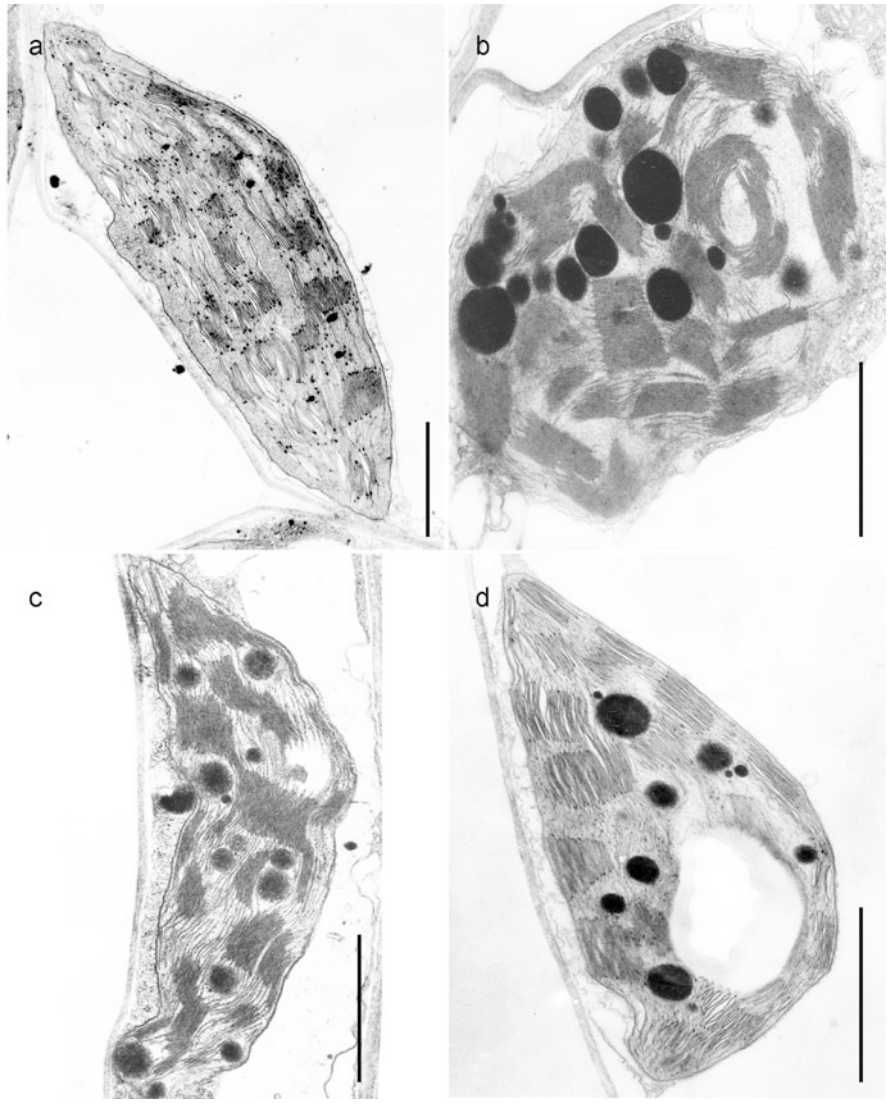

Fig. 26.1. Regreening of a *Nicotiana rustica* yellow leaf after removal of all buds and green leaves. (**a**) Chloroplast ultrastructure in a green leaf; (**b**) ultrastructure of plastid from a yellow leaf 2–3 days after the removal; (**c**) chloroplast of the yellow leaf in the following 5 days; (**d**) recovered chloroplast with large starch 14–21 days after the initiation of regreening. *Bar* = 1 μm.

Recovery started after 2–3 days of the removal of younger leaves and shoots and visible changes could be observed on the remaining yellow leaf (Fig. 26.1b). Processes of yellowing were slowed down while the turgor of the leaf cells gradually increased. In the following 5 days, the leaf recovered further and changed its color to yellow-green. The chlorophyll amount reached 80% of that observed in green leaves. Light microscopy revealed enlargement of leaf cells, while the number of chloroplasts in palisade cells increased and reached the number found in green leaves. Chloroplasts increased in size, although great variations were observed. Ultrastructural analyses (Fig. 26.1c) corroborated enlargement of chloroplasts which could be attributed to the *de novo* synthesis of stroma. Grana stacks started to reconnect with stroma thylakoids

and the number of plastoglobuli slightly decreased. Fourteen to twenty-one days after the initiation of regreening (Fig. 26.1b), the fully recovered leaf had the phenotype of a normal green leaf. Only few large plastoglobuli remained. In the last stage, the chlorophyll amount increased for more than 10% above the normal levels and marked accumulation of assimilatory starch was observed. About 80 days after the initiation of the experiment, yellowing started to reappear. In this study, regreening could not take place when the majority of grana stacks were already in the advanced stages of degeneration. Opposite to this observation are the results of Sveshnikova et al. (1966) who, after the treatment with cytokinins, managed to get full recovery of the thylakoid system in plastids which had completely degraded grana stacks. The last stage of the regreening process is characterized by enhanced accumulation of assimilatory starch. It seems that this is the consequence of increased deposition of assimilates caused by the absence of young and developing tissues which normally act as sinks.

Decapitation induced regreening of *Nicotiana rustica* requires low light illumination (Zavaleta-Mancrea et al. 1999a) This is probably so because thylakoid light harvesting structures are mostly damaged during yellowing and leaves have a poor ability to cope with high-light stress. Chlorophyll *b* appears to be more resistant to degradation during yellowing. It has been postulated that increased chlorophyll *b* may not be the same to that which was bound to antennae of pre-senescent leaves. Instead, unbalanced operation of the *a*-*b* interconversion cycle was postulated (Zavaleta-Mancrea et al. 1999a). During senescence, LHCP was almost entirely degraded, probably due to increased protease activity. In the course of regreening, protease activity declined, total protein levels recovered, and LHCP reappeared. The resynthesis of chlorophyll in regreening leaves coincided with renewed expression of genes encoding NADPH-POR. POR gene expression was accelerated by cytokinin treatment, thus demonstrating that hormones are important regulators of chlorophyll biosynthesis during rejuvenation (Zavaleta-Mancrea et al. 1999a). Important roles of kinetin and gibberelic acid have also been demonstrated in regreening of blackberry leaf discs (Ljubešić 1976). After 2–3 weeks treatment with kinetin or gibberelic acid the yellow-green discs became green. Visible changes of the chloroplast ultrastructure could be demonstrated, most notably the disappearance of phytoferritin inclusions (Ljubešić 1976). Detailed analyses of ultrastructural changes taking place during regreening-associated plastid conversion indicate that all rejuvenated plastids arise from gerontoplasts found in yellow leaves (Zavaleta-Mancrea et al. 1999b). The plastid number per cell declined during senescence and did not increase again during regreening. Thus it is likely that all chloroplasts of regreening leaves arise by redifferentiation of gerontoplasts. Opposite to these findings are the results from regreening studies of soybean cotyledons (Tuquet and Newman 1980). Ultrastructural data indicated that chloroplasts of regreened soybean cotyledons originate either from proplastids or from intermediate stages of slightly senescent plastids (Tuquet and Newman 1980).

Furthermore, morphological differences between plastoglobuli isolated from senescent tissues and those from mature tissues were found by using scanning electron microscopy (Tuquet and Newman 1980). Senescing cotyledons contained large and wrinkled plastoglobuli, while those from mature tissues were small and spherical. It is possible that soybean cotyledons contain substantially more proplastids than the mature leaves of *Nicotiana rustica*, and that those proplastids differentiate into mature chloroplasts during regreening. However, in the previous study (Huber and Newman 1976) it was shown that plastids in regreened soybean cotyledons originated from chromoplast-chloroplast conversion. An important role of galactolipid synthesis for the capability of soybean cotyledons to regreen was also proposed (Huber and Newman 1976; Wetteraua et al. 1978). Interestingly, senescence was accompanied by more

pronounced changes of cytoplasmatic lipids, while the chloroplast lipids experienced less change. Labeling of the galactolipids indicated that the level of monogalactosyl diacylglycerides (MGDG) more closely paralleled plastid structure than did that of digalactosyl diacylglyceride (DGDG). A relationship between chlorophyll data and lamellar arrangement was found. Futhermore, a role of the envelope in the dynamic reconstitution of lamellar membranes was demonstrated (Huber and Newman 1976). It can be concluded that leaf regreening is a result of plastid conversion and not of *de novo* synthesis or of plastid division (Greening et al. 1982).

A comparative study of the photosynthetic parameters associated with regreening and rejuvenation of decapitated and defoliated bean plants was carried out (Yordanov et al. 2008). Unlike previous studies, decapitations were performed during different phases of plant ontogenesis, while for defoliation studies, plants with primary leaves and a stem with an apical bud, but without composite leaves were used. Decapitation of bean plants above the primary leaves reversed or considerably delayed their senescence. Analysis of chlorophyll fluorescence, millisecond delayed fluorescence and absorption at 830 nm was utilized to investigate the alterations in photosystem II and I electron transport during the decapitation-induced delayed senescence in the non-detached leaves. Both decapitation and defoliation caused changes in the anatomy of primary leaves, suggesting alteration in metabolism. Interestingly, regreening was more evident when decapitation was carried out in old plants while senescence occurred more easily in younger leaves than in older leaves. A PI index (performance index) was used to characterize PSII structural and functional alterations in control and decapitated bean plants. PI values decreased with the aging of the primary leaves. Decapitation applied after emergence of the primary leaves and of the first trifoliate leaves caused a visible increase in PI. PI remained relatively unchanged during the entire period of regreening. No significant structural changes in PS II were observed during leaf senescence, as evaluated by the F_v/F_m ratio. Linear electron transport was increased in decapitated plants, which was explained by higher activity of the Calvin-Benson cycle reactions in the remaining leaf. Most likely, changed sink to source relationships in decapitated/defoliated plants were responsible for this effect. It was concluded that decapitation of the plants modified the leaf senescence program, as do other internal and external stimuli such as light, temperature, drought, elevated CO_2, age, reproduction, plant growth regulators, defoliation, and others.

III. Rejuvenation of Aged Leaves Under the Effect of Low-Dose Stressors

Many chemical stressors, like heavy metals and herbicides, impose heavy damage on plant tissues when applied in high concentrations. Applied in low-concentrations, chemical stressors may exert beneficial and stimulative effects, such as an increase in the chlorophyll content, changes in the composition of chlorophyll-protein complexes, elevation of CO_2 incorporation, and changes in the ultrastructure of chloroplasts (Nyitrai et al. 2003, 2004, 2007). For example, delayed ageing of detached leaves of cucumber, bean, and barley was observed after treatment with low-dose stressors (Kovács et al. 2007, 2009; Nyitrai et al. 2008). Electron microscopy revealed differences between the stressor treated and control leaves mostly in the size of plastoglobuli. Rejuvenated plastids contained small plastoglobuli, similar to those found in young leaves (Kovács et al. 2008, 2009). Degradation of chlorophyll was also inhibited which was an indication of delayed ageing (Kovács et al. 2007). It was hypothesized that stressors, such as cadmium and 3-(3,4-diclorophenyl)-1,1-dimethylurea (DCMU), increase the amount of active cytokinins in roots of treated barley seedlings. Cytokinins transported to the leaves were shown to have a stimulatory effect on

chloroplast functionality (Kovács et al. 2009). Stimulatory effects are most likely non-specific, since chemically different stressors had the same effect (Kovács et al. 2008). PIP_2-IP_3/DAG and MAPK signaling pathways were also implicated in the rejuvenation processes (Kovács et al. 2008; Nyitrai et al. 2009). Measurements of superoxide dismutase (SOD) activity and the amount of malonyldialdehyde (MDA) showed that the increased amounts of cadmium did neither cause oxidative stress in roots nor in the leaves (Kovács et al. 2009). DCMU slightly increased the activity of SOD, but did not cause lipid peroxidation, while in leaves there was no oxidative stress upon treatment with DCMU (Kovács et al. 2009). It was thus concluded that the oxidative stress cannot be responsible for the stimulation with low-concentration stressors (Kovács et al. 2009).

IV. Regreening of *Aurea*-Type Leaves

Leaves of *aurea*, "golden" varieties of some vascular plants are extremely sensitive to high-light intensities and can remain normally green only in deep shade. Extended exposure of *aurea*-type leaves to strong sunlight leads to their bleaching. When shaded, or during prolonged periods of low-light illumination, yellow and bleached leaves can regain green coloration. This adaptation ability is limited to younger leaves, and in general declines with leaf age. Conditional *aurea* mutants are especially suitable for ultrastructural studies of chloroplast conversions (Fulgosi et al. 2008), structure and function of PS I and II (Okabe et al. 1977; Kawata and Cheung 1990), plastid signaling (Oelmüller and Kendrick 1991) leading to changes in expression of nuclear *cab* genes (Oelmüller et al. 1989; Palomares et al. 1991). Additionally, some conditional mutants have considerably diminished biosynthesis of carotenoids, which in high-light leads to photooxidative damage, destruction of chloroplast ultrastructure and termination of mRNA biosynthesis of some additional photosynthesis associated genes. Most of the *aurea* mutants that are characterized on molecular level are deficient in the light receptor phytochrome.

Differentiation of chloroplasts of *aurea* varieties has been studied in a number of species e.g. *Acer negundo* Hassk. var. *Odessanum*, *Fraxinus excelsior* L. var. *aurea*, *Euonymus fortune* var. *radicans*, and *Ligustrum ovalifolium* Hassk. var. *aureum* (Wrischer et al. 1975a, b, 1976; Antica and Wrischer 1982; Kunst 1983; Kunst and Wrischer 1984). Chloroplasts of green *aurea* leaves are similar to wild-type ones (Fig. 26.2a), except more grana thylakoids are present since the leaves usually grow under low light illumination or in the shade. When exposed to high light intensities, green *aurea* leaves start to lose green coloration and eventually turn yellow. Plastids of yellow leaves contain only a few separate thylakoid membranes, while the grana are degraded (Fig. 26.2b). As a leftover of this process aggregated, concentric structures of plastid lipids, as well as the plastoglobules are present in the stroma (Fig. 26.2c). Ribosome number is much reduced. Yellow *aurea* leaves are severely deficient in all pigments and particularly, in chlorophyll *a* and *b*. The effect is largely increased at the end of the vegetation period. HPLC analysis of carotenoids reveals a great reduction in the content of some xanthophylls in green *aurea* leaves (Fulgosi H. unpublished data). In yellow leaves this effect is even more evident and is concomitant with reduction of α as well as β carotene. Protein analyses have revealed a gross deficiency of LHCII apoproteins (Fulgosi et al. 2008). Photosynthetic activity, as measured by O_2 evolution is highest in light-green leaves, when expressed on the fresh weight of leaf tissue. However, when calculated on a chlorophyll basis, the photosynthetic activity of yellow leaves is very high; in *A. negundo* var. *Odessanum* it is about five times higher than of green leaves from deep shade (Wrischer et al. 1986).

Sun-grown yellow leaves, when shaded, green in a few days, and green leaves, when exposed to the sunlight, yellow again in about 10–14 days. The promptness of these transformations depends on the leaf age and on

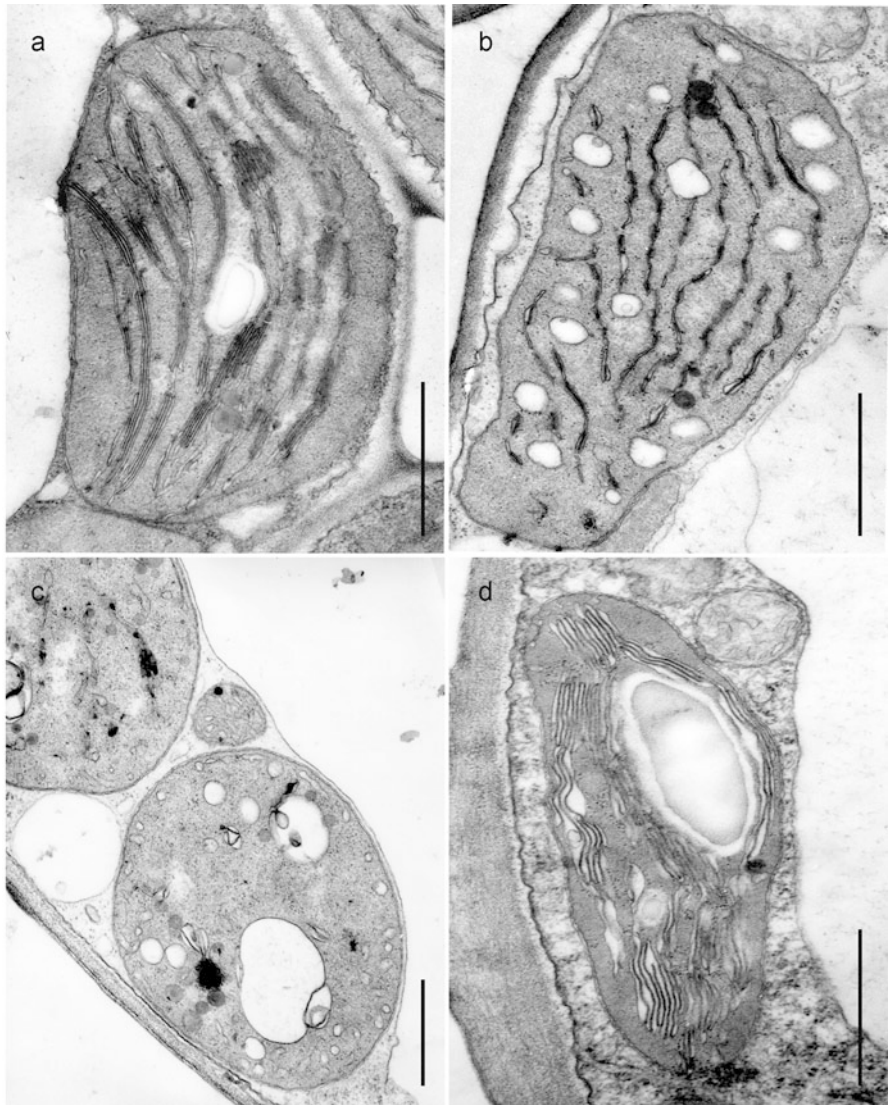

Fig. 26.2. Regreening of *aurea* mutant *Acer negundo* var *Odessanum* leaf. (**a**) Chloroplast found in *aurea* leaves grown in shade; (**b**) ultrastructure of plastid from yellow *aurea* leaves; (**c**) deteriorated ultrastructure of bleached *aurea* leaf plastids; (**d**) regreened chloroplast from shaded conditions. *Bar* = 1 μm.

the plant species examined. Simultaneously with these adaptations, changes in pigment content and in plastid fine structure appeared (Fig. 26.2d). Electron microscopic analyses revealed that during degreening of the leaves in full sunlight peculiar membranous structures, like coils or cup-shaped appressed thylakoid stacks, appear in the plastids (Fig. 26.2). These structures, representing thylakoids in degradation, are seldomly found in plastids of *A. negundo* var. *Odessanum*, but very often in those of *F. excelsior* var. *aurea*. These structures disappear completely in old yellow and in bleached leaves (Fig. 26.2c), but large plastoglobules are found in the stroma instead. The plastoglobules can in turn be partially resorbed when the leaves regreen again (Fig. 26.2d).

To conclude, chloroplasts of *aurea* plants adapt well to alterations in light intensity.

There are some differences in this ability among the plant species. *A. negundo* var. *Odessanum* adapts most promptly, other plants with some delay, whereas *F. excelsior* var. *aurea* is especially sensitive to strong illumination. The membranous coil-like or cup-shaped inclusions found in plastids of degreening leaves, are transient structures, which represent a way to eliminate parts of the thylakoid system, when the leaves are transferred from low to high light. Under favorable low-light conditions, plastids in yellow leaves are able to resynthesize a normal thylakoid apparatus. Therefore, yellowing of the *aurea* plants does not represent an usual process of chloroplast degeneration and subsequent regreening.

Acknowledgments

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Chapter 27

Modulation of Chlorophyll Biosynthesis by Environmental Cues

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Summary

Environmental signals control diverse physiological processes in plant growth and development. Plants tend to adapt the structure of photosynthetic apparatus and pigment composition in response to several environmental factors. Tetrapyrroles play vital roles in various biological processes, including photosynthesis and respiration. Expression of genes encoding enzymes of tetrapyrrol biosynthesis as well as the abundances and activities of the enzymes

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are severely impacted by availability of water, soil salinity, low or high temperature and low or high light intensity. Plastids share many cellular metabolic pathways and alterations of plastid functions by environmental signals are known to affect various aspects of plant development. The generation of reactive oxygen species (ROS) in plants is triggered by different kinds of environmental parameters, such as high light, high or low temperature, salinity, drought and nutrient deficiency. Imbalance between production of ROS and their detoxification by enzymatic and non-enzymatic reactions causes oxidative stress. Suitable genetic manipulation of the chlorophyll (Chl) biosynthetic pathway might lead to tolerance towards environmental stresses leading to oxidative stress at the cellular level, and efficient adaptation of the photosynthetic apparatus to low and high light intensities. The present review deals with environmental modulation of Chl biosynthesis and its impact on plant productivity.

I. Introduction

Environmental cues including light, water, temperature, soil and nutrient content have a profound impact on plant growth and development. For example, light has significant effects on morphogenesis of seedlings during the transition from heterotrophic to photoautotrophic growth. Plants tend to adapt the structure of the photosynthetic apparatus and the pigment composition to light quality and quantity and other environmental factors. Tetrapyrroles play vital roles in various biological processes, including photosynthesis and respiration (Rebeiz et al. 1994; Papenbrock and Grimm 2001; Block et al. 2007; Tanaka and Tanaka 2007; Masuda 2008; Biswal et al. 2012; Phung et al. 2012). Plants are exposed to various abiotic stresses such as low temperature, high temperature, salinity, drought, flooding, oxidative stress and heavy metal toxicity etc. either during their entire life cycle or a part thereof. Plant growth, development, photosynthesis and productivity are severely affected due to environmental stresses, particularly during early seedling growth. When seeds germinate beneath the soil, their seedlings remain in near-darkness for a while. Therefore, etiolated rice seedlings beneath the soil do not synthesize Chl and contain a special form of plastids called etioplasts or etiochloroplasts. As seedlings come out of soil, they are exposed to light and light-mediated Chl biosynthesis and other associated greening processes are initiated resulting in transformation of etioplasts to chloroplasts (See Solymosi and Aronsson, Chap. 3). During chloroplast biogenesis in the light, proplastids in meristematic tissue and etioplasts in dark-grown seedlings develop into the mature, photosynthetic chloroplast of the green leaf (Waters and Pyke 2005).

Chloroplast biogenesis and development in seedlings can be described as the differentiation process from the plastid progenitor, a proplastid, to a mature chloroplast. Plastids carry out many essential metabolic pathways and alteration of plastid functions affects various

Abbreviations: ALA – 5-Aminolevulinic acid; ALAD – 5-Aminolevulinic acid dehydratase; CAO – Chlorophyllide A oxygenase; Chl – Chlorophyll; Chlide – Chlorophyllide; Coprox – Coproporphyrinogen oxidase; CPO – Coproporphyrinogen oxidase; DV – Divinyl; DV-Pchlide – Divinyl protochlorophyllide; DVR – Divinyl reductase; FLU – Fluorescence; GGPP – Geranyl geranyl pyrophosphate; GluRS – Glutamyl-tRNA synthetase; GluTR – Glutamyl-tRNA reductase; GSA – Glutamate 1-semialdehyde; GSA-AT – Glutamate 1-semialdehyde aminotransferase; GUN – Genome uncoupled; LHC II – Light-harvesting complex II; Lin2 – Lesion initiation 2; lip1 – Light-independent photomorphogenesis 1; MPE – Mg-protoporphyrin IX monomethylester; MTF – Mg-Protoporphyrin IX methyltransferase; MV – Monovinyl; MV-Pchlide – Monovinyl protochlorophyllide; PBG – Porphobilinogen; PBGD – Porphobilinogen deaminase; Pchlide – Protochlorophyllide; PhPP – Phytyl diphosphate; PLBs – Prolamellar bodies; POR – Protochlorophyllide oxidoreductase; PPIX – Protoporphyrin IX; Protogen IX – Protoporphyrinogen IX; Protox – Protoporphyrinogen oxidase; ROS – Reactive oxygen species; SAM – *S*-adenosyl-methionine; SDR – Short chain dehydrogenases/reductases; tRNAglu – Glutamate conjugated tRNA; Urogen III – Uroporphyrinogen III; UROS – Uroporphyrinogen III synthase

aspects of plant growth and development. Chloroplasts are responsible for the biosynthesis of carbohydrates, fatty acids, pigments, and the synthesis of amino acids and proteins from inorganic nitrogen (Staehelin and Newcomb 2000). Chloroplast development involves the biosynthesis of components of the photosynthetic apparatus involving synthesis of Chl and carotenoids, lipids and proteins which is governed in a coordinated manner by chloroplast and nuclear genomes (Leon et al. 1998; Gray et al. 2003; Nott et al. 2006). Biosynthesis of porphyrins, particularly that of Chl, during early greening stages of seedlings is elucidated in detail (Tripathy and Rebeiz 1985, 1986, 1988; Meskauskiene et al. 2001; Goslings et al. 2004; Bollivar 2006; Tanaka and Tanaka 2007; Wu et al. 2007; Wang et al. 2010; Tripathy and Pattanayak 2012).

Chl biosynthesis and chloroplast development during irradiation of dark-grown plants is impacted by external and internal factors such as light quality, temperature, nutrition, leaf age, leaf water potential, salt etc. as they influence transcription, translation and post-translational modification of proteins involved in chloroplast biogenesis (Virgin 1965; Bengtson et al. 1978; Bhardwaj and Singhal 1981; Eskins et al. 1986; Tewari and Tripathy 1998, 1999; Le Lay et al. 2000, 2001; Sood et al. 2004, 2005; Mohanty et al. 2006; Dutta et al. 2009; Mohanty and Tripathy 2011; Dalal and Tripathy 2012).

All enzymes of the Chl biosynthetic pathway are nuclear encoded and post-translationally imported into chloroplasts. Chl synthesis is synchronized with the formation of other pigments such as carotenoids and with pigment-binding proteins; Chl synthesis is also involved in the coordination between chloroplast and nucleus (Nott et al. 2006).

II. Chlorophyll Biosynthetic Enzymes and Their Modulation by Environment

A. Biosynthesis of 5-Aminolevulinic Acid

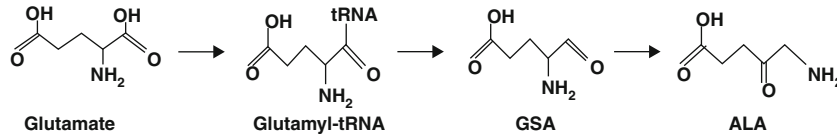
Unlike animals where one enzyme 5-aminolevulinic acid (ALA) synthase could form ALA by condensation and decarboxylation of succinyl-CoA and glycine, the synthesis of ALA in plants requires three different enzymes located in the chloroplast stroma. These are glutamyl-tRNA synthetase (GluRS) [EC 6.1.1.17], glutamyl-tRNA reductase (GluTR) [EC 1.2.1.70] and glutamate semialdehyde aminotransferase (GSA-AT) [EC 5.4.3.8] (Fig. 27.1a).

GluRS, also known as aminoacyl-tRNA synthetase, ligates glutamate to $tRNA^{GLU}$ (Huang et al. 1984; Kannangara et al. 1984, 1994) (Fig. 27.1a). Unlike class I aminoacyl-tRNA synthetases, GluRS avoids the aminoacyl-AMP formation in the absence of tRNA. In eukaryotic cells chloroplastic GluRS is post-translationally imported into the chloroplast where it ligates glutamate to $tRNA^{GLU}$ that contains the UUC anticodon (Schön et al. 1986, 1988).

GluTR, the second enzyme of the pathway, uses NADPH to reduce the activated α-carboxyl group of glutamyl-tRNA (Glu-tRNA) to synthesize glutamate 1-semialdehyde (GSA) (Hoober et al. 1988). The GluTR is a homopentamer of identical subunits of 54 kDa (Pontoppodian and Kannangara 1994). This enzyme is subject to feedback regulation by heme and appears to be a major control point of porphyrin biosynthesis (Kannangara et al. 1988). In *A. thaliana* GluTR interacts with FLU, a negative regulator of the Chl biosynthesis pathway (Meskauskiene et al. 2001; Meskauskiene and Apel 2002). FLU is a nuclear-encoded chloroplast protein, and the *flu* mutant has a higher level of ALA synthesis and protochlorophyllide (Pchlide) accumulation than that of wild-type plants. Probably FLU is a component of negative regulatory system for ALA synthesis when cells have high Pchlide contents. A FLU-like protein is also present in barley (Lee et al. 2003). GluTR is encoded by the *HEMA* gene. It has two isoforms in barley and cucumber, whereas in *A. thaliana* it has three isoforms.

The formation of 5-aminolevulinate/ALA from GSA is catalyzed by GSA-AT, the third and the last enzyme required for ALA biosynthesis. This enzyme is functionally an aminomutase, which transfers the amino group from carbon 2 of GSA to the neighboring carbon atom i.e., carbon 5 to form ALA (Fig. 27.1a). The enzyme is inhibited by gabaculine (Gough et al. 1992).

a



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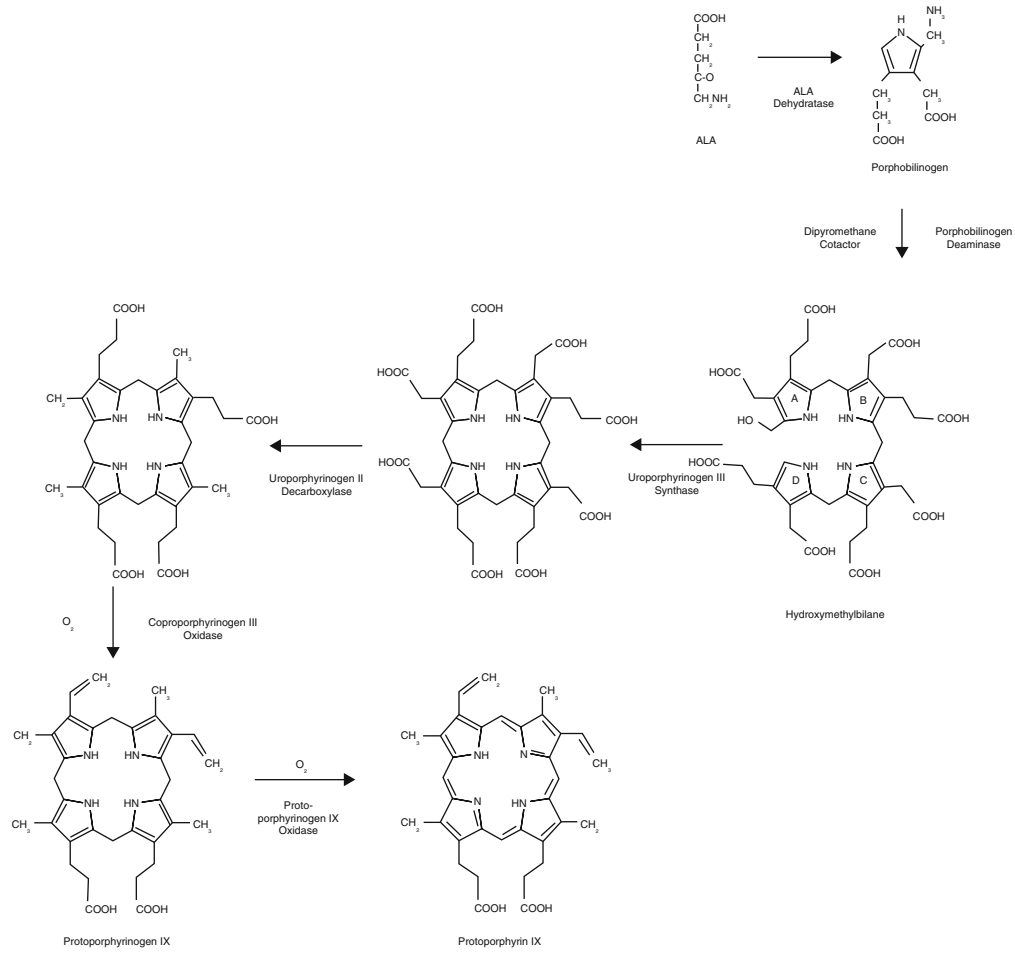

Fig. 27.1. (continued)

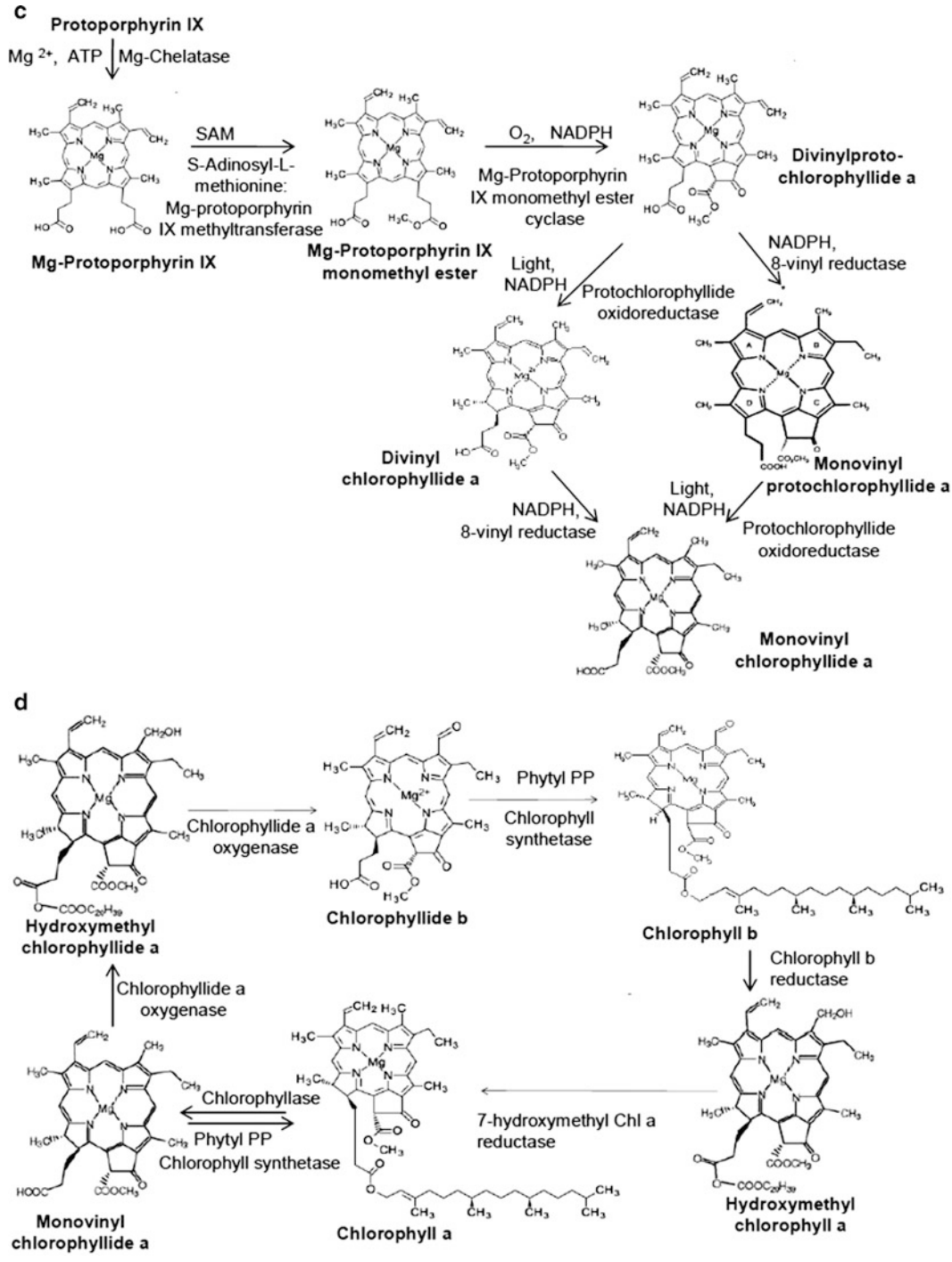

Fig. 27.1. Chl biosynthesis pathway in higher plants. (**a**) Biosynthesis of ALA from glutamic acid (**b**) Biosynthesis of protoporphyrin IX from ALA (**c**) Biosynthesis of monovinyl chlorophylide a from protoporphyrin IX (**d**) Biosynthesis of Chl a and Chl b from monovinyl chlorophylide a.

1. Environmental Modulation of ALA Biosynthesis

a. Light Regulation of ALA Biosynthesis

In cucumber and *Arabidopsis thaliana*, the *HEMA1* gene is expressed in photosynthetic tissues and is induced by illumination, but no transcripts were detectable in roots (Tanaka et al. 1996; Ilag et al. 1994). Gene expression of *HEMA1,* and the corresponding protein abundance, increases in response to light treatment of dark-grown seedlings suggesting that an increased demand for Chl biosynthesis stimulates its expression and the gene promoter may have light-responsive elements (Mohanty et al. 2006). On the other hand, *HEMA2* is preferentially expressed in non-photosynthetic tissues, and its expression is light-independent (Tanaka et al. 1996; Nagai et al. 2007). A third *HEMA* gene, *HEMA3*, has been identified in *A. thaliana*, but its expression is low (Matsumoto et al. 2004).

In *A. thaliana* light stimulates transcription of *GSA* (Ilag et al. 1994). The gene expression is also activated by the hormone kinetin (Yaronskaya et al. 2006). The expression of *GSA* and protein abundance of GSA-AT increases when etiolated seedlings are transferred to light demonstrating that it is a light-inducible gene that significantly contributes to Chl synthesis (Mohanty et al. 2006). In soybean also, the *GSA* is light inducible. It contains a light-regulated cis element (containing GAGA) that is found to be involved in transcriptional control (Frustaci et al. 1995). The mRNA level is high in soybean leaves (Sangwan and O'Brian 1993) whereas the mRNA is not detectable in roots (Frustaci et al. 1995).

b. Modulation of ALA Biosynthesis by Temperature

Environmental factors such as chill- or heat-stress influence gene expression, translation and post-translational modification of proteins involved in chloroplast biogenesis (Tewari and Tripathy 1998, 1999; Mohanty et al. 2006; Abdelkader et al. 2007a, b; Dutta et al. 2009). When 5-day old etiolated wheat seedlings grown at 25°C are transferred to 7°C (chill-stress), 42°C (heat-stress) or 25°C (control) and exposed to cool white fluorescent light (50 μmoles m^{-2} s^{-1}) for 24 h, the Chl content gradually increases in control seedlings. In chill- and heat-stressed seedlings Chl biosynthesis is severely down-regulated. A lag period up to 12 h is observed, both in chill- and heat-stressed wheat seedlings before Chl accumulation accelerates (Fig. 27.2).

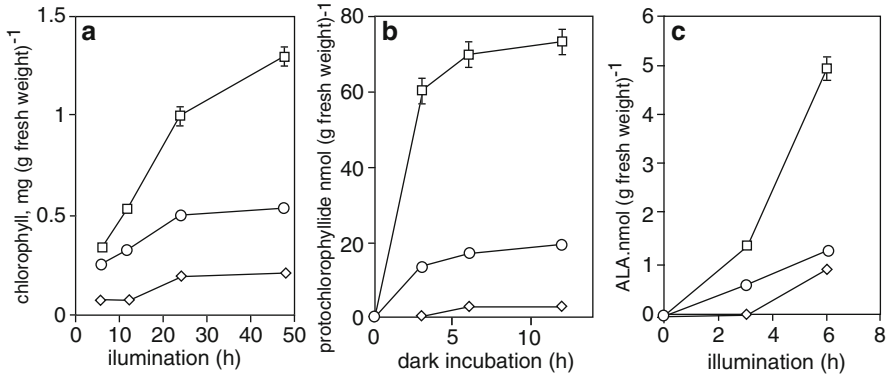

Fig. 27.2. Biosynthesis of Chl (*left panel* (**a**)), Pchlide (*central panel* (**b**)), and ALA (*right panel* (**c**)) in control (25°C, squares), chill-stressed (7°C, diamonds), or heat-stressed (42°C, circles) cucumber seedlings. Each data point is the mean of three replicates; error *bars* represent SD. Missing error bars indicate that they are smaller than the symbols (Tewari and Tripathy 1998).

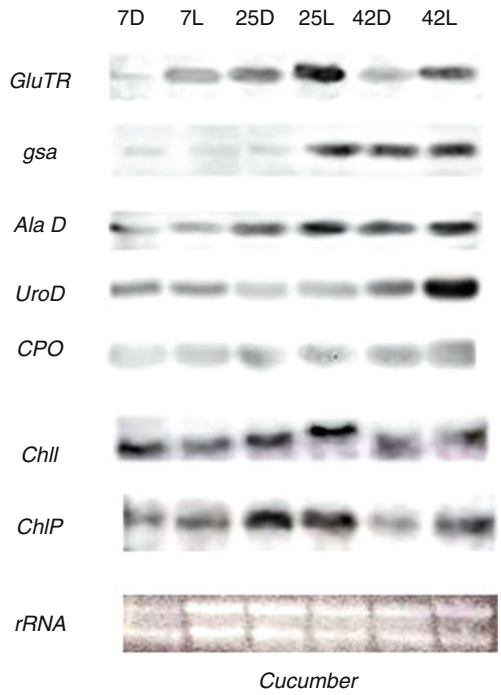

Fig. 27.3. Modulation of gene expression (Northern blot) of Chl biosynthesis pathway enzymes by temperature stress in cucumber. Five-day old etiolated seedlings grown at 25°C were transferred to light (50 mmol m^{-2} s^{-1}) or dark at 7°C, 25°C or 42°C for 24 h and Northern blotting was performed. *Numbers* denote temperature and D and L denote *dark* and *light* respectively (Mohanty et al. 2006).

ALA synthesis in the presence of LA is almost linear up to 6 h of illumination in control and heat-stressed seedlings (Tewari and Tripathy 1998). For the first 3 h, ALA synthesis is completely inhibited in chill-stressed cucumber seedlings. As compared with the controls, the net synthesis of ALA is severely reduced in chill- and heat-stressed seedlings, respectively (Fig. 27.2) (Tewari and Tripathy 1998). Among ALA biosynthetic enzymes, the expression of *HEMA* is light-inducible in cucumber i.e., its expression increases in response to light in cucumber seedlings (Fig. 27.3). Its expression was down-regulated both in chill- and heat-stressed seedlings (Mohanty et al. 2006).

The expression of *GSA* increases upon light exposure of etiolated control seedlings. However, heat-stressed etiolated seedlings display a higher *GSA* expression level than etiolated control seedlings. *GSA* expression further increases in illuminated heat-stressed seedlings and is significantly reduced in cold-treated cucumber seedlings (Fig. 27.3) (Mohanty et al. 2006).

Reduced ALA biosynthesis in cucumber at low temperature could be due to reduced gene and/or protein expression of two ALA biosynthetic enzymes GluTR and GSA-AT.

The reduced Chl synthesis was also reported in temperature-stressed maize/Pinus seedlings mostly due to down-regulation of early intermediates of Chl biosynthesis i.e., GSA and ALA (Hodgins and Van Huystee 1986; Hodgins and Oquist 2006).

c. Regulation of ALA Biosynthesis by Salinity

The Chl biosynthesis and chloroplast biogenesis are substantially regulated by salt-stress. ALA content was reduced in sunflower leaves on treatment with salt stress (Santos 2004) that may be due to reduction in the ALA precursor glutamate (Santos and Caldeira 1999; Santos et al. 2001).

d. Water-Stress and ALA Biosynthesis

In response to water-stress, Chl biosynthesis is down-regulated. The reduced Chl synthesis in water-stressed seedlings is mostly due to down-regulation of early intermediates of Chl biosynthesis i.e., GSA and ALA (Dalal and Tripathy 2012).

Reduced GSA synthesis in water-stressed rice seedlings is due to down-regulation of *HEMA1* transcript abundance (Fig. 27.4). The protein/transcript abundance of GSA-AT increased (Fig. 27.4) in water-stressed rice seedlings, however the ALA contents declined suggesting that the GSA-AT, the next enzyme involved in ALA biosynthesis, may be inactivated by post-translational modification. These results show that the Chl biosynthesis pathway is down-regulated at the early steps under stress conditions to prevent the accumulation of harmful singlet oxygen generating tetrapyrroles (Dalal and Tripathy 2012).

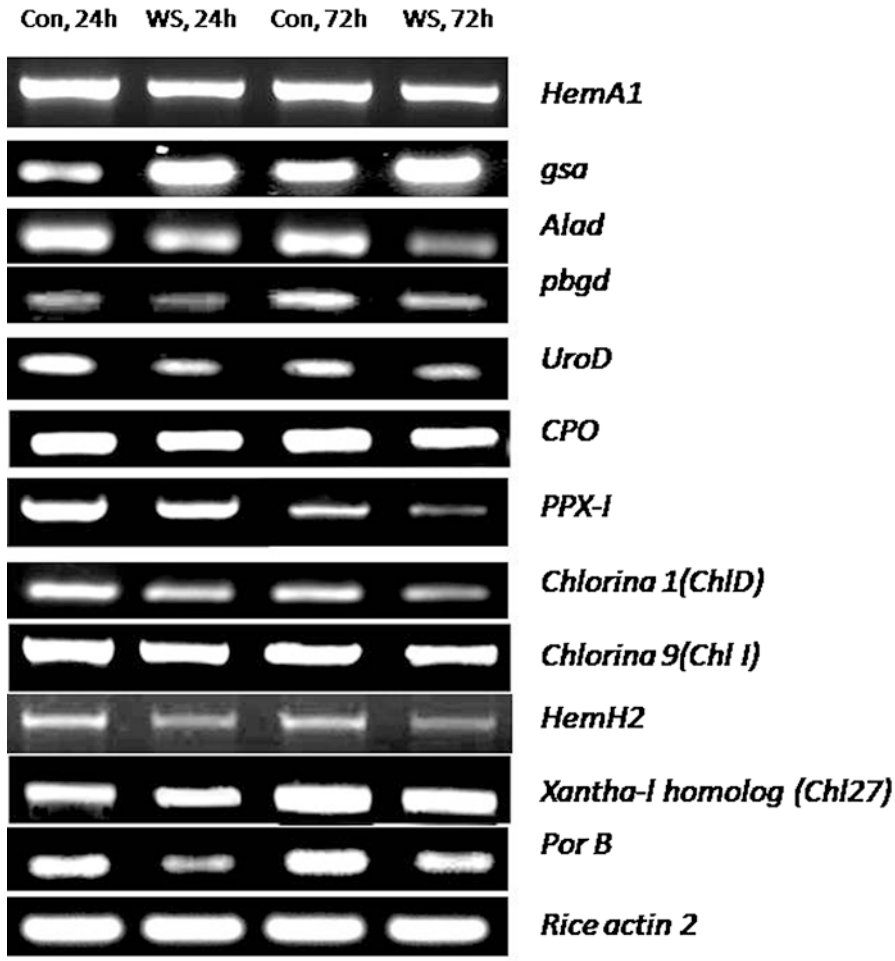

Fig. 27.4. Modulation of gene expression of chlorophyll biosynthetic enzymes due to water stress in seedlings of drought sensitive rice cultivar Pusa Basmati 1, after 24 h and 72 h of greening. Con denotes control and WS denotes water-stressed seedlings, respectively, that were treated with 50 mM PEG 6000, dissolved in nutrient solution, 16 h prior to transfer to 100 μmol m^{-2} s^{-1} white light (Dalal and Tripathy 2012).

Micromolar concentrations of Zn^{2+}, Cu^{2+} and Cd^{2+} inhibit ALA biosynthesis in barley by impairing the activity of GluTR (Pontoppidan and Kannangara 1994).

B. Biosynthesis of Protoporphyrin IX

Protoporphyrin IX (PPIX) is synthesized from eight molecules of ALA by a series of enzymatic biochemical reactions that are largely common to plants and animals. PPIX synthesis involves several enzymes.

5-Aminolevulinic acid dehydratase (ALAD), also known as porphobilinogen (PBG) synthase is a homo-octameric metalloenzyme that catalyzes the condensation of two ALA molecules to form PBG (Fig. 27.1b) (Shemin 1976; Spencer and Jordan 1995). The ALAD of spinach is a hexamer with molecular weight of 300 kDa (Liedgens et al. 1980). The enzyme from radish cotyledons has a pH optimum of 8.0 (Shibata and Ochiai 1977) and requires Mg^{2+} and Mn^{2+} for activity. It is most active at slightly alkaline pH and shows a maximal binding of three Mg (II) per subunit (Kervinen et al. 2000).

The gene encoding ALAD is cloned from various plant sources. It has been isolated from pea, spinach, soybean and tomato.

The enzyme porphobilinogen deaminase (PBGD) is a soluble chloroplast protein (Castelfranco et al. 1988) that catalyzes the formation of the linear tetrapyrrole, hydroxymethylbilane, from four molecules of PBG (Fig. 27.1). Molecular weights from different plants range from 34 to 44 kDa. The *PBGD* gene has been isolated and cloned from pea (Witty et al. 1993) and *A. thaliana* (Lim et al. 1994). In *A. thaliana*, PBGD was found in both leaves and roots (Lim et al. 1994). Sequence comparison from different species shows that specific Arg and Cys residues are well conserved, and that these are implicated in catalysis and dipyrromethane cofactor binding (Witty et al. 1993). The synthesis and activity of PBGD are regulated by light and differ among cell types (Smith 1988; Shashidhara and Smith 1991; Spano and Timko 1991; He et al. 1994).

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

The Uroporphyrinogen III Decarboxylase (UROD) enzyme catalyzes stepwise decarboxylation of Urogen III to yield coproporphyrinogen III (Coprogen III). The enzyme catalyzes decarboxylation of all four carboxyl residues of Urogen III to yield Coprogen III. The order of Urogen III decarboxylation is substrate concentration dependent and under normal conditions enzymatic decarboxylation begins at the ring-D acetate group, in a clockwise manner (Luo and Lim 1993). Although all four isomers of Urogen are accepted by the enzyme, aromatic porphyrins are not decarboxylated (Castelfranco and Beale 1981). The discrimination between isomers, Urogen I and Urogen III for conversion into Coprogen occurs principally at the first step. Porphyrins, especially oxidation products of the substrates, have been shown to inhibit the enzyme (Smith and Francis 1981). The *UROD* was cloned from tobacco and barley (Mock et al. 1995). The in vitro translational product of *UROD* was imported into pea chloroplasts and processed into a 39 kDa product (Mock et al. 1995). Martin et al. (2001) reported the first crystal structure of a plant (tobacco) UROD.

Coproporphyrinogen oxidase (Coprox, CPOX) catalyses the oxidative decarboxylation of propionate side chains on ring A and B of Coprogen III to yield protoporphyrinogen IX (Protogen IX). In aerobic organisms, oxygen is utilized as the sole electron acceptor for enzymatic activity. The enzyme activity was found to be activated by Fe^{2+}, Mn^{2+} and inhibited by EDTA and o-phenanthroline (Hsu and Miller 1970). The corresponding gene, *CPOX*, was isolated and characterized from soybean (Madsen et al. 1993), tobacco and barley (Kruse et al. 1995a, b), *A. thaliana* (Ishikawa et al. 2001) and maize (Williams et al. 2006). The *CPOX* mRNA is highly expressed in soybean root nodules followed by leaves, but no mRNA for *CPOX* was detectable in soybean roots (Madsen et al. 1993). The level of mRNA reached its maximum in developing cells and decreased drastically when cells were completely differentiated. The Coprox isoform, CPO1 fused with green fluorescent protein showed that it was localized in the plastids, whereas CPO2 appeared to localize to mitochondria (Williams et al. 2006). However, mitochondria lack CPOX activity (Smith 1988).

Protoporphyrinogen oxidase (Protox) catalyzes the oxygen-dependent aromatization of Protogen IX to protoporphyrin IX (PPIX, Proto IX). This enzyme catalyzes the six-electron oxidation of Protogen using a flavin cofactor, and molecular oxygen as terminal electron acceptor (Poulson and Polglasse 1974). Protogen is unstable and spontaneously undergoes oxidation in the presence of oxygen and its oxidation is enhanced by light (Jacobs and Jacobs 1979). Koch et al. (2004) reported on the crystal structure of mitochondrial Protox from tobacco and revealed that it contains an FAD-binding domain, a substrate-binding domain and a membrane-binding domain (Koch et al. 2004). Protox forms a loosely associated dimer that folds into an FAD-binding and substrate-binding domain. The substrate-binding domain of Protox also helps in forming a complex with the ferrochelatase enzyme. Protox has been purified from barley etioplasts (Jacobs and Jacobs 1987) and found to be localized in the envelope (stromal side) and thylakoid membranes (stromal side) of chloroplasts (Matringe et al. 1992a; Che et al. 2000). The envelope and thylakoid membranes fail to synthesize Proto IX from the substrate ALA, whereas the stromal fraction could synthesize a small amount. When however, all three components were mixed together the PPIX synthesizing capacity increased. The PPIX synthesizing capacity was reduced by oxidizing agents, and increased in the presence of reductants like dithiothreitol (DTT). ATP also increased PPIX synthesis (Manohara and Tripathy 2000).

Protox has been isolated from spinach, tobacco and *A. thaliana* (Narita et al. 1996; Lermontova et al. 1997; Che et al. 2000; Watanabe et al. 2001). In all these plant species, Protox was encoded by two genes, namely *PPOX1* and *PPOX2*, and was found in both the chloroplast and mitochondria, respectively. In *A. thaliana* the levels of transcripts of plastid Protox were very high in leaves, whereas it was low in roots and floral buds (Narita et al. 1996). In tobacco, both transcripts were accumulated synchronously during diurnal and circadian growth (Lermontova et al. 1997). The spinach PPOX1 are preferentially localized to the stromal side of the thylakoid membrane and inner envelope membrane (Che et al. 2000). The spinach *PPOX2* codes for two proteins of 59 kDa (PPOX2 L) and a 55 kDa (PPOX2 S) by using two in-frame start codons. PPOX2 L is associated with the chloroplast inner envelope membrane and PPOX2 S is associated with inner mitochondrial membranes (Watanabe et al. 2001). As it is folded into an extremely compact form, the Protox is highly resistant to proteases i.e., trypsin, endoproteinase Glu-C, or carboxypeptidases (Arnould and Camadro 1998). However, structurally bicyclic herbicides i.e., diphenyl ether-type herbicides, were shown to inhibit Protox activity in chloroplasts (Camadro et al. 1991; Matringe et al. 1992b). It has also been shown that the fungal toxin cyperin inhibits Protox activity (Dayan et al. 2008).

1. Developmental Modulation of ALAD

In cucumber and wheat *ALAD* expression increases upon transfer of etiolated seedlings to light (Mohanty et al. 2006). However, in pea, expression of *ALAD* is high in dark-grown samples as compared to light-grown samples (Li et al. 1991). In contrast, the corresponding protein level is significantly lower in dark-grown seedlings as compared to light-grown seedlings (He et al. 1994). ALAD is detectable in embryonic leaves whether the plants are grown in darkness or under continuous white-light illumination (He et al. 1994). In pea, *ALAD* transcript abundance is highly dependent on leaf developmental age; the transcript abundance increases with time until the leaf is fully expanded. Subsequently, its mRNA abundance decreases sharply (He et al. 1994). However, a significant amount of the protein is detected even in the matured leaves despite the mRNA abundance of *ALAD* being extremely low. The activity of ALAD significantly decreases during senescence (Hukmani and Tripathy 1994).

ALAD from tobacco leaves and radish cotyledons is inhibited by Zn^{2+} and Fe^{2+} (Shetty and Miller 1969; Shibata and Ochiai 1977), and arsenic inhibits its activity in

maize leaves (Jain and Gadre 2004). $PbCl_2$ and $CdCl_2$ inhibit ALAD in *Amaranthus lividus* (Bhattacharjee and Mukherjee 2003).

2. Developmental Regulation of PBGD

The *PBGD* mRNA is slightly higher in the dark than in the light, even though the protein level is significantly lower in dark (He et al. 1994). The *PBGD* transcript abundance is dependent on leaf developmental age; i.e., the transcript abundance increases with increased age until the leaf is fully expanded and after that its mRNA level declines (He et al. 1994). Despite an extremely low level of *PBGD* mRNA, a significant amount of protein is detected even in matured leaves. Furthermore, PBGD activity rapidly declines during senescence (Hukmani and Tripathy 1994).

The enzyme is heat stable and maintains its activity at temperatures ranging from 55°C to 70°C. The PBGD enzyme from pea chloroplasts was inhibited by Fe^{2+}, Mn^{2+} and Zn^{2+} whereas Ca^{2+} and Mg^{2+} were only weakly inhibitory at physiological concentrations (Spano and Timko 1991).

3. Environmental Regulation of UROD

The expression of the *UROD* gene and the corresponding protein level increase during illumination in barley (Mock et al. 1995) and cucumber (Mohanty et al. 2006).

In heat-stressed plants UROD activity, its gene and protein expression are substantially upregulated in heat-stressed seedlings whereas it is downregulated in chill-stressed plants (Tewari and Tripathy 1998; Mohanty et al. 2006) (Fig. 27.3).

The enzyme activity is inhibited by metals such as Fe^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+} and Mg^{2+} (Chen and Miller 1974), but stimulated by ATP (Manohara and Tripathy 2000).

4. Modulation of CPO

The *CPO* gene expression is not affected by light or heat stress in wheat and cucumber, however the gene expression is down-regulated by chill stress (Mohanty et al. 2006) (Fig. 27.3). In vitro protein import assays of tobacco and barley CPO protein showed that it was imported into the pea chloroplast and accumulated in the stroma. There are two isoforms of CPO (CPO1and CPO2) in maize. *A. thaliana* mutant defective in the *lesion initiation 2* (*LIN2)* gene encoding CPO develops lesions on leaves, in a developmentally regulated and light-dependent manner (Ishikawa et al. 2001).

5. Environmental Modulation of Protox

The Protox activity substantially decreases in response to chill-stress (Tewari and Tripathy 1998). Its transcript and protein abundance decrease in water- stressed seedlings (Fig. 27.4) (Dalal and Tripathy 2012).

6. Environmental Regulation of Proto IX Biosynthesis

Proto IX biosynthesis is modulated by several environmental factors. In chill-stressed seedlings Proto IX synthesis from Urogen III is severely reduced whereas in heat-stressed seedlings the activity is substantially increased (Tewari and Tripathy 1998). Proto-IX synthesis from Coprogen III is reduced in chill-stressed seedlings, however in heat-stressed seedlings, Proto IX synthesis from Coprogen III is not affected (Tewari and Tripathy 1998). In heat-stressed seedlings, ALAD and PBGD were partially inhibited.

Reduced ALAD activity and gene expression were observed in water-stressed rice and chill- and heat-stressed cucumber seedlings (Figs. 27.3 and 27.4) (Mohanty et al. 2006; Dalal and Tripathy 2012). Limitation of ALA, a substrate for ALAD probably reduced its gene expression in water stress and other stress conditions. The increased or decreased availability of the substrate of the enzyme could positively or negatively regulate the gene expression of the enzyme. The enzymatic activity of PBGD that deaminates PBG to form Urogen III is reduced (Dalal and Tripathy 2012) due to down-regulation of its transcript abundance in water-stressed rice seedlings (Fig. 27.4).

UROD protein abundance decreases in water-stressed seedlings, which well

correlates with the declined message abundance of *UROD* in response to water stress (Fig. 27.4). The UROD protein and transcript abundance also decline in chill-stressed wheat seedlings (Mohanty et al. 2006). This is in contrast to the earlier observations in cucumber and wheat where UROD activity and its transcript/protein abundance increased (Fig. 27.3) in response to heat-stress (Tewari and Tripathy 1998; Mohanty et al. 2006). The next two enzymes involved in Proto IX biosynthesis are Coprox and Protox. The enzyme activity of Coprox and Protox decreased in water-stressed rice and chill-stressed cucumber seedlings (Dalal and Tripathy 2012; Tewari and Tripathy 1998) due to down-regulation of their gene/protein abundance (Figs. 27.3 and 27.4).

C. Biosynthesis of Protochlorophyllide

The PPIX synthesis reactions are common to plants and animals. In green organisms the tetrapyrrole biosynthesis pathway branches to the formation of Fe-tetrapyrrole leading to synthesis of heme and Mg-tetrapyrrole that results in the synthesis of Chl. In green plants the Mg-branch involves insertion of Mg to PPIX by Mg-chelatase to synthesize Mg-PPIX, esterification of Mg-PPIX to Mg-protoporphyrin IX monomethylester (MPE) mediated by Mg-Protoporphyrin IX: S-adenosyl methione methyl transferase and formation of the isocyclic ring to synthesize Pchlide by MPE cyclase (Fig. 27.1c).

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

The *CHLI/Chlorina9* has been cloned from soybean, barley (Jensen et al. 1996), *A. thaliana* (Gibson et al. 1996; Rissler et al. 2002), maize and rice (Zhang et al. 2006). This protein is localized to the stroma. In *A. thaliana*, most of the *CHLI* homozygous mutants have a pale green phenotype (Rissler et al. 2002). A second *CHLI* gene, *CHLI-2* has been reported from *A. thaliana* (Rissler et al. 2002; Huang and Li 2009).

The N-terminus of CHLD shows structural similarities with the AAA domain of CHLI and therefore it is believed to contribute in complex formation and interaction with CHLH (Fodje et al. 2001). The *CHLD/Chlorina1* cDNA sequence has been isolated and cloned from tobacco (Papenbrock et al. 1997) and rice (Zhang et al. 2006). The *CHLD* gene expression changes with respect to the diurnal changes in tobacco (Papenbrock et al. 1999). Virus-induced gene silencing of *CHLH* in tobacco led to the lowering of *CHLD* and *CHLI* mRNAs along with reduction in the Chl contents (Hiriart et al. 2002).

In tobacco, *CHLH* is strongly expressed in young leaves and less expressed in mature leaves and only traces of its transcripts were found in flowering organs (Kruse et al. 1997). *CHLH* expression was found to be light inducible in soybean and rice and its transcript levels were under the control of a circadian oscillation (Nakayama et al. 1998; Jung et al. 2003). CHLH protein was also found to be induced on transferring the Arabidopsis seedlings to white light from dark (Stephenson and Terry 2008). The *CHLH* transcripts undergo diurnal variation in *A. thaliana* and tobacco (Gibson et al. 1996; Papenbrock et al. 1999). Depending upon the concentration of Mg^{2+} in the lysis buffer, the CHLH protein migrated between stroma and the envelope membranes and was localized in the envelope membrane at very high concentrations of Mg^{2+} (Nakayama et al. 1998).

Mutants of *CHLH* have been isolated from *A. thaliana* (Mochizuki et al. 2001). *Genome uncoupled 5* (*GUN5*) gene codes for CHLH subunits of Mg-chelatase. The rice *CHLH* mutants also showed a Chl-deficient phenotype (Jung et al. 2003; Zhang et al. 2006). In *A. thaliana*, the Mg-chelatase subunit CHLH regulates retrograde signaling (Mochizuki et al. 2001). Mutation in the *CHLH* gene results in the repressed expression of *LHCB*. Transgenic tobacco plants expressing antisense RNA for Mg-chelatase *CHLH* were Chl deficient (Papenbrock et al. 2000b). In these plants, less PPIX and heme accumulated, and a decrease in ALA synthesizing capacity was observed. *A. thaliana* protein GUN4 regulates Mg-chelatase activity (Larkin et al. 2003; Davison et al. 2005), and promotes the interactions between CHLH and chloroplast membranes and Chl biosynthesis (Adhikari et al. 2009, 2011).

S-adenosyl-L-methionine:Mg-PPIX methyltransferase (SAM-MgProtoMTF) catalyzes the conversion of Mg-PPIX to Mg-protoporphyrin monomethyl ester (MPE). It transfers a methyl group to the carboxyl group of the C13-propionate side chain of Mg-PPIX (Gibson et al. 1963) where SAM acts as a methyl group donor. The gene (*CHLM*) encoding for the SAM-MgProtoMT is cloned from *A. thaliana* (Block et al. 2002) and tobacco (Alawady and Grimm 2005). In tobacco, the methyltransferase physically interacts with the CHLH subunit of Mg-chelatases (Alawady et al. 2005).

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

Mg-protoporphyrin IX monomethylester cyclase catalyzes the formation of the isocyclic ring E of the Mg-protoporphyrins and converts MPE to Pchlide. There are two pathways for the formation of the isocyclic ring, i.e., aerobic cyclization and anaerobic cyclization. The former pathway is predominant in plants, green algae and cyanobacteria where the ketone oxygen of divinyl Pchlide (DV-Pchlide) is derived from molecular oxygen (Walker et al. 1989). The pH optimum of the cyclase activity is approximately 9.0 and the enzyme activity was found to be inhibited by CN^- and N_3^- (Whyte and Castelfranco 1993). The cyclase reaction with the two barley mutants *xantha l* and *viridis K* revealed that at least two plastid proteins (a membrane bound protein and a soluble protein) are required for cyclization (Walker et al. 1991; Walker and Willows 1997). Biochemical and genetic studies have demonstrated that the gene responsible for the *xantha-l* mutant encodes a membrane-bound cyclase subunit and it needs a soluble fraction for the cyclization reaction (Rzeznicka et al. 2005). *Xantha l* mutants accumulated less Chl, high MPE and had no cyclase activity (Rzeznicka et al. 2005). Three *Xantha l* mutants were characterized. In the leaky mutant *xantha-l*^{35}, a C-to-T point mutation resulted in an exchange of amino acid residue Ser-181 to Phe. In the non-leaky mutant *xantha-l*^{81}, a G-to-A point mutation resulted in the exchange of Gly-155 to Glu. Sequence alignment showed that Gly-155 is a highly conserved residue. In the non-leaky mutant *xantha-l*^{82}, point mutation resulted in truncation as a TGG codon corresponding to Trp-291 turned into a TGA stop codon and the truncated protein was not stable. However, the level of the protein found in the *xantha-l*^{35} and *xantha-l*^{81} mutants was similar as in the wild-type (Rzeznicka et al. 2005).

The gene responsible for the aerobic cyclization reaction has been isolated and characterized from different plants i.e., *CHL27* from *A. thaliana* and *xantha l* from barley (Tottey et al. 2003; Rzeznicka et al. 2005). Antisense *A. thaliana* and tobacco

plants with reduced amounts of CHL27 show chlorotic leaves with reduced abundance of all Chl-proteins and accumulate MPE (Tottey et al. 2003; Peter et al. 2010). The *A. thaliana chl27* T-DNA mutant is pale green with an elevated Chl *a*/*b* ratio, and has unstacked thylakoid membranes with reduced LHCII protein. Their photosynthetic activity is reduced due to damaged Photosystem II (PS II) reaction centers (Bang et al. 2008; Hansson and Jensen 2009). In tobacco, the co-suppression of the *NTZIP,* which includes coding region for a di-iron motif, resulted in reduced Chl level and lower photosynthetic activity (Liu et al. 2004).

1. Environmental Regulation of Protochlorophyllide Synthesis

The accumulation of Mg-PPIX and pheophorbide inhibits Mg-chelatase activity in pea (Popperl et al. 1997). Mg-chelatase activity and the expression of the genes encoding this enzyme are up-regulated by light (Mohanty et al. 2006) (Fig. 27.3). The *CHLI* mRNA is induced by light (Gibson et al. 1996; Jensen et al. 1996; Nakayama et al. 1998) and constitutively expressed in matured leaves. It is also regulated by diurnal rhythm but not regulated by circadian rhythm (Matsumoto et al. 2004). CHLI could be a target for chloroplastic thioredoxin and the in vivo reduction process is light dependent (Ikegami et al. 2007).

The Mg-chelatase activity is severely down-regulated in chill- and heat- stressed cucumber seedlings (Tewari and Tripathy 1998). In wheat light treatment in control and heat-stressed seedlings leads to higher accumulation of *CHLI/Chlorina9* transcripts. Its expression diminishes in cold- and heat-treated wheat seedlings. *CHLI/Chlorina9* expression is also down-regulated by salt-stress in cucumber/wheat/rice seedlings.

Mg-chelatase activity is down-regulated in water-stressed rice seedlings (Dalal and Tripathy 2012) (Fig. 27.4). Moreover, the gene/protein expression of *CHLI/Chlorina9* and *CHLD/Chlorina1* subunits of Mg-chelatase (Zhang et al. 2006) partially declined in water-stressed seedlings. CHLI1 subunit is post-translationally regulated by chloroplastic thioredoxin (Ikegami et al. 2007) and therefore could have impaired function in altered redox environment in water-stressed seedlings. Stoichiometric imbalance among the subunits of Mg-Chelatase decreases the Mg-chelatase activity as seen in CHLI over-expressing or under-expressing transgenic Arabidopsis plants (Papenbrock et al. 2000a). Inadequate proportion of all subunits is known to hamper the correct assembly of active Mg-chelatase (Guo et al. 1998; Hansson et al. 1999; Jensen et al. 1999); therefore, nonstoichiometric abundance among its subunits may have led to decreased enzyme activity of Mg-chelatase in water stressed seedlings.

D. Phototransformation of Protochlorophyllide to Chlorophyllide

In angiosperms, Protochlorophyllide oxidoreductase (POR) has the obligate requirement of light for photo-converting Pchlide to chlorophyllide (Chlide). It catalyzes the conversion of Pchlide to Chlide in light using NADPH as reductant (Fig. 27.1c). POR converts Pchlide to Chlide, by adding two hydrogen atoms at C17 and C18 on ring D. In the POR catalytic cycle, a ternary enzyme-NADPH-Pchlide complex is formed. Light energy absorbed by the Pchlide in the complex may produce torsional strain in the molecule that provides a favorable condition for hydride/hydrogen transfer from NADPH (Begley and Young 1989). POR is a member of a large family of enzymes known as short chain dehydrogenases/reductases (SDR) (Wilks and Timko 1995), which generally catalyze NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers. A tyrosine (Tyr) and a lysine (Lys) residues are both conserved throughout all members of the SDR family. In POR, it was also seen that Tyr and Lys residues are important for its activity (Wilks and Timko 1995; Lebedev et al. 2001). The Tyr may be deprotonated, acting as a general acid to facilitate hydride transfer to or from $NAD(P)^+/H$ (Bohren et al. 1994). The proton at the C-18 position of Pchlide is derived from Tyr and the hydride transferred to the C-17 position is

derived from the *pro-S* face of NADPH. The close proximity of the Lys residue is thought to allow the deprotonation step to occur at physiological pH by lowering the apparent pK_a of the phenolic group of the Tyr (Wilks and Timko 1995). A light-activated conformational change of the protein is necessary to activate catalysis (Heyes et al. 2008; Sytina et al. 2008). The fact that POR is light activated means that the enzyme–substrate complex can be formed in the dark. This has recently been exploited by studying Pchlide reduction at low temperatures to trap intermediates in the reaction pathway (Heyes et al. 2002, 2003; Heyes and Hunter 2004). As a result, the reaction has been shown to consist of at least three distinct steps: an initial light-driven step, followed by a series of 'dark' reactions. An initial photochemical step can occur below 200 K (Heyes et al. 2002), whereas two 'dark' steps were identified for *Synechocystis* sp. PCC 6803 POR, which can only occur close to or above the 'glass transition' temperature of proteins (Heyes et al. 2003). First, $NADP^+$ is released from the enzyme and then replaced by NADPH, before release of the product (Chlide) and subsequent binding of Pchlide have taken place (Heyes and Hunter 2004). Monovinyl Pchlide (MV-Pchlide) and DV-Pchlide don't influence differentially the enzyme kinetics or the steps involved in the reaction pathway (Heyes et al. 2006). The secondary structure analysis of POR reveals that it has 33% alpha helix, 19% beta-sheets, 20% turn and 28% random coil.

Mutation studies by Dahlin et al. (1999) showed that, mutation in predicted α-helical regions of the protein showed the least effect on enzyme activity, whereas mutations in the predicted β-sheet regions showed an adverse effect on enzyme function. The replacement of charged amino acids by alanine in the N- and C-terminal regions of the mature protein did not affect POR assembly, whereas mutations within the central core prevent proper attachment to the thylakoid. It is a peripheral membrane protein that accumulates to a high level in PLBs, where it forms a ternary complex with Pchlide and NADPH (Oliver and Griffiths 1982) and is present at low levels in the thylakoid membranes of developing and mature plastids. It is observed that the Cys residues of POR are crucial for its membrane association (Aronsson et al. 2001) and for NADPH and pigment binding (Townley et al. 2001; Reinbothe et al. 2006). The association of POR with Pchilde results in three different spectral forms of Pchlide based on their fluorescence emission maximum (in nm): Pchlide F631 (due to the pigment structural arrangements), Pchlide F644 (due to association of POR), and Pchlide F655 (due to localization in PLBs and/or prothylakoids) (Böddi et al. 1992, 1993). Spectroscopic studies of dark-grown bean seedlings indicated the existence of two forms of Pchlide, a main component with a red absorption band at 650 nm and a minor component absorbing at 636 nm (Shibata 1957). On the basis of flash illumination, two kinds of Pchlide can be categorized: one is transformed into Chlide and is called photoactive Pchlide, whereas the other remains unchanged and is called nonphotoactive Pchlide. The latter is assembled into various complexes with different molecular structure and spectral properties (Schoefs and Franck 2003; Masuda and Takamiya 2004). Plastids isolated from dark-grown wheat seedlings exhibit a smaller 77 K fluorescence emission peak at 632 nm due to non-phototransformable Pchlide and a larger peak at 657 nm due to phototransformable Pchlide. The non-phototransformable Pchlide emitting at 632 nm is due to a monomeric Pchlide complex or esterified Pchlide i.e., Protochlorophyll (Lindsten et al. 1988), which spontaneously dimerizes to form $(POR\text{-}Pchlide\text{-}NADPH)_2$. The short-wavelength, monomeric Pchlide is not flash-photoactive: instead, it regenerates the long wavelength Pchlide forms (Schoefs and Franck 1993; He et al. 1994; Schoefs et al. 1994, 2000a, b). The dimer has an absorption maximum at 638 nm and an emission maximum at 645 nm (Lebedev and Timko 1999). The dimeric POR-Pchlide-NADPH complex further polymerizes to form 16-mer or larger aggregates of POR-Pchlide-NADPH complex i.e., $(POR\text{-}Pchlide\text{-}NADPH)_n$ having absorption maximum at 650 nm and emission maximum at 657 nm (Böddi et al. 1989; Wiktorsson et al. 1993) and is flash photoactive (Böddi

et al. 1991). However, illumination for more than a minute usually converts non-active Pchlide to photo-active Pchlide.

Full-length cDNA clones of *POR* were isolated from barley (Holtorf et al. 1995), pea (Spano et al. 1992), *A. thaliana* (Armstrong et al. 1995; Oosawa et al. 2000), tobacco (Masuda et al. 2002), cucumber (Kuroda et al. 1995) and many other higher plants. The high degree of sequence similarity among PORs from different taxonomic groups implies a common mechanism of enzyme action.

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

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

POR gene expression is also organ specific. *A. thaliana PORB* and *PORC* are expressed in all photosynthetic tissues of the mature plants but not in the root (Armstrong et al. 1995; Oosawa et al. 2000). Cucumber *POR* gene expression is also observed in photosynthetic tissues (Kuroda et al. 1995). Plant age plays a crucial role in *POR* gene expression. In *A. thaliana* and barley *PORA* expression is only observed in young seedlings whereas *PORB* is expressed both in young and matured green tissue (Armstrong et al. 1995). In *A. thaliana* both *PORB* and *PORC* expression is observed in green tissue (Oosawa et al. 2000; Su et al. 2001). In the leaves of dark-grown seedlings, the highest level of expression is observed 8–10 days

after germination of seedlings (Spano et al. 1992). The transcript level of pea *POR* did not decrease after 48 h of light exposure. Immunoblot analysis showed that there was no POR protein detectable after 48 h of light exposure. These results suggested that pchlide reductase activity in pea is primarily regulated post-transcriptionally, most likely at the level of translation initiation/elongation or protein turnover (Spano et al. 1992).

Far-red-light modulation of POR: Etiolated seedlings of *A. thaliana* grown under continuous far-red light are unable to green when subsequently transferred to white light; this is called far-red blocking of the greening (Buhr et al. 2008). This process involves depletion of PORA, partial depletion of PORB and the concomitant loss of PLBs resulting in photo-oxidative damage (Barnes et al. 1996; Runge et al. 1996). From these studies, PORA has been proposed to play a special role in the formation of POR ternary complexes containing photoactive Pchlide-F655, PLB assembly, and protection against photo-oxidative damage caused by non-photoactive Pchlide (Reinbothe et al. 1999). Overexpression of *PORA* and *PORB* in specific mutants overcame the photooxidative damage (Sperling et al. 1997, 1998).

Overexpression of a cyanobacterial POR protein in the *A. thaliana porA* mutant could restore prolamellar body formation. However, the amount of photoactive Pchlide in the etioplasts of the complementing lines was retained at a low level as in the parent *PORA* knockdown mutant (Masuda et al. 2009). The *lip1* mutant of pea lacked PLBs but could form prolamellar bodies if treated with cytokinin (Seyedi et al. 2001a): however unlike the *A. thaliana* mutant, it did not undergo photooxidative damage (Seyedi et al. 2001b). The physiological function of specific POR isoforms in vivo has been well characterized in knockout mutants of *A. thaliana* (Frick et al. 2003; Masuda et al. 2003). Single *POR* mutants display no obvious phenotypes at the whole plant or chloroplast ultra-structural levels, except that etiolated *PORB* mutants have less extensive inner membranes. However, the *PORB/PORC* double mutant, which displayed a seedling-lethal *xantha* phenotype at the cotyledon stage, contained only a small amount of Chl *a*, and possessed chloroplasts with mostly unstacked thylakoid membranes (Frick et al. 2003). Masuda et al. (2003) focused on the greening process of *por* mutants, and showed that the etiolated *PORB* mutant seedlings were able to green to a similar extent as the wild type, and the greening of the *PORC* mutant was repressed under high light conditions.

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

In all photosynthetic organisms Pchlide and Chlide are originally formed as 3,8-divinyl derivates. The 8-vinyl reductase reduces the 8-vinyl group on the tetrapyrrole to an ethyl group using NADPH as the reductant. This enzymatic activity has been detected in isolated chloroplasts of barley (Tripathy and Rebeiz 1988), plastid membranes from cucumber (Parham and Rebeiz 1995), and also in solubilized crude extracts derived from etiolated barley leaves (Kolossov and Rebeiz 2001). It has been demonstrated in vitro that the monovinyl (MV) and divinyl (DV) Chl biosynthesis reactions may operate in parallel (Tripathy and Rebeiz 1986). However, the mutant of maize (*Zea mays*) that accumulated only DV-Chl instead of MV-Chl and capable of photosynthetic growth with DV-Chl suggests that a single gene product is responsible for the reduction of the vinyl group of Chlide (Bazzaz 1981). Nagata et al. (2005) followed by Nakanishi et al. (2005) isolated a mutant of *A. thaliana* which accumulates DV- Chl. By map-based cloning they detected that the gene is 8-vinyl

reductase. The recombinant protein was successfully tested for the conversion of the C8-vinyl group of Chlide to an ethyl group on ring B. The 3,8-divinyl-chlide *a* is the major substrate of divinyl reductase (DVR) (Nagata et al. 2007). Starch granules were not found in the mutant chloroplasts, suggesting the reduction of photosynthetic activity in the mutant (Nakanishi et al. 2005). The transcript level of *DVR* expression is high in leaves, stems and flower buds, and low in roots.

The mutant is pale green and the Chl *a*/*b* ratio varies in between 6 and 10 depending on the developmental stage and growth conditions. This mutant is capable of photosynthesizing and growing under low-light conditions (70–90 μmol photons m^{-2} s^{-1}); but it rapidly dies under high light conditions (1,000 μmoles photons m^{-2} s^{-1}) (Nagata et al. 2005). The thylakoid membranes were in a disorderly fashion having no distinct grana stacks in the mutant and no significant differences in the size and the number of chloroplasts between the wild type and the mutants were observed.

1. Role of POR in Combating Oxidative Stress

Light absorbed by colored intermediates of Chl biosynthesis is not utilized in photosynthesis. Instead, it is transferred to molecular oxygen, generating singlet oxygen ($^1O_2$) (Chakraborty and Tripathy 1992). As there is no enzymatic detoxification mechanism available in plants to destroy $^1O_2$, its generation should be minimized. The concentration of a major Chl biosynthetic intermediate, i.e., Pchlide in Arabidopsis was manipulated by overexpressing the light-inducible PORC that effectively phototransforms endogenous Pchlide to Chlide leading to minimal accumulation of the photosensitizer Pchlide in light-grown plants (Pattanayak and Tripathy 2011). In *PORC* overexpressing (*PORCx*) plants exposed to high-light, the $^1O_2$ generation and consequent malonedialdehyde production was minimal and the maximum quantum efficiency of photosystem II remained unaffected (Fig. 27.5) demonstrating that their photosynthetic apparatus and cellular organization were intact.

Further, *PORCx* plants treated with 5-aminolevulinicacid, when exposed to light, photo-converted over-accumulated Pchlide to Chlide, reduced the generation of $^1O_2$ and malonedialdehyde production and reduced plasma membrane damage (Fig. 27.6). So *PORCx* plants survived and bolted whereas, the ALA-treated wild type plants perished. Thus, overexpression of *PORC* could be biotechnologically exploited in crop plants for tolerance to $^1O_2$-induced oxidative stress, paving the use of ALA as a selective commercial light-activated biodegradable herbicide (Pattanayak and Tripathy 2011).

Reduced Pchlide content in *PORCx* plants released the Pchlide-mediated feed-back inhibition of ALA biosynthesis that resulted in higher ALA production. Increase of ALA synthesis up-regulated gene expression and protein level of several downstream Chl biosynthetic enzymes elucidating a regulatory net work of expression of genes involved in ALA and tetrapyrrole biosynthesis (Pattanayak and Tripathy 2011).

2. Environmental Modulation of Shibata Shift

In etiolated wheat seedlings, the phototransformable Pchlide peak (F657) is substantially higher than the non-phototransformable peak (F632) (Fig. 27.7a) demonstrating the presence of large aggregates of POR-Pchlide-NADPH ternary complexes. Although chilling arrested *de novo* synthesis of the Chl biosynthetic intermediate Pchlide (Tiwari and Tripathy 1998), it did not affect the ratio of non-phototransformable to phototransformable Pchlide suggesting that low temperature did not affect formation of large aggregates of POR-Pchlide-NADPH ternary complexes (Fig. 27.7a) (Mohanty and Tripathy 2011). In contrast, although heat-stress partially arrested Pchlide synthesis (Fig. 27.7a) in etiolated seedlings (Tewari and Tripathy 1998), it substantially affected the aggregation state of POR-Pchlide-NADPH

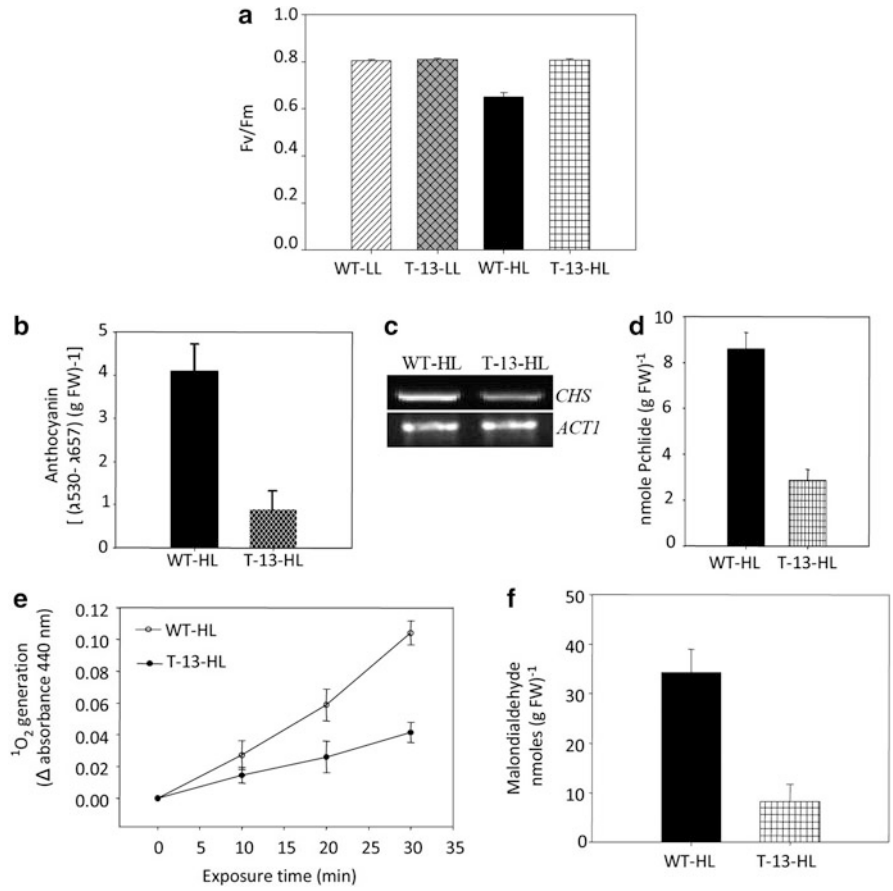

Fig. 27.5. Morphological and physiological responses of WT and PORCx (T-13) plants to light stress. Both WT and T-13 plants were grown in moderate light (100 μmoles photons m^{-2} s^{-1}) for 22–24 days and subsequently transferred to low-light (LL) (50 μmoles photons m^{-2} s^{-1}, 16 h light/8 h dark) or high-light (HL) (330 μmoles photons m^{-2} s^{-1}, 16 h light/8 h dark) regimes for 6-7 d as described in experimental procedures. (**a**) Photosynthetic efficiency (Fv/Fm) of leaves of LL- and HL-exposed plants was monitored by PAM 2100 fluorometer. Values are mean ± SD (n = 20). (**b**) Anthocyanin contents of WT and T-13 plants grown under HL. (**c**) The gene expression study of CHS in HL-grown WT and T-13 plants was done by RT-PCR as described in experimental procedures. *AtACT1* was used as an internal control. (**d**) Pchlide contents of HL-treated WT and T-13 plants measured 10 min after the end of dark period. (**e**) Singlet oxygen ($^1O_2$) contents in WT and T-13 plants. Thylakoid membranes were isolated in complete darkness from HL-exposed plants and the $^1O_2$ production were determined in terms of RNO bleaching using histidine as a trap. (**f**) Malondialdehyde (MDA) production in HL-treated WT and T-13 plants. Each data point represented in all the above experiments is the average of six replicates. The error *bar* represents SD. (Adopted from Pattanayak and Tripathy 2011).

complex as indicated by near absence of 657 nm peak in etiolated heat- stressed seedlings. Within hours of exposure of etiolated seedlings grown at 25°C to heat-stress, a progressive decline of the 657 nm phototransformable peak was observed suggesting the disaggregation of existing large aggregates of POR-Pchlide-NADPH polymeric complexes present in prolamellar bodies to monomeric or dimeric forms. The rate of degradation of polymeric complexes must exceed their formation, if any, in heat-stressed samples. Unlike heat-stress, in vivo application of salt-stress to excised wheat leaves reduced the peak of non-photo-transformable Pchlide and did not affect the

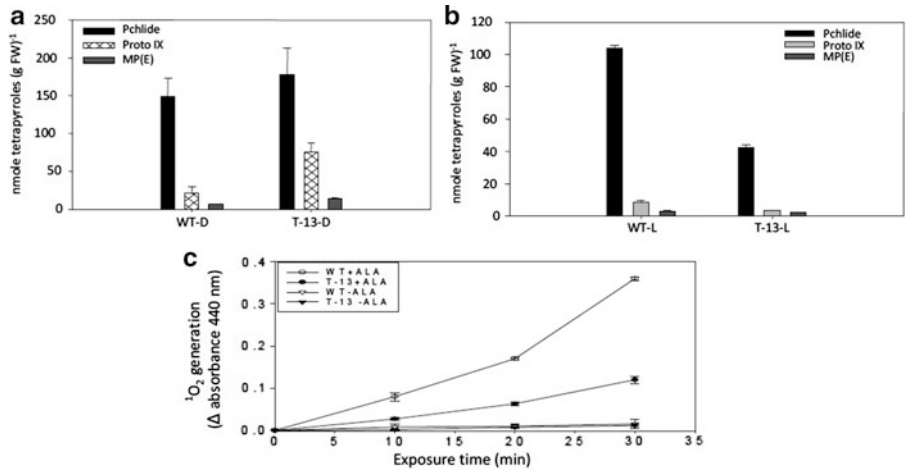

Fig. 27.6. Contents of chlorophyll biosynthetic pathway intermediates and singlet oxygen production in ALA-treated WT and PORCx (T-13) plants. WT and T-13 plants grown for 28–32 days at 220C ± 20C under 14 h L/10 h D photoperiod (100 μmoles photons m^{-2} s^{-1}) were sprayed with ALA (3 mM), dark incubated for 14 h and exposed to light (100 μmoles photons m^{-2} s^{-1}) for 10 min. Leaves were harvested both from dark incubated and light exposed plants, homogenized and their tetrapyrrole contents were monitored spectrofluorometrically. (**a**) Pchlide, Proto IX and MP(E) contents of ALA-treated (3 mM) and 14 h-dark-incubated WT and T-13 plants. (**b**) After dark incubation both WT and T-13 plants were exposed to light (10 min) and their Pchlide, Proto IX and MP(E) were determined. (**c**) $^1O_2$ contents in ALA-treated (+ALA) and untreated (−ALA) WT and T-13 plants. The experiments were repeated five times and each data point is the average of five replicates. The bars represents ± SD (Pattanayak and Tripathy 2011).

longer wavelength phototransformable forms suggesting that prothylakoids rather than prolamellar bodies were affected by salinity (Abdelkader et al. 2007a).

In addition to changes in the aggregation status of polymeric POR-Pchlide-NADPH complexes, the flash-induced photo-transformation and the Shibata shift (Shibata 1957) leading to chloroplast biogenesis is substantially affected in temperature-stressed samples. Upon flash illumination (0.2 s) of etioplasts isolated from control seedlings the phototransformable Pchlide peak at 657 nm emanating from large aggregates of polymeric POR-Pchlide-NADPH complexes almost disappeared due to photo-reduction of Pchlide to Chlide. Transformation of $Pchlide_{655}$ into $Chlide_{692}$ was observed by exposing the leaf primordia of common ash (*Fraxinus excelsior* L.) and Hungarian ash (*Fraxinus angustifolia* Vahl.) (Solymosi and Böddi 2006) and that of Horse chestnut (*Aesculus hippocastanum*) (Solymosi et al. 2006) to a white light flash of 10 s. After 1 and 10 min of illumination, the peak at 692 nm slowly blue shifted to 676 nm (Shibata shift) (Fig. 27.7b). In chill-stressed and heat-stressed seedlings the Shibata shift was significantly arrested (Fig. 27.7a) (Mohanty and Tripathy 2011) probably due to the disaggregation of the PLB membrane particles or of the POR units as well as by their conformational changes (Böddi et al. 1990; Smeller et al. 2003).

Heating of excised etiolated barley leaves resulted in decreased accumulation of Pchlide(650), and a flash could trigger the formation of Chlide(672) instead of the formation of Chlide(684) (Eullaffroy and Popovic 1997). Similar to heat-stress, water-stress affects the Shibata shift, although the phototransformation of Pchlide to Chlide was not impaired by water deficit (Fig. 27.7b) (Le Lay et al. 2001). Both chill-stress and heat-stress affected the Shibata shift (Fig. 27.7a). This is variance to in vitro heating (40°C) of excised barley leaves where the Shibata shift was not affected (Eullaffroy et al. 1995).

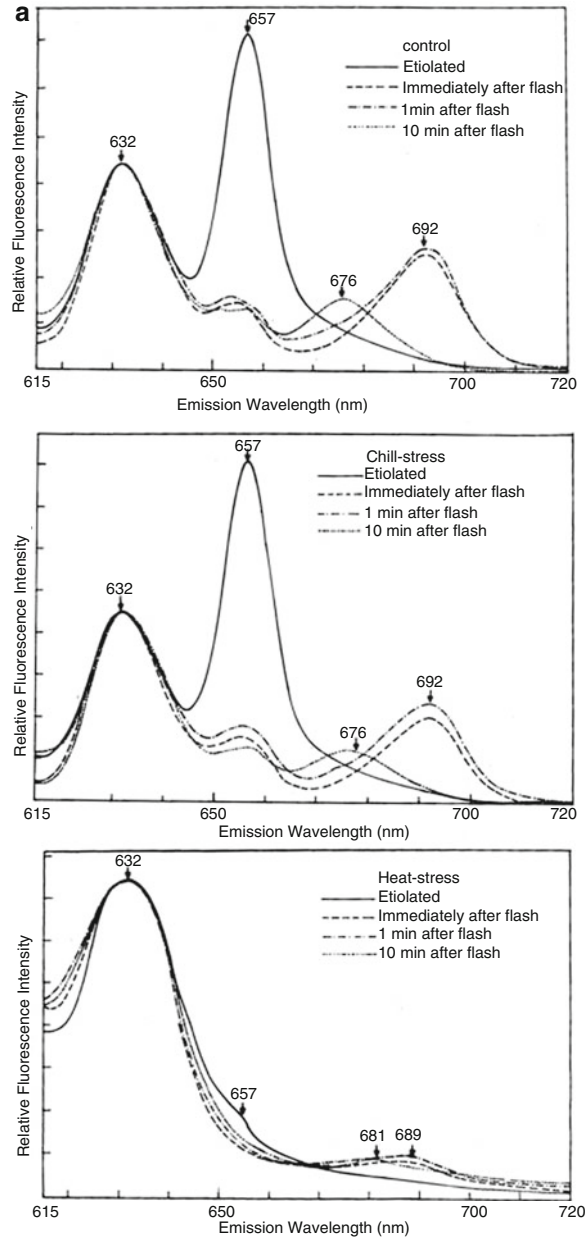

Fig. 27.7. (continued)

Upon 16 h of water-stress treatment, the etiolated seedlings displayed an emission fluorescence peak (77 K) at 632 nm due to non-phototransformable Pchlide and a peak at 657 nm due to phototransformable Pchlide (Fig. 27.7b). However, as compared to the control, the ratio of non-photo-transformable/photo-transformable Pchlide (F632/F657) increased from 0.10 to 0.15 in stressed seedlings suggesting an impairment of aggregation of monomeric POR-Pchlide-NADPH to 16-mer or larger aggregates of POR-Pchlide-NADPH complex, i.e. $(\text{POR-Pchlide-NADPH})_n$. This may be due to reduced assembly or due to degradation of polymeric complexes in the stressed environment (Dalal and Tripathy 2012).

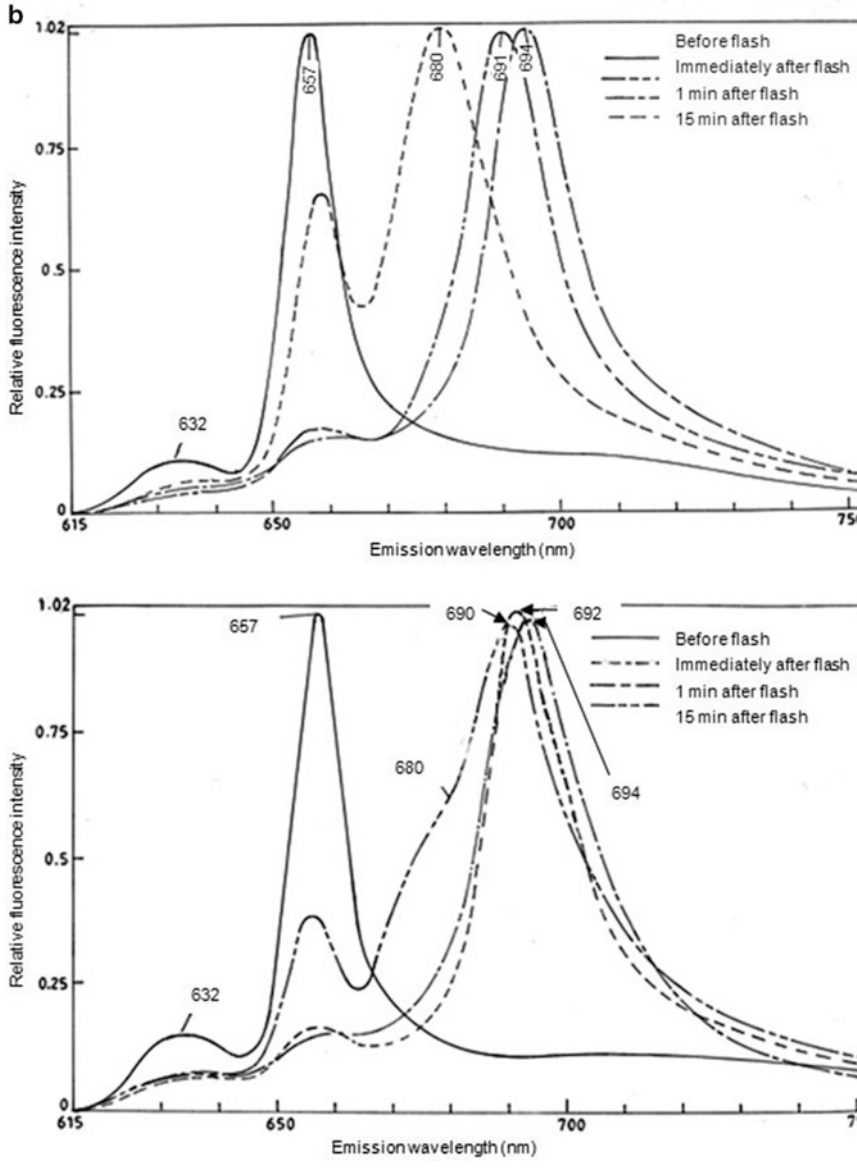

Fig. 27.7. (**a**) Low temperature (77 K) fluorescence emission spectra (E440) of plastids isolated from 6-day old etiolated control, (*upper panel*) chill-stressed (*middle panel*) and heat-stressed (*lower panel*) wheat seedlings showing Shibata-shift. Five-day old seedlings grown at 25°C were transferred to 7°C and 42°C in dark for 24 h. Low temperature fluorescence emission spectra were recorded before flash, immediately after flash (0.2 s) and after 1 min and 15 min post-flash incubation (Mohanty and Tripathy 2011). (**b**) Low temperature (77 K) fluorescence emission spectra (E440) of leaves from 6-d old etiolated control (*upper panel*) and water-stressed (*lower panel*) rice (PB1) seedlings, showing Shibata-shift. For water-stress, seedlings were treated with 50 mM PEG 6000, dissolved in nutrient solution, 16 h prior to taking spectra. Low temperature fluorescence emission spectra were recorded before the flash, immediately after a flash of 0.2 s and after 1 min and 15 min post-flash incubation (Dalal and Tripathy 2012).

The flash-induced photo-transformation and Shibata shift leading to chloroplast biogenesis was substantially affected in 16 h-water stressed samples. Upon red light flash illumination (0.2 s) of control leaves the phototransformable Pchlide peak at 657 nm emanating from large aggregates of polymeric $(POR\text{-}Pchlide\text{-}NADPH)_n$ complexes almost disappeared due to photo-reduction of Pchlide to Chlide, and a new peak appeared at 691 nm due to formation of Chlide-LPOR-$NADP^+$ complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982; Franck 1993; Wiktorsson et al. 1993; Franck et al. 1999). Transformation of Pchlide 658 into Chlide692 was observed by exposing the leaf primordia of common ash (*Fraxinus excelsior* L.) and Hungarian ash, *Fraxinus angustifolia* Vahl. (Solymosi et al. 2006), wheat (Franck et al. 1999) and that of Horse chestnut (*Aesculus hippocastanum*) (Solymosi et al. 2006) to light flash. One min after flash, 691 nm-peak shifted to 694 nm (Fig. 27.7b) in control leaves due to the formation of Chlide-LPOR-NADPH complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982; Franck et al. 1999). Subsequently, this peak blue-shifted to 680 nm after 15 min post-flash incubation of control leaves due to the release of Chlide from the active site of LPOR and disaggregation of multimeric complexes, a process called Shibata shift (Shibata 1957; Böddi et al. 1990; Franck 1993).

In water-stressed leaves, upon red light flash illumination of etiolated leaves the phototransformable pchlide peak at 657 nm disappeared and a new peak appeared at 692 nm due to formation of Chlide-LPOR-$NADP^+$ complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982) demonstrating that phototransformation of Pchlide to Chlide could still take place in 16 h-water-stressed samples (Dalal and Tripathy 2012). After 1 min post-flash incubation this peak shifted to 694 nm due to the formation of Chlide-LPOR-NADPH complexes (Fig. 27.7b). In water stressed leaves the shift to lower wavelengths was substantially delayed. A shoulder appeared at 680 nm after 15 min of dark incubation, in contrast to complete shift to 680 nm in control seedlings, suggesting a slow release of Chlide from the active site of LPOR (Shibata 1957; Böddi et al. 1990). In a non-physiological environment i.e. after desiccation of detached barley leaves a slow-down of Shibata was earlier reported (Le Lay et al. 2000, 2001). Upon 15 min of dark incubation after flash illumination, a good amount of phototransformable Pchlide (F657) was regenerated in control seedlings (Fig. 27.7b) and substantially less in water-stressed seedlings (Fig. 27.7b) demonstrating the down-regulation of synthesis of Pchlide and its conversion to photo-transformable form.

E. Synthesis of Chlorophyll a and Chlorophyll b

Chlorophyllide *a* oxygenase (CAO) catalyzes the oxidation of Chlide *a* to Chlide *b* (Fig. 27.1d). During conversion of Chlide *a* to Chlide *b* the electron is transferred from the Rieske center to the mononuclear iron with subsequent activation of molecular oxygen for oxygenation of the Chlide *a* methyl group (Beale and Weinstein 1990; Porra et al. 1993). Chlide *b* is synthesized by oxidation/conversion of the methyl group on the D ring of the porphyrin molecule to a formyl group at that position. The CAO enzyme contains domains for a [2Fe-2S] Rieske center and for a mononuclear nonheme iron-binding site and has a tyrosine radical (Eggink et al. 2004). The conserved Rieske center and non-heme-iron binding motifs of CAO are likely to be involved in electron transport from ferredoxin to molecular oxygen. The recombinant CAO protein catalyzes chlide *a* to chlide *b* in the presence of NADPH and reduced ferredoxin (Oster et al. 2000). However, Pchlide *a* is not a substrate for the CAO enzyme (Oster et al. 2000).

The *CAO* was first cloned by Tanaka et al. (1998) from Chlamydomonas and also has been cloned from *A. thaliana* (Espineda et al. 1999) and rice (Lee et al. 2005). Both transcript and protein level of CAO increased when *A. thaliana* plants were transferred from moderate to shade light (Harper et al. 2004). Rice has two CAO isoforms namely *OsCAO1*,

OsCAO2 that are differentially regulated in light and dark. The level of the *OsCAO1* transcript is less in the dark and is higher in the light whereas the *OsCAO2* mRNA levels are higher in dark conditions and are reduced by exposure to light (Lee et al. 2005).

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

Chl synthetase encoded by *CHLG* catalyzes the esterification of Chlide *a* and Chlide *b* to Chl (Fig. 27.1d) (Rüdiger et al. 1980). Pchlide is not the substrate for this enzyme, which indicates that reduction of the 17, 18 double bond on ring D is essential for esterification (Benz and Rüdiger 1981b). Compounds which have the 13(2)-carbomethoxy group at the same side of the macrocycle as the propionic side chain of ring D are neither substrates nor competitive inhibitors (Helfrich et al. 1994). Only compounds having the 13(2)-carbomethoxy group at the opposite site are substrates for the enzyme. Esterification of Chlide is a rapid phase, leading to esterification of 15% of total Chlide within 15–30 s, followed by a lag-phase of nearly 2 min and a subsequent main phase (Schmid et al. 2002). It has been shown that the conversion of Chlide to Chl is a four-step process including three intermediates, i.e., Chlide geranylgeraniol, Chlide dihydrogeranylgeraniol and Chlide tetrahydrogeranylgeraniol before the formation of Chlide phytol or Chl (Schoefs et al. 2000a, b).

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

The *CHLG* gene was isolated from *A. thaliana*, *Avena sativa*, rice and tobacco (Gaubier et al. 1995; Schmid et al. 2001; Wu et al. 2007; Shalygo et al. 2009). In *A. thaliana*, the *CHLG* transcript has been detected in green or greening tissues (Gaubier et al. 1995), whereas in *A. sativa*, the gene is expressed equally both in dark- and light-grown seedlings (Schmid et al. 2001). Sequence analysis of cDNAs from rice yielded a putative Chl synthase homolog (Scolnik and Bartley 1996); however, the biochemical properties and physiological functions remained unknown until Wu et al. (2007) characterized a rice mutant with inactivated *CHLG*. The young rice Chl synthase mutant plants have yellow-green leaves with decreased Chl synthesis (Wu et al. 2007). In the mutated plants, there is accumulation of tetrapyrrole intermediates, reduced expression of *LHCB1* and delayed chloroplast development.

1. Modulation of Chlorophyll b Synthesis Confers Tolerance to Low Light and High Light

Overexpression of *CAO* in tobacco plants resulted in a decreased Chl *a*/*b* ratio i.e., from 3.38 in wild-type plants to 2.33 in transgenic plants when grown in high light and from 2.8 to 2.4 in low light-grown plants (Pattanayak et al. 2005). The overexpression of full length *CAO* in tobacco (*Nicotiana tabacum*) resulted in an increased Chl synthesis and a decreased Chl *a/b* ratio in low-light-grown(LL)aswellasinhigh-light-grown (HL) tobacco plants; this effect was more pronounced in HL-plants. The potential of Chl biosynthesis and the POR activity increased compensating for the usual loss of Chl when plants were grown in high light. Increased Chl *b* synthesis in CAOx plants was accompanied by an increased abundance of light-harvesting chlorophyll-proteins (LHCPs) and other proteins of electron transport chain that led to an increase in capture of light, as well as enhanced (40–80%) electron transport rates of Photosystem I and Photosystem II at both limiting and saturating light intensities. However, the increase in the whole chain electron transport was somewhat lower (20–50%). The light-saturated photosynthetic carbon assimilation, starch content and the dry matter accumulation increased in CAOx plants grown in both low and high-light regimes (Figs. 27.8 and 27.9).

These results from the laboratory of the author (Biswal et al. 2012) demonstrate that controlled up-regulation of Chl b biosynthesis co-modulates the expression of chloroplast proteins that increase the antenna size and electron transport rates and enhances CO_2 assimilation, starch contents and dry matter accumulation.

Chl *b* reductase catalyzes the conversion of Chl *b* to Chl *a*. It reduces the formyl group of Chl *b* to a hydroxymethyl group. It was observed that barley etioplast had Chlide *b* reductase activity and the enzyme needs NADPH and reduced ferredoxin for its activity (Scheumann et al. 1996, 1999). The gene encoding Chl *b* reductase was isolated from rice and it belongs to a family of short-chain dehydrogenase/reductases (Kusaba et al. 2007). It encodes a protein of 504 amino acids and contains a dinucleotide binding motif (TGXXXGXG) and a catalytic site (YXXXK) and uses NADPH as a cofactor. Interestingly, two genes for Chl *b* reductase were found in the genomes of *A. thaliana* and rice (Kusaba et al. 2007; Sato et al. 2009). It was also observed that disruption of the genes encoding Chl *b* reductase in *A. thaliana* resulted in non-degradation of Chl *b* and LHC II (Horie et al. 2009). In the presence of recombinant CAO enzyme, the Chlide *a* gets converted to Chlide *b* using NADPH, molecular oxygen and ferredoxin (Oster et al. 2000). In this in vitro assay, a small amount of 7-hydroxymehtyl Chlide *a* was also formed. When the 7-hydroxymethyl Chlide *a* was used as a substrate for the in vitro enzymatic assay, the recombinant enzyme also efficiently converted 7-hydroxymehtyl Chlide *a* to Chlide *b* (Oster et al. 2000). Then, Chl synthase converts Chlide *b* into Chl *b*. Chl *b* is further converted to hydroxymethyl Chl *a* by the enzyme Chl *b* reductase (Kusaba et al. 2007). This enzyme converts the formyl group of Chl *b* to a hydroxymethyl group using NADPH as a reductant.

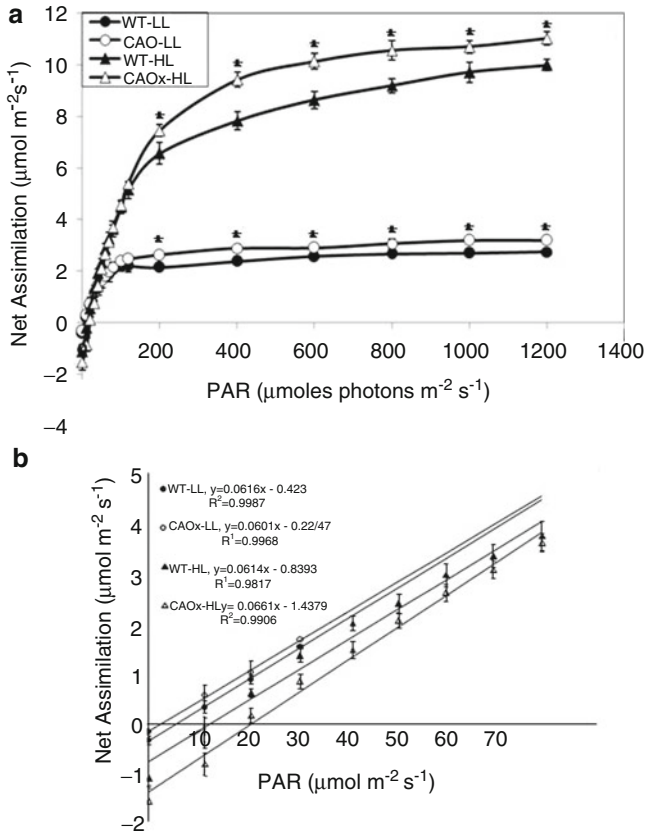

Fig. 27.8. Photosynthesis (net CO_2 assimilation rate) light response curves and quantum yield of leaves from attached WT and CAOx plants grown in LL and HL intensities. (**a**) Net CO_2 assimilation rates of attached leaves of WT and CAOx plants were monitored by IRGA (Licor 6400-XT portable photosynthetic system) in ambient CO_2 at different light intensities. Light response curves were measured up to 1,800 µmol of photons m^{-2} s^{-1} at 28°C. (**b**) Relative quantum yield of CO_2 fixation by leaves from WT and CAOx plants grown in LL or HL regimes. Quantum yield was measured from the above photosynthetic rate after the chamber reached to a steady-state. Light intensity curves at LL-intensities upto 80 µmol of photons m^{-2} s^{-1}; the slopes of these curves provide relative quantum yield of CO_2 fixation by leaves. Leaves were pre-exposed for 15 min at 700 µmol photons m^{-2} s^{-1} and 200 µmol photons m^{-2} s^{-1} for LL and HL grown plants respectively prior to CO_2 assimilation measurement. These experiments were done three times with similar results. Each data point is the average of five replicates and the error *bar* represents SE. *Asterisks* indicate significant differences determined by ANOVA followed by Tukey's test ($^*P < 0.05$) (Biswal et al. 2012).

Geranyl-geranyl reductase mediates the reduction of geranylgeranyl diphosphate to phytyl diphosphate. The cDNA encoding a pre-geranyl-geranyl reductase from *A. thaliana* has been isolated and characterized (Keller et al. 1998). The recombinant protein catalyzes the reduction of geranyl-geranyl-Chl *a* into phytyl-Chl *a,* as well as the reduction of free geranyl-geranyl diphosphate to phytyl diphosphate, suggesting this is a multifunctional enzyme.

2. Modulation of Phytol Synthesis and Its Impact on Plant Development, Photosynthesis, Tocopherol Contents and Oxidative Stress

a. Modulation by CHLP

Antisense expression of *CHLP* coding for geranyl-geranyl reductase affects the Chl and tocopherol contents in tobacco (Tanaka et al. 1999). The reduced tocopherol and Chl

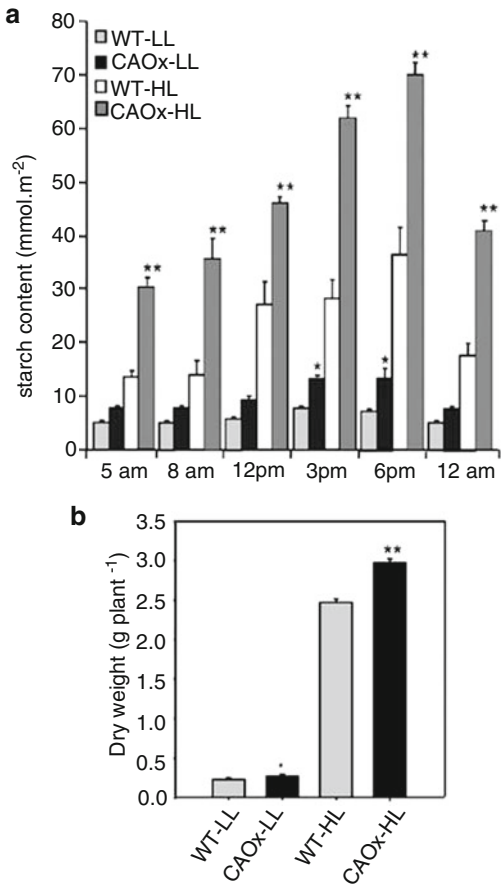

Fig. 27.9. Diurnal starch content and dry weight measurement in WT and CAOx plants. (**a**) Starch content was measured from mature leaves of WT and CAOx plants grown under LL and HL at various times over a diurnal cycle as described in methods. Note the diurnal starch accumulation was maximum between 3 and 6 PM and CAOx-HL plants showed maximum starch accumulation. (**b**) Dry weight of WT and CAOx plants was measured after aerial parts of the plant were dried at 70°C for 5 days. HL-grown WT and CAOx plants showed significant increase in dry matter accumulation in comparison to WT-LL and CAOx-LL plants. *Asterisks* indicate significant differences determined by ANOVA followed by Tukey's test ($^{*}P<0.05$; $^{**}P<0.001$). These experiments were done three times with similar results. Each data point is the average of four replicates in (**a**) and 15 replicate in (**b**) and the error *bars* represent SD (Biswal et al. 2012).

contents in *CHLP* antisense plants resulted in the reduction of electron transport chains and PS II activity. There are also more lipid peroxidation products in *CHLP* antisense plants. Havaux et al. (2003) found accumulation of xanthophylls cycle pigments in *CHLP* antisense plants which could be a compensatory mechanism for tochopherol deficiency. The *CHLP* transcript levels in peach were abundant in Chl-containing tissues and flower organs however barely detected in roots and mesocarp of the ripening fruits (Giannino et al. 2004). Its transcript level is up-regulated during etioplast to chloroplast and chloroplast to chromoplast development (Keller et al. 1998).

The responses of Chl biosynthetic enzymes to various environmental stresses are examined and summarized in Table 27.1. These stresses broadly downregulate most of the enzymes of Chl biosynthesis pathway. However, gene/protein expression of a certain enzyme i.e., GSA-AT is upregulated in most stresses, i.e., heat, water, salt etc. The expression of UroD is upregulated in high-temperature. As GSA-AT is a crucial enzyme involved in the last step of synthesis of ALA, plants most likely upregulate its expression to compensate for the reduced expression of earlier enzymes of the ALA biosynthesis.

III. Future Prospects

Plant tetrapyrroles play an important role in plant development, growth, productivity and modulation of their biosynthesis and protect plants from environmental stresses. Therefore, genetic manipulation of tetrapyrrole biosynthesis either via molecular marker assisted breeding programs or transgenic approaches will have a potential to protect crop plants from environmental stresses and increase yield.

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Table 27.1. Impact of various stress-induced alterations in activity, protein and transcript levels of enzymes involved in Chl biosynthesis (Dalal and Tripathy 2012)

	Water stress	Salt	Chill	Heat
GluTR				
Protein			–	–
Transcript	–	+	–	–
GSA-AT				
Protein	+	+	+	+
Transcript	+	+	–	+
ALAD				
Enzyme activity	–	–	–	–
Transcript	–	–	–	–
PBGD				
Enzyme activity	–	–	–	–
Transcript	–	–	–	0
UROD				
Enzyme activity			–	+
Protein	–	–	–	+
Transcript	–	–	–	+
CPO				
Enzyme activity	–	–	–	0
Protein	–		0	0
Transcript	–	–	0	0
Protox				
Enzyme activity	–	–	–	0
Protein	–	–		
Transcript	–	–		
Mg-chelatase				
Enzyme activity	–	–	–	–
Protein	0	–		
Transcript	–	–	–	–
MPE cyclase				
Enzyme activity			–	–
Transcript	–	–	–	–
POR				
Enzyme activity	–	–	0	–
Protein	–		0	0
Transcript	–			
CAO				
Transcript		–		
ChlP				
Protein	–	–		
Transcript			–	–

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Chapter 28

Response of Mature, Developing and Senescing Chloroplasts to Environmental Stress

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Summary

The composition, organization and function of the photosynthetic apparatus of higher plants change in response to different environmental stress conditions. The changes include disorganization of thylakoid membranes, alteration in the composition and function of light-harvesting complexes (LHC), impairment of electron transport chain and inactivation of reaction centers of photosystems (PSI and PSII), ATP synthase and enzymes of the Calvin-Benson cycle. It is believed that these changes bring about imbalances in photostasis of photosynthesis, redox homeostasis, and endogenous sugar status. These imbalances are responsible for the metabolism of reactive oxygen species (ROS) and play important regulatory roles in the process of acclimation of plants to various environments. There is convincing evidence that such imbalances modulate the expression of many plastid and nuclear genes encoding photosynthetic components. However, with respect to both damage and acclimation, the responses to environmental stress factors of mature, developing and senescing chloroplasts are different. This chapter discusses the evidences for the different aspects of acclimation and provides an understanding of the underlying mechanisms of damage and acclimation of chloroplasts during different phases of leaf development.

Abbreviations: ABA – Abscisic acid; b Zip – Basic leucine zipper; Car – Carotenoids; Chl – Chlorophyll; GSH – Glutathione; JA – Jasmonic acid; LHC – Light harvesting complex; LHCP – Light harvesting chlorophyll protein complex; PLB – Prolamellar body; POR – Protochlorophyllide oxidoreductase; PSI (II) – Photosystem I (II); ROS – Reactive oxygen species; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; SA – Salicylic acid; *SAGs* – Senescence associated genes; *SDG* – Senescence down-regulating genes; SnRK 1 – Sucrose non-fermenting 1-related kinase 1

I. Introduction

A remarkable advance has been made to understand various domains of plant stress biology in general and the response of chloroplasts to environmental stress in particular. The environmental stresses like low light (Lovisolo et al. 1996; Biswal et al. 2003; Shaohui et al. 2008), high light (Anderson and Andersson 1988; Andersson and Barber 1996; Biswal et al. 2003, 2011; Tyystjärvi

2008; Sarvikas et al. 2010), water deficit (Bourque et al. 1975; Giardi et al. 1996; Deo and Biswal 2001; Chaves et al. 2002; Deo et al. 2006; Singh and Reddy 2011), flooding (Mauchamp and Methy 2004; Mishra et al. 2008), ultraviolet radiation (Greenberg et al. 1989; Joshi et al. 1994, 1997, 2011; Jordan 1996, 2002; Vass et al. 2002), salinity (Munns and Termaat 1986; Munns 2005), and metal ion variation (Larbi et al. 2006; Krasensky and Jonak 2012) have been demonstrated to act primarily at the level of the photochemical reactions and the activity of the enzymes of the Calvin-Benson cycle. The damages induced by these stresses include loss in photosynthetic pigments, changes in the composition of carotenoids (Car), breakdown of proteins, impairment of electron transport chain and disorganization of thylakoid membranes (Biswal 1997; Biswal et al. 2003, 2008; Pareek et al. 2010). The alterations in structural organization and functional efficiency of the photosynthetic apparatus in response to environmental signals have been reviewed (Biswal et al. 2003, 2008, 2011; Baena-Gonzalez and Sheen 2008; Lawlor 2009; Pfannschmidt et al. 2009).

Plants can perceive stress and elicit appropriate responses to adapt to the stressful condition. The course of adaptation is such that chloroplasts, after being fully developed, carry on the coordinated interactions among light harvesting, energy conversion, electron transport, proton translocation, and carbon fixation in the altered environment. Chloroplasts, at different stages of leaf development, adopt different strategies to cope with the stress factors. A developing chloroplast has the flexibility for structural modifications in response to a stress, whereas a mature one, which is structurally stable, has to invoke functional changes to cope with the adversities like adjustments to stress-induced metabolic disparities such as losses in photostasis of photosynthesis, redox homeostasis in electron transport chain between PSII and PSI and/or sugar imbalance. The literature on the mechanisms of damage and adaptive responses of chloroplast to several environmental stresses is rich (see Rai and Takabe 2006; Jenks and Wood 2010; Pareek et al. 2010; Shanker and Venkateswarlu 2011). The current chapter, however, aims at integrating the views of the past in the field of stress-induced changes in the structure and the function of chloroplasts during different phases of leaf development and to put forward directions for future investigations.

A. On the Boundaries and Sphere of the Photosynthetic Apparatus

Photosynthesis is the primary function of chloroplasts. It is the overall process by which plants use light energy to synthesize energy rich organic compounds. The process includes a complex series of reactions involving light absorption, energy conversion, electron transfer and a multistep enzymatic pathway that finally converts CO_2 and water into carbohydrates (for basic information, see Rabinowitch and Govindjee 1969 and Blankenship 2002). The chloroplast of eukaryotes, where all steps of photosynthesis take place, contains an internal membrane system with several multisubunit protein complexes including PSI, PSII, Cyt b_6/f complex and ATP synthase. In nature, PSI and PSII are known to generate the most negative redox potential and the most positive redox potential, respectively (Nelson 2011). Thus PSII is capable of extracting electrons from water. PSI receives electrons from the PSII via plastoquinone, Cyt b_6/f complex and plastocyanin and transfers them to $NADP^+$ to produce NADPH. In both linear and cyclic electron transport pathways, a H^+ gradient (ΔpH) is built up across the thylakoid membrane by the coupled transport of e^- and H^+. The resulting potential gradient across the thylakoid membrane (proton motive force) is used by ATP synthase for the production of ATP.

The fixation of CO_2, on the other hand, involves a number of enzymes operating in the Calvin-Benson cycle. Primary photochemistry in thylakoids and CO_2 fixation in stroma are integrated to complete the

process effectively inspite of their spatial separation and temporal differences in their activities.

II. Sensitivity of Chloroplasts to Environmental Signals: Structural and Functional Limitations

The structure and function of the photosynthetic apparatus besides being very complex are ever changing with leaf age. The built-in complexity of the apparatus is the root of its susceptibility to stress while the structural flexibility makes it a primary target for stress-induced damage and adaptation. In this section we have attempted to provide a clear picture of these features of the apparatus, which make it vulnerable to environmental stress.

A. Unique Features of Photosystem II (PSII)

The PSII of photosynthetic apparatus is a specially designed protein complex that harvests light energy, oxidizes the water molecule and reduces Q_B plastoquinone. The photosystem is composed of several subunits of intrinsic and extrinsic polypeptides including CP43 and CP47 core antenna complexes and several chemical moieties. The D1 and D2 proteins with a special pair of Chl (P680) form the enzymatic heart of the reaction center of the photosystem. The pigments associated with the light harvesting Chl protein complex of PSII absorb photons and pass them to the reaction center as excitons. The photoredox reactions within PSII result in the evolution of O_2 by oxidation of water at Mn_4Ca-metal complex at the donor side and reduction of PQ to PQH_2 at the acceptor side of PSII. The PSII is connected to PSI, which contains the special pair reaction center Chl (P700), a core complex and an antenna system (Amunts et al. 2010), through a series of electron transport carriers (Anderson and Andersson 1988).

The excited state of Chl, formed due to the absorption of light energy, is exposed to an environment of molecular oxygen which inevitably leads to the generation of reactive oxygen species (ROS). Further, the oxidizing potential of P_{680}^+ is very high (i.e., 1.17 V), a requirement for oxidation of water. However, P_{680}^+ is short-lived. Once its lifetime is enhanced, which occurs under stress conditions, it oxidizes the pigments and proteins in its vicinity (Barber 1998; Dau et al. 2001). Inhibition at the acceptor side of PSII, on the other hand, leads to the formation of $^3P_{680}$ which can transfer its energy to O_2, available in the vicinity of PSII, forming the toxic singlet oxygen ($^1O_2$) (Andersson and Barber 1996). Thus the molecule P_{680} that is central to the photosynthetic activity of green plants may become the source of ROS, the radicals mostly accountable for the breakdown of the apparatus under environmental stress. The relatively slow rate of electron transport associated with the Cyt b_6/f complex is an inherent limitation of the chloroplasts. The condition leads to over-reduction of PSII (Vass and Cser 2009) culminating in the generation of ROS.

B. Highly Sophisticated Oxygen Evolving Complex (OEC) Structure and Function

The oxygen evolving complex, responsible for water splitting, is a highly sophisticated metallo-protein complex of PSII. When the subtle structure of the complex is disturbed, which by and large occurs under environmental stresses, it may prove harmful for the chloroplast.

The electrons released from P_{680} are replaced from tyrosine Z and ultimately from water molecules, which are oxidized by the OEC of PSII with the release of molecular oxygen. The OEC complex consists of the catalytically important inorganic manganese, calcium, and chloride ions in PSII as Mn_4Ca cluster. The metal complex in OEC undergoes a series of oxidation steps in succession that results in the accumulation of positive charges (for a review on PSII, see Govindjee et al. 2010). After storing four positive charges, OEC oxidizes two water molecules and releases one oxygen molecule and four protons to the thylakoid lumen. Hence OEC participates in the generation of a proton motive force (PMF), an electrochemical

gradient of protons across the thylakoid membrane, which is used to drive ATP synthesis (Nield and Barber 2006).

Manganese (Mn) in the metal center of OEC exists in various oxidation states during the light reactions of photosynthesis. Under stress, the population of higher oxidation states is likely to increase. Higher oxidation states oxidize the adjoining proteins and chemical species and get reduced to oxidation state II. In oxidation state II, the metal complex under stress is unstable and it breaks down leading to the release of Mn from the OEC (Antal et al. 2009; Biswal et al. 2011). Antal et al. (2009) have critically discussed the photosensitivity of the Mn complex and its possible association with photoinhibition. Thus OEC, because of its innate structure and function, is the most susceptible part of photosynthetic apparatus to environmental stresses (Biswal et al. 2011). However, the mechanism of water oxidation in OEC has not been completely understood. The crystallographic study with 1.9 Å resolutions of PSII by Umena et al. (2011) is providing further insight into the structure of water oxidation and possible stress sensitive components.

C. Topology of Pigments in the Light Harvesting Chlorophyll Protein Complex (LHCP)

The pigments and proteins are the essential components of light harvesting chlorophyll protein complex (LHCP). The effective functioning of LHCP not only depends on the presence of these components but also on their proper configuration. In addition to harvesting light to drive photochemical reactions, an essential feature of LHCP is to allow the transfer of excess excitation energy to different quenching species of carotenoids (Car) including components of the xanthophyll cycle. The structural organization of the LHCP of thylakoid membrane has been elucidated from the studies with high resolution electron microscopy and image analysis (Yakushevska et al. 2003; Ruban et al. 2012). The three dimensional structure of LHCII has revealed the possible molecular mechanisms of the energy transfer (Liu et al. 2004; Barros et al. 2009). Cars, besides transferring their absorbed energy through singlet–singlet transfer mechanism for light harvesting process, quench the excess quanta absorbed through several known mechanisms (Young and Frank 1996; Havaux et al. 2007; Mozzo et al. 2008; Ruban et al. 2012). Both these functions require a suitable proximity and orientation of these pigments. Stresses are known to distort the geometry of the complex resulting in the loss of their proximity and orientation that hinder the energy transfer from excited Chl to Car and lead to generation of ROS (Biswal et al. 2003)

Further, photosynthetic pigments constitute a part of LHCP. While Chl b is associated only with the light harvesting complex of green plants, Chl a is associated with this complex in addition to its binding with the reaction centers of the photosystems. During biosynthesis of Chl, intermediates like protochlorophyllide (Pchlide) and protoporphyrin IX (Proto IX) are formed and are loosely bound to thylakoid membranes (Mohapatra and Tripathy 2007). They absorb light energy but cannot transfer it to the reaction center. Unutilized and unquenched because of its spatial separation from pigment protein complexes, this excitation energy becomes a potential source for the formation of singlet oxygen. Similarly, during senescence-mediated degradation of chloroplasts, many intermediates of the Chl degradation pathway with ability to absorb light energy are formed. But the energy absorbed by these intermediates cannot be used for photosynthesis and instead is transferred to molecular O_2. This leads to the formation of ROS and subsequently to the oxidative damage of chloroplasts. Thus individual components of the LHCP without co-ordinated association may pull towards disruption but once united properly the complex becomes stable.

D. The Telescopic Time Scale

The light reaction, culminating in charge separation as P_{680}^{+} $Pheo^{-}$, is initiated with the absorption of photons in the pigment bed of PSII (Wydrzynski 2008). The intrinsic rate

of the entire process including this primary charge separation, an ultrafast energy conversion process (Zinth and Wachtveitl 2005) is in the time scale of femtoseconds (10^{-15} s) to picoseconds (10^{-12} s), while each step of the electron transport chain has its own time span which varies from step to step and ranges from nanoseconds (10^{-9} s) to milliseconds (10^{-3} s). Additionally, the time scale of the enzymatic reactions during Calvin-Benson cycle is in the milliseconds to seconds range. Physical constraints determine an increasing order of time span for different steps in the electron transport chain from the initiation of charge separation to its final consumption to make the system more efficient. But when the enzyme-mediated electron sink is deteriorated under stressful conditions, a factor incompatible with higher efficient primary photochemistry, the excitation pressure at the source is created and the energy balance is lost. The loss in turn leads to ROS production.

III. The Environmental Conditions that Limit the Function of Mature Chloroplasts

A. Responses to Different Environmental Stress and Adaptational Measures

Most of the environmental stresses are connected to anthropogenic activities which are clearly causing major changes in atmospheric chemistry and climate (Reddy et al. 2004) and almost all stressors affect either directly or indirectly the photosynthetic performance of green plants (Lichtenthaler and Babani 2000). A decline in photosynthesis is common under these stresses (Earl and Tollenaar 1999; Biswal et al. 2011). In response to these stresses, plants develop a host of mechanisms to cope and to survive. A scheme (Fig. 28.1) attempts to provide current understanding of the changes in the fully functional mature chloroplast induced by stress and on the adaptive responses of the organelle to meet the challenges.

1. Degradation of Reaction Center Proteins Under Light Stress

Both low and high light affect the photosynthesis of green plants (Lovisolo et al. 1996; Zhang et al. 2003; Shaohui et al. 2008). When the seedlings grown under relatively low light intensity are exposed to high light, photosynthetic activity declines (Zhang et al. 2003). Impairment of PSII has been demonstrated to be the primary cause of this decline (Takahashi and Murata 2008). Under high light environment, the excess absorbed quanta, beyond that required for optimum photochemistry, may damage OEC and generate ROS leading to photo-oxidative damage of pigments, lipids and amino acids of proteins in the vicinity of PSII (Takahashi and Murata 2008; Tyystjärvi 2008). The photodamage of PSII occurs due to participation of strong oxidant P_{680}^{+} through two different routes: (1) the acceptor side mechanism involving the recombination of P_{680}^{+} with $Pheo^{-}$ (Pheo; pheophytin, the primary electron acceptor of PSII) (2) the donor side mechanism caused by an increase in the lifetime of P_{680}^{+}. The acceptor side photoinhibition occurs under high light when Q_A is doubly reduced. The recombination of P_{680}^{+} with $Pheo^{-}$ leads to the formation of triplet P_{680} and subsequently highly toxic $^1O_2$ (as discussed in Sect. II.A). The recombination of these pairs is also possible under low light due to the back reaction of partially reduced Q_B^{-} and S_2/S_3 state of OEC. On the other hand, the donor side mechanism engages long lived P_{680}^{+} for oxidation of pigments and proteins which destabilize the D1 and D2 complex. The D1 protein gets oxidized and subsequently undergoes proteolytic degradation (Jegerschold and Styring 1996; Barber and Sharma 2000). Thus PSII is the main target of high-light stress (Andersson and Barber 1996; Sarvikas et al. 2010; Biswal et al. 2011).

Plants exhibit various adaptation strategies in response to high light stress. As a means of long-term adaptation, prolonged exposure to high light leads to the formation of sun type chloroplasts with a reduced light harvesting antenna and higher rate of

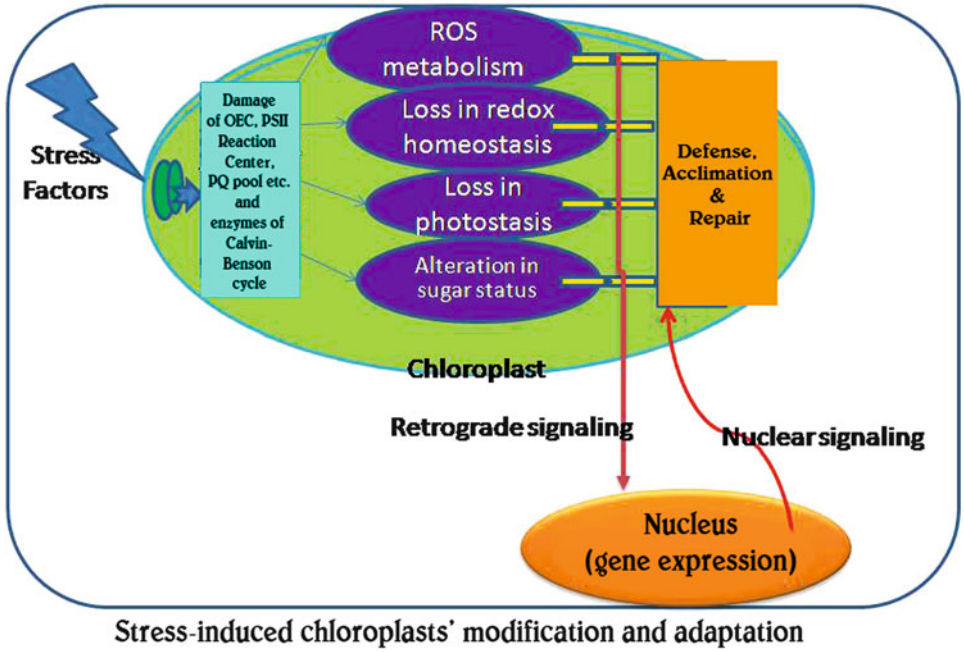

Fig. 28.1. A schematic representation of stress induced alterations and adaptations in fully functional mature chloroplast. Stress induced damages of different functional components of photosynthetic apparatus lead to ROS metabolism and/or alteration in redox/energy/sugar status. These changes are signaled from chloroplast to the nucleus for gene expression (retrograde signaling) and other appropriate changes in genetic and cellular metabolic network for development of defense, repair and acclimation as indicated by arrows. The scheme is based upon the work of Foyer and Noctor (2005a, b); Wingler and Roitsch (2008); Pfannschmidt et al. (2009); Biswal et al. (2011) and De Pinto et al. (2012).

photosynthetic quantum conversion efficiency (Lichtenthaler and Burkart 1999). The major short term regulatory mechanism exhibited by plants during high irradiance is the harmless dissipation of excess light energy in the form of heat. The involvement of the xanthophyll cycle during such non-radiative dissipation has been extensively reported (Gilmore 1997; Demmig-Adams and Adams 2000, 2006). The accumulation of zeaxanthin, a component of the xanthophyll cycle, in a strain of *Chlamydomonas reinhardtii* has been reported to protect it from photooxidative stress (Baroli et al. 2003).

2. Inhibition of PSII Under Water Stress

Plants in nature experience stress under excessive water (flood) as well as water deficit (drought) conditions. These two stresses are injurious to the photosynthetic apparatus but in two different ways. Flooding stress induces damage to the photosynthetic apparatus by affecting mainly the cellular respiration due to submergence within an O_2 deficit environment (Mauchamp and Methy 2004; Panda et al. 2006). PSII has been identified to be the major target of the stress factor from the observation of higher loss in photochemical potential and O_2 evolution compared to the overall loss in pigment content (Panda et al. 2006; Mishra et al. 2008).

Drought, on the other hand, perturbs the biochemical processes (Graan and Boyer 1990) besides damaging the OEC (Canaani et al. 1986; Toivonen and Vidaver 1988) and reaction center of PSII (He et al. 1995; Giardi

et al. 1996). Reduction in leaf photosynthesis during drought, however, is attributed to stomatal closure-induced decline in carbon uptake (Chaves et al. 2002; Flexas and Medrano 2002) in addition to down-regulation of PSII activity (Pieters et al. 2003) and reduction in the activities of the enzymes associated with Calvin-Benson cycle (Reddy et al. 2004). In contrast, reports that water stress hardly affects PSII photochemistry are also available in the literature (Genty et al. 1987; Lu and Zhang 1999). Predisposition of PSII of water-stressed leaves to photoinhibition even in moderate light intensity could provide an explanation for such a contradiction (Deo and Biswal 2001; Behera et al. 2003; Pastenes et al. 2005). Singh and Reddy (2011), however, correlated the occurrence of oxidative stress with drought-induced reduction in total Chl and Car along with increased leaf wax contents.

The ability of plants to respond to water deficit depends on all mechanisms involving the use of water that integrate cellular responses by triggering a cellular signal transduction pathway (Lawlor and Tezara 2009). Drought triggers the production of abscisic acid (ABA), which in turn induces various genes involved in a signaling cascade for the regulation of downstream biochemical protective mechanisms (Shinozaki and Yamaguchi-Shinozaki 1997), besides promoting stomatal closure. In general there is a higher concentration of glutathione (GSH) and ascorbate in water deficit plants. An elevated GSH content is usually correlated with the increased adaptive response of plants to environmental stress (Biswal et al. 2008). Moussa and Mohamed (2011) have critically discussed the development of antioxidant defense systems during drought stress in pea.

3. Energy Imbalance Under Chilling Stress

Photosynthesis is susceptible to both chilling and high temperature stresses. However, the mode of damage of chloroplasts in response to chilling stress is different from that of high temperature stress. Low temperature inhibits the activity of PSI much more than that of PSII (Terashima et al. 1994). Lehner and Lutz (2003) did not observe any change in PSII function even though the xanthophyll cycle was affected by chilling stress. However, a report demonstrating the loss in D1 protein of the PSII reaction center is available (Ensminger et al. 2006). Loss in thylakoid membrane fluidity because of low temperature-induced hardening of membrane lipids could be the basis for chloroplast breakdown and thus maintenance of membrane fluidity may be identified to be a major metabolic factor influencing growth. Inhibition of electron transport from Q_A^- to Q_B due to change in redox potential of Q_A during low temperature stress is reported to exacerbate the imbalance between the source of energy and metabolic sink resulting in generation of an excitation pressure (Ensminger et al. 2006).

4. Inactivation of PSII Reaction Centers Under High Temperature Stress

High temperature stress is known to affect specifically PSII (Carpentier 1999; Pospisil and Tyystjärvi 1999; Vani et al. 2001) besides altering the thylakoid membrane fluidity (Kim and Portis 2005). The stress during damage response dismantles the thylakoid membrane, uncouples the LHCII and reduces oxygen evolution (Weis and Berry 1988; Wen et al. 2005). It is believed that increasing temperature leads to a blockage of PSII reaction centers which is followed by dissociation of antenna pigment protein complexes from the reaction centers (Gounaris et al. 1984). On the other hand, PSI activity, activity of Calvin-Benson cycle enzymes, and the structure of the chloroplast envelope are reported to remain relatively stable at higher temperature (Krause and Santarius 1975; Berry and Bjorkman 1980).

5. UV-Induced Loss in Photochemistry of Chloroplasts

The UV spectrum of solar radiation is known to modulate the structure and the primary photochemical reactions of thylakoid

membranes. While UV-C (200–280 nm) and UV-B (280–320 nm) radiations have adverse effects on chloroplasts (Greenberg et al. 1989; Jordan 1996, 2002; Kovacs and Keresztes 2002; Brösche and Strid 2003; Lidon et al. 2012), UV-A (320–400 nm) radiation exhibits both damaging (Joshi et al. 1991; Turcasanyi and Vass 2000; Nayak et al. 2003) and non-damaging (Shiozaki et al. 1999; Gartia et al. 2003; Helsper et al. 2003) effects. As UV-C is almost filtered out by stratospheric ozone, we have preferred here to describe the effects of only UV-B and UV-A on the photosynthetic apparatus of green leaves.

UV-B induced damage of the photosynthetic apparatus causes declines in photosynthetic pigment content, exciton transfer efficiency, PSII photochemistry and in the rate of O_2 evolution (Joshi et al. 2011). The D1 protein of the PSII reaction center is sensitive to UV-B radiation (Friso et al. 1994a, b; Barbato et al. 1995) and the degradation of this protein is believed to occur via the plastoquinone anion Q_B^- (Friso et al. 1994a). The acceptor side of PSII is also affected by the radiation either due to modification of quinone binding sites or due to direct damage to plastoquinone molecules (Rodrigues et al. 2006). The radiation induces losses in photostasis of photosynthesis and redox homeostasis of the electron transfer chain (Joshi et al. 2011). UV-B radiation is also reported to affect the activity of Rubisco (Jordan et al. 1992; Bischof et al. 2002), ATP synthase and violaxanthin de-epoxidase (Pfündel et al. 1992; Bischof et al. 2002). However, interestingly, many studies reveal that PSII inhibition under natural UV-B radiation is small and transitory (Kolb et al. 2001; Xiong and Day 2001).

Inhibition of photosynthesis by UV-A radiation in higher plants has been documented (Joshi et al. 1994; Vass et al. 2002; Nayak et al. 2003). The adverse effects of UV-A include decline in energy transfer efficiency, impairment of electron transport between PSII and PSI, loss in maximum photochemical efficiency (as inferred from F_v/F_m ratio measurements) and damage of OEC (Joshi et al. 1994; Nayak et al. 2003). However, some positive effects of UV-A have also been reported (Shiozaki et al. 1999; Kolb et al. 2001; Helsper et al. 2003). Biswal et al. (1997) have shown that UV-A provides stability to PSI during senescence. This radiation was observed to mitigate the damaging effects of UV-B radiation (Flint and Caldwell 1996; Pradhan et al. 2006; Joshi et al. 2007).

6. Inactivation of Calvin-Benson Cycle and Creation of Excitation Pressure

Environmental stresses are known to affect the enzymes of the Calvin-Benson cycle (Carmo-Silva et al. 2012). Extreme temperatures may slow down the energy utilization in the cycle, without significantly disturbing the primary photochemistry of thylakoids (Huner et al. 1993, 1996). Similarly, water stress may result in a situation when the amount of light absorbed becomes more than its utilization in CO_2 fixation in the Calvin-Benson cycle (Biswal 1997; Biswal and Biswal 1999). Alterations in CO_2 concentration that repress or enhance the Calvin-Benson cycle may also alter the rate of electron flow through the photosynthetic electron transport chain (Wormuth et al. 2006) and could create excitation pressure in the PSII. The stress induced imbalance between energy source and sink of photosynthesis may result in the creation of excitation or redox pressure on the photosystems of thylakoids leading to production of ROS as a stress response. The plants, however, readjust the metabolic processes and develop several mechanisms of adaptation as discussed subsequently.

B. The Limitations and the Possibilities

As already discussed, all detrimental effects arising from environmental stresses are likely to induce energy imbalance, alteration in redox homeostasis and metabolic networks associated with mature chloroplast of green plants. In this section we provide a list of some basic stress-induced imbalances that limit photosynthesis and consequently develop adaptive mechanisms during the stress response.

1. The Loss in Photostasis of Photosynthesis

From the point of view of light absorption and utilization, the functional features of chloroplasts may be grouped into three basic processes: (1) light energy harvesting system (energy source); (2) channeling of the energy from the source to energy utilizing carbon, nitrogen and sulfur assimilation processes (energy sink); and (3) a series of redox reactions associated with the electron transport chain of thylakoid (electron transport systems). In a normal leaf there exists an equilibrium amongst these three processes for optimum photosynthesis. The equilibrium can be best described in terms of photostasis, the balance in source and sink, of photosynthesis. Falkowski and Chen (2003) have used the mathematical relation $\sigma_{PSII} E_K = \tau^{-1}$ to describe this energy balance condition. [In this equation, σ_{PSII} = absorption cross section of PSII, E_K = the irradiance at which the maximum quantum yield balances the photosynthetic capacity and τ^{-1} = the rate of utilization of photosynthetic electrons.] Stress induced alteration in any of these parameters results in a loss in photostasis leading to a decline in photosynthesis. Plants experiencing stress, however, develop several adaptive mechanisms to modulate the sink, source or both to maintain the photostasis of photosynthesis (Biswal et al. 2011).

2. Loss in Redox Homeostasis in Electron Transport Chain

The photosynthetic energy source – sink equilibrium condition determines and is determined by the redox chemistry of thylakoids. A redox homeostasis in the electron transport chain between PSII and PSI is maintained by the efficient charge transfer mechanism amongst the redox active charge carriers and PQ pool plays a pivotal role in maintaining homeostasis. Any change in the cellular energy may induce a loss in redox homeostasis which begets the appropriate signals for its restoration through cellular readjustment. The process of cellular adjustment could be better understood from the example of the so-called "state transitions". The loss in redox homeostasis creates either reduced or oxidized state of the PQ pool. The reduced state of the pool activates a redox sensitive kinase that phosphorylates a mobile LHCII causing the migration and attachment of LHCII to PSI (Wollman 2001). On the other hand, the oxidized PQ pool activates a phosphatase that dephosphorylates the mobile LHCII. Thus the dephosphorylated LHCII migrates to PSII (Wollman 2001). Thereby, chloroplasts proportionately manage the distribution of excitation energy to balance the redox state of the electron transport chain between PSII and PSI. The role of kinases like STT7 in *Chlamydomonas reinhardtii* and STN7 in *A. thaliana* during state transitions have been demonstrated by Depege et al. (2003) and Bellafiore et al. (2005), respectively. However, the mechanism involved in the activation/deactivation process has not been properly understood. Further, the redox state of PQ pool is known to modulate the gene expression in both chloroplasts and nucleus for suitable adjustment of the stoichiometry of PSII and PSI (Biswal et al. 2003; De Pinto et al. 2012).

Stress usually disrupts and disengages the electron transfer pathways and/or energy transfer pathways. When electrons are transferred to molecular oxygen (O_2), ROS such as $^1O_2$, H_2O_2, O_2^-, and OH^- are formed (Mittler 2002; Suzuki and Mittler 2006). In plant and algal cells, chloroplasts are the sites where both electrons from electron transport carriers and molecular oxygen are available in close vicinity. Further, stress-induced limitation of CO_2 fixation coupled with over-reduction of the electron transport chain is also a cause of ROS metabolism in chloroplasts. Even under normal state, these redox active species are produced at a low level, but under stress their production increases. The scheme in Fig. 28.1 describes stress-induced alterations in these processes and adaptive responses of chloroplasts to survive.

Furthermore, the cyclic electron flow is an essential component of chloroplasts that serves to coordinate energy metabolism and to balance the redox status. There exists

another mechanism of redox regulation for photosynthesis that prevents over-reduction of the acceptor side of PSI and the chloroplast stroma: it is the "malate valve" system (Scheibe 2004). This system modulates the metabolite distribution and the export of excess reducing power that reduces electron pressure in the chloroplast.

3. Involvement of Sugar and Hormone Signaling

Stresses that affect the photosynthetic efficiency of chloroplasts may induce an alteration in the sugar status which regulates plant metabolism and development either through its effect on primary carbon metabolism or by altering the source-sink relation. Under stressful environment, the decline in photosynthetic efficiency may result in a sugar-limiting condition, which generates a signaling system to up-regulate many catabolic processes for replenishment of deficiency with nutrients available from other sources (Lee et al. 2004; Mohapatra et al. 2010). For example, enzymes like β-glucosidase and β-glucanase mediate the breakdown of cell wall polysaccharides to sugars under sugar limiting condition to supply energy for the survival of plants. Genes responsible for enhancing catabolic pathways in dark-induced sugar starvation conditions have been identified (Fujiki et al. 2001). The sucrose non-fermenting 1-related kinase 1 (SNRK1) is thought to trigger the expression of the genes for these processes (Baena-Gonzalez and Sheen 2008). Even under sugar deficit condition, SnRK1 in Arabidopsis down-regulates the synthesis of lipids, proteins, sucrose, starch, amino acids and nucleotides to conserve energy by repressing many associated genes responsible for their biosynthesis (Rolland et al. 2006).

On the other hand, in the event of an enhancement of the sugar level in response to the stress, the mode of sugar signaling becomes different. Increase in the sugar level is known to occur under elevated concentration of atmospheric CO_2, nitrogen deficiency and/or by changes in phloem loading/unloading for transport of sugar under different stress conditions. The increase in the sugar level is sensed by hexokinase (HXK) that suppresses many photosynthetic genes, resulting in a decrease in sugar production (Rolland et al. 2006; Baena-Gonzalez and Sheen 2008). Once the production of sugar is reduced due to activities of these genes, the recovery of loss, however, may not be possible. Further, different multiple stresses ultimately converge as an energy-deficiency signal in the cell by triggering the activation of kinase 10/11 of SNRK1 family (KIN10/11), which in turn drives the transcriptional reprogramming to modulate many cellular processes for energy conservation. This is partly mediated by the S-group of basic leucine zipper (bZIP) transcription factors. KIN10/11 also influences the growth, viability, reproduction and senescence processes besides playing a role in the maintenance of cellular energy homeostasis and stress tolerance, and is thus proposed to be the central integrator of metabolic, stress and developmental signals (Baena-González et al. 2007).

Many responses of chloroplasts to environmental stress are known to be mediated through hormones like ABA, jasmonic acid (JA) and salicylic acid (SA), which are partly or completely synthesized inside the plastid (Bouvier et al. 2009; Dempsey et al. 2011; see also Pfannschmidt and Munné-Bosch, Chap. 22). Any alteration induced by stress in their biosynthesis pathways may also upset the response mechanisms.

IV. Adaptational Features of Developing Chloroplasts to Variations in Environmental Conditions

Plants can correctly sense and recognize the environmental signals and make use of the signals for appropriate modifications at various levels including changes in morphological structures, cellular organization, physiological behavior, and regulation of gene activity (Misra et al. 2001, 2002; Shulaev et al. 2008). Dynamic adaptational responses also find

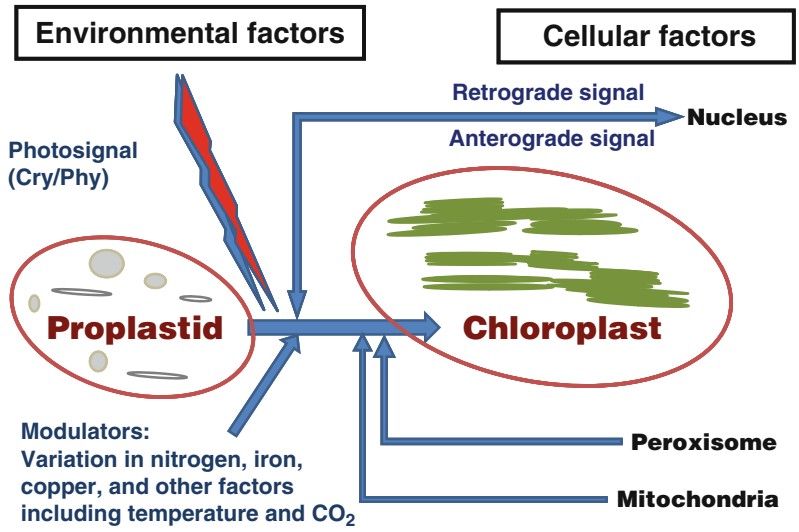

Fig. 28.2. Factors regulating chloroplast development. *Left*: Photosignals regulating chloroplast development through phytochrome (Phy) and cryptochrome (Cry) and modulation of the development by other environmental factors. *Right*: Interaction of chloroplast with other cellular organelles during development. The scheme is based on the work of Biswal et al. (2003); Pogson and Albrecht (2011) and Lepisto and Rintamaki (2012).

their expression during development of the cellular organelles including chloroplasts (Sakamoto et al. 2008; Pogson and Albrecht 2011); further, they have made the plants adapt to prevailing environments (Shulaev et al. 2008; Lorkovic 2009).

Chloroplast development is controlled by both cellular and environmental factors (Fig. 28.2). The development of chloroplasts starts from the 'proplastid' level, and upon exposure to light the proplastid differentiates into mature chloroplast with functional photoelectron transport carriers, membrane channel proteins and stromal enzymes. On the other hand, in darkness the etioplast has characteristic structural features with the membranous structures called the prolamellar body (PLB). The PLB contains protochlorophyllide, the precursor of Chl, bound to its reducing enzyme protochlorophyllide oxidoreductase (POR), NADPH, lipids, a few proteins and two carotenoids, namely lutein and violaxanthin (see Solymosi and Aronsson, Chap. 3 in this volume). In white light, the light dependent POR enzyme activity converts protochlorophyllide into chlorophyllide *a*, which is subsequently converted into Chl *a* and *b* (Forreiter and Apel 1993). In addition to the synthesis of pigments, the differentiation process involves the synthesis of thylakoids and stromal multimeric protein complexes.

The modifications of chloroplasts during leaf development in response to the changing environment are quite well known (Yano and Terashima 2001; Biswal et al. 2003). The synthesis of pigments and assembly of internal membrane structures are impaired under environmental stress during the development of chloroplasts (Mc Donald et al. 2011). It is also important to mention that the nature and mode of the response of chloroplasts to various environmental stress factors may be different at different developmental stages of the organelle (Biswal et al. 2003).

A. Environmental Modulation of Developing Chloroplasts

The major environmental stress factors monitored either in the laboratory or in the field to examine their effects on the developmental

characteristics of the photosynthetic organelle include changes in the quality and quantity of light, temperature extremes, drought, elevated CO_2, nutritional deficiency, and heavy metal stress. These changes bring about wide variations in the shape, size and internal structures including thylakoid organization (Lichtenthaler and Burkart 1999; Yano and Terashima 2001; López-Juez and Pyke 2005). A mature chloroplast with specific features is considered as the adaptational response of the developing organelle in a particular environmental setting. The factors regulating chloroplast development are shown in Fig. 28.2. In this section we present the modulation of structure and function of developing chloroplast along with its adaptational measures against environmental factors experienced by plants during the transformation of proplastids into chloroplasts.

1. Light, the Major Environmental Factor for Chloroplast Development

The profound influence of light on the development of the proplastid is evident from a comparative study of the organelle during skotomorphogenesis and photomorphogenesis. The changes in quality, quantity, duration and direction of light may significantly modulate the structure of plants, leaf orientation, internal structure, and the geometry of the canopy to optimize light conditions for plants (Lichtenthaler and Burkart 1999; Yano and Terashima 2001; López-Juez and Pyke 2005). The type of the light signal received by the green leaves determines the type of chloroplast to be developed with the structural features that would permit the mature and developed organelle to function efficiently (see Lichtenthaler, Chap. 15 in this volume).

a. Variation in the Intensity of Light

Plants can develop sun leaves with sun type chloroplasts under high light or the shade leaves with characteristic shade type chloroplasts at low light conditions (Lichtenthaler and Burkart 1999; Yano and Terashima 2001; López-Juez and Pyke 2005). The structural variations in the chloroplasts of both types are primarily the adaptive responses to capture the required number of photons in a well regulated manner. Variations in the organelle ultrastructure during high or low light irradiation have been examined in different plant systems. Greater thickness of grana stacks and increase in the number of thylakoids per grana stack with well formed extensive grana structures reflect the basic features of lamellar systems of chloroplasts of the leaves experiencing low irradiation (Lichtenthaler and Burkart 1999; Yano and Terashima 2001; López-Juez and Pyke 2005). In contrast, a different ultrastructure of the membrane system, with quantitatively less number of thylakoids per granum and relatively narrow thylakoids, is the structural feature of chloroplasts of high light adapted leaves (Lichtenthaler and Burkart 1999). The changes in thylakoid membrane composition, the levels of pigments, electron transport carriers, alterations in the efficiency of energy transfer, redox reactions as well as the activity of carbon fixation enzymes have been demonstrated (Lichtenthaler and Burkart 1999; Yano and Terashima 2001; López-Juez and Pyke 2005).

b. Variation in the Quality of Light

In darkness, the seedlings undergo skotomorphogenesis to develop etioplasts from proplastids and on exposure to light they undergo photomorphogenesis (de-etiolation) to develop into green mature chloroplasts (Biswal et al. 2003; Li et al. 2011). Chloroplast biogenesis from proplastids requires coordination of many events including the expression of nuclear and chloroplast genes (Valkov et al. 2009; Waters and Langdale 2009; Jung and Chory 2010; Zhou et al. 2011), and the process is largely regulated by developmental and environmental cues like light (Pogson and Albrecht 2011; Qiao et al. 2011; Zhang et al. 2011). The light signaling pathways involve the participation of photoreceptors, transcription factors and several other factors

regulating the network for chloroplast development (Castillon et al. 2007; Bu et al. 2011; Zhou et al. 2011; also see Burman and Khurana, Chap. 25 in this volume). Studies on photomorphogenic mutants have substantiated the involvement of phytochrome and blue light photoreceptors in the process (Vinti et al. 2005; Bae and Choi 2008; Albrecht et al. 2011). The precise mechanism of their action on the plastid biogenesis and development is still unclear, even though several possible mechanisms of photoreceptors mediated plastid development have been proposed. However, the photoreceptors are suggested to regulate the expression of specific genes through low fluence and very low fluence response modes (Biswal et al. 2003; Peschke and Kretsch 2011; Lepistö and Rintamäki 2012).

2. Development of Chloroplasts Under Limited Nitrogen, Low Iron and Excess Copper Environment

Chloroplast development is reported to be regulated by nutrients. Both deficiency and excess of nutrients affect the ultrastructure of developing chloroplasts. Nitrogen (N) nutrition is one of the major determinants of chloroplasts development, because about 75 % of the total N in a plant is required for normal chloroplast formation, synthesis of components of the photosynthetic apparatus including thylakoid membranes (Terashima and Evans 1988; Kutik et al. 1995) and photosynthetic enzymes (Bondada and Syverstein 2003). A low N supply induces an accumulation of starch granules in chloroplasts and changes in the chloroplast ultrastructure. The deficiency of N also affects Chl concentration, photosynthesis and growth (Kutik et al. 1995). On the other hand, total Chl concentration increases linearly with increasing leaf N, even if the Chl a/b ratio declines. Net assimilation of CO_2 and leaf water-use efficiency (WUE) reach maximum values in leaves with high N concentration (Bondada and Syverstein 2003). A N limiting condition, in addition to affecting the development of chloroplasts by producing lower Chl content and reduced capacity for photosynthesis, alters the composition of xanthophylls in spinach (Terashima and Evans 1988; Verhoeven et al. 1997).

Besides N, iron (Fe) is known to modulate the development of chloroplasts. Most of the mobilized Fe in plants exists in the form of phytoferritin and 80 % of this complex is contained in chloroplasts (Lambers et al. 2008). The effect of Fe deficiency results in decreased concentrations of photosynthetic pigments and many other components of the thylakoid membrane (Larbi et al. 2006). Iron-deficient plants have a reduced number of Chl molecules per unit of chloroplasts. The deficiency alters the structure and function of the chloroplasts and modifies the thylakoid organization and the degree of stacking of the membranes (Larbi et al. 2006; Lambers et al. 2008). Iron being a constituent of many electron transport components between PSII and PSI, its deficiency may reduce the rate of photosynthetic electron transport resulting in a loss in photosynthetic capacity (Larbi et al. 2006). Although the precise mechanism of Fe deficiency-induced changes in chloroplasts during its development is not known, the deficiency induced photodestruction of pigments and activation of chlorophyllase could be responsible for the organelle damage (Larbi et al. 2006). Proteome analysis of the thylakoid membrane of Fe deficient *Arabidopsis thaliana* plants shows the presence of many new and several modified proteins (Laganowsky et al. 2009). Based on the gene expression pattern, Jeong and Connolly (2009) proposed that FRO7, a new class of proteins, is involved in supplying iron to chloroplasts.

Copper in excess is a well known environmental stress factor for green plants. Excess of copper induces modification(s) in the ultrastructure of thylakoid membrane during development of the chloroplast (Maksymiec et al. 1995). In addition to the primary stress induced by excess copper, its interaction with other factors may lead to symptoms similar to those induced by light stress or photo-oxidative damage (Cohu and Pilon 2007; Palmer and Guerinot 2009;

Tuncz-Ozdemir et al. 2009; Estavillo et al. 2011; Suzuki et al. 2011).

3. Development of Chloroplasts Under Temperature Stress, Drought and Excess CO_2

Temperature is one of the major environmental factors that significantly modulates chloroplast development (Mohanty and Tripathy 2011; see Tripathy and Dalal, Chap. 27). The variation in temperature brings about changes in the lipid composition leading to alterations in membrane fluidity and ultimately altering the geometry of lipo-protein complexes. Chilling stress has been reported to induce accumulation of ROS in chloroplasts and mitochondria of cucumber resulting in a decreased net photosynthesis and alteration of the respiratory pathway with, of course, development of protective mechanisms such as thermal dissipation and ROS scavenging systems (Hu et al. 2008). Low temperature alters the levels of proteins such as Cyt f and subunits of ATP synthase (Robertson et al. 1993), and also activates a number of cold inducible genes (Murata et al. 1992; Moon et al. 1995) which are responsible for protecting thylakoid membrane integrity.

Heat stress, on the other hand, acts differently and is known to activate gene expression for the synthesis of stress induced proteins. These proteins are classified as heat-shock proteins (HSPs) (Gupta et al. 2010). In plants, chilling, metal deficiency, drought or osmotic stress and high light induce, in addition to other effects, the synthesis of HSPs (Swindell et al. 2007). This response is amplified under natural conditions when plants experience two or more stresses in combination (Mittler 2006). The expression of HSPs is limited in the absence of environmental stress but these proteins participate in several stages of growth and development of plants (Sun et al. 2002). Timperio et al. (2008) suggested that under heat stress, the role of HSPs as molecular chaperones is certain but their function in non-thermal stress could be different. The function of HSPs is not only restricted to acclimation of plants to high temperature, but also a specific member of the family of this protein provides housekeeping functions that are essential for chloroplast development (Bosl et al. 2006; Lee et al. 2006).

Like temperature stress, drought also brings about several modifications during development of chloroplasts. The low relative humidity or decreased root zone osmotic potential causes an increase in the time period of the lag phase of Chl accumulation and a decrease in the Chl a/b ratio of greening etiolated tissues (Misra and Misra 1987) resulting in pronounced effects on the production rate and accumulation of the major light-harvesting Chl a/b protein (Alberte et al. 1975). Modulation of the formation of thylakoids by relative humidity is supported by the work of Bourque et al. (1975) in jack bean leaves. A study on the development of chloroplasts from severe soil drought stressed oak seedlings by Kwak et al. (2011) demonstrated the swelling and disruption of the thylakoids, formation of large starch grains and increase in the population of plastoglobuli. Although thylakoids are poorly developed under severe atmospheric moisture stress, their normal development has been demonstrated during greening of bean leaves floated on 0.2 M NaCl solutions (Siew and Klein 1968). These results suggest that the salinity stress is less toxic for chloroplast development than the water stress. Probably, this is one of the reasons why most of the plants can grow in saline conditions, but do not survive under xeric environments.

High concentration of atmospheric CO_2 in this century is one of our major concerns for the future of our environment (Zuo et al. 2002; Zhang et al. 2012). Since chloroplast is the site for CO_2 assimilation, excess CO_2 can affect the development of the organelle itself and, in turn, can affect the growth and development of plants in general. In this background, several studies have been conducted on the possible effects of CO_2 enrichment on plant and chloroplast development (Robertson and Leech 1995; Griffin et al. 2001; Zuo et al. 2002; Zhang et al. 2012). Although total Chl

content and Chl per unit leaf fresh weight are reduced under elevated CO_2, the rate of photosynthesis has been demonstrated to increase (Zhang et al. 2012). Higher CO_2 level is also known to bring specific changes in the structure and composition of thylakoid membranes, namely quantitative alteration in the accumulation of the 33kD protein and Cyt f without any significant changes in the accumulation of other chloroplast proteins during development of the organelle (Robertson and Leech 1995).

An elevated CO_2 level brings about changes in starch inclusions and in the density of thylakoids (Kutik et al. 1995). It causes an increase in the number and size of starch grains in chloroplasts (Zhang et al. 2012), besides increasing the ratio of stroma to grana thylakoids (Griffin et al. 2001). In addition to starch grains, more plastoglobuli are observed in chloroplasts with elevated CO_2 concentration (Velikova et al. 2009). However, CO_2 enrichment promotes growth by raising the photosynthetic rate and the ratio of stroma to grana thylakoids, probably, as a result of enhanced antioxidant capacity of plants (Rao et al. 1995; Ghasemzadeh et al. 2010). The changes in the ultrastructure of chloroplasts under elevated CO_2 environment, as observed by Zhang et al. (2012), are shown in Fig. 28.3.

The precise adaptational mechanisms to high concentration of CO_2 is not known. However, a high concentration of CO_2 has been demonstrated to improve the activities of antioxidant enzymes and reduce the accumulation of malondialdehyde (Zhang et al. 2012).

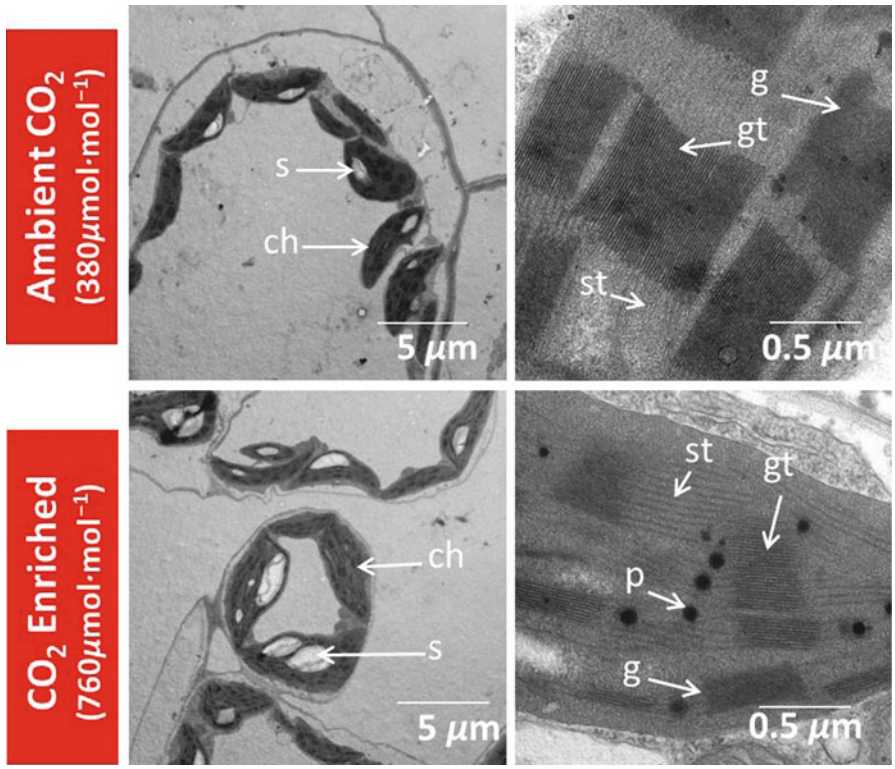

Fig. 28.3. Changes in chloroplast ultrastructure (transmission electron micrograph, TEM) by elevated CO_2. The elevated CO_2 causes an increase in number and size of starch grains, enhancement in the ratio of stroma to grana thylakoids and results in rearrangement of thylakoids with the formation of loosely aligned grana (Zhang et al. 2012). The labels abbreviated in the figure are: *ch* chloroplast, *g* grana, *gt* grana thylakoid, *p* plastoglobuli, *s* starch, *st* stroma thylakoids

V. Response of Senescing Chloroplasts to Environmental Stress

Leaf senescence, the last phase of leaf development, is a well regulated process under the active control of specific genes. The remobilization of nutrient resources from the senescing leaves to other young and growing parts or the storage organs of the plants is a purposeful event accomplished during this phase. Endogenous factors like age, hormones and ROS are implicated in regulating leaf senescence. However, these factors are, in turn, influenced by several environmental factors, and thus senescence is subject to modulation by environmental stresses like drought, salinity, nutrient deprivation, extreme temperatures, UV radiation, heavy metal toxicity, high light and darkness (Biswal 1997; Biswal and Biswal 1999; Biswal et al. 2003; Buchanan-Wollaston et al. 2003; Lim et al. 2007). The chloroplast is the primary organelle that is targeted by both stress and senescence. Further, this organelle bears two other distinctions pertinent to the study of stress induced modulation of senescence: (1) Chloroplasts are potential source of ROS, the ROS being generated from the various photo-redox reactions associated with photosystems of the thylakoid membrane; (2) Almost 75 % of the proteins of the cell are available in the chloroplasts for remobilization to other growing parts during leaf senescence.

Under optimal growth and favorable environmental conditions, growth and development of leaves occur in an age-dependent manner. However, environmental stresses are known to induce premature leaf senescence leading to transformation of chloroplasts into gerontoplasts (Biswal et al. 2003; Buchanan-Wollaston et al. 2003; Lim et al. 2007).

The expression of genes and pathways for induction and progress of senescence induced by environmental stresses overlap with the age-dependent gene expression associated senescence pathway (Becker and Apel 1993; Buchanan-Wollaston et al. 2005; Munns 2005). Thus the stress mediated transformation of chloroplasts into gerontoplasts warrants further attention.

A. The Beginning of the End: Stress Induced Initiation of Senescence

The precise timing and the appropriate conditions when the chloroplast "commits" to become a gerontoplast are not clearly understood inspite of rapid progress made in the area of molecular biology of leaf senescence. However, there is mounting evidence that ROS are the key inducers of programmed cell death in plants and the balance between ROS and their scavengers modulates the signaling pathways (Zapata et al. 2005; Procházková and Wilhelmová 2007; De Pinto et al. 2012). Studies reveal that the production of ROS is genetically programmed and is an essential component of plant development and response to environmental cues, and to this effect, the term 'oxidative stress' could be a misnomer (Foyer and Noctor 2005a).

Chloroplasts are the sites for operation of a cascade of photoredox reactions leading to the production of molecular oxygen, the process that provides ample scope for production of ROS species like $O_2^{\cdot-}$, H_2O_2, $HO\cdot$ and $^1O_2$ in the thylakoid membrane (Apel and Hirt 2004; Biswal et al. 2011). The decline in photochemical potential, so commonly observed during stress and senescence, may affect the redox homeostasis of the chloroplast by promoting ROS metabolism. The role of H_2O_2 as a signaling molecule has long been established. The work of Apel and co-workers in the Arabidopsis *flu* mutant demonstrated that $^1O_2$ can induce damage by triggering the cell death program rather than by direct oxidation of molecules (Op den Camp et al. 2003). Further, Wagner et al. (2004) have shown that the inactivation of a single gene, *EXECUTER1* (*EX1*), is sufficient to prevent $^1O_2$ induced damage in *flu* mutants. Although we know that singlet oxygen has a signaling role, yet we do not know how this molecule, with extremely short life-time, can transmit the signal. However, it is thought that degradation products of Chl, lipids or D1 protein might be somehow involved in the signaling process (Foyer and Noctor 2005b).

B. Dismantling of the Fabric: Structural and Functional Modifications of Chloroplasts During Stress Induced Senescence

Many of the stress induced changes in the chloroplast resemble changes that would occur during developmental senescence. Loss of photosynthetic pigments, lipid damage, degradation of proteins, decline in the photosynthetic potential, increase in ROS levels and decreasing activities of antioxidant enzymes have been reported to occur during high light (Haendeler and Klotz 2008), UV radiation (Joshi et al. 2007) and drought (Deo and Biswal 2001; Munné-Bosch et al. 2001) induced leaf senescence. The degradation of Chl allows the proteases to degrade the light harvesting complex apoproteins thus making them available for remobilization during the process. The changes in the chromatin condensation in nuclear matrix and nucleolus, cell ultrastructure including membrane whirling, swelling of chloroplasts, accumulation of plastoglobuli in the stroma have been observed during senescence induced by stress factors such as drought (Munné-Bosch et al. 2001) and ozone (Vollenweider et al. 2003). It is likely that the stress induced loss of chloroplast integrity is well regulated, with the active involvement of many of the genes associated with natural senescence (John et al. 2001).

C. Acceleration of Stress Induced Senescence

The working models for developmental senescence divide the process into three phases: initiation, progression and termination of senescence. It is believed that the initiation of senescence is triggered by specific signals that influence the expression of genes. The expression of senescence associated genes (*SAGs*) may be regulated by oxidative stress (Navabpour et al. 2003). Generation of oxidative stress has been reported in several adverse conditions such as high light (Haendeler and Klotz 2008), UV exposure (Joshi et al. 2011), drought and nitrogen deficiency (Agüera et al. 2010); it appears that environmental stresses regulate *SAGs* by an enhanced level of ROS. Besides ROS, multiple signaling pathways mediated by sugars, nitric oxide, JA, ABA, ethylene and cytokinin have been identified. The complex interactions of ROS signaling pathway with other signals during stress induced senescence have not been fully understood (Biswal et al. 2003). It is during the progression phase that the major changes in structure, function, and metabolism take place in the senescing chloroplast. Several studies suggest that the progression of senescence is accelerated in response to a stress (Biswal et al. 2003). The senescence-mediated loss in Chl and photochemical potential has been observed to be hastened during exposure to stresses such as high light, UV and nutrient deficiency (Biswal et al. 2003). Acceleration of senescence along with a faster rate of nitrogen remobilization has been observed in sunflower plants under nitrogen stress (Agüera et al. 2010). It has been demonstrated in Arabidopsis that the loss of function of one of the aminopeptidases renders the plants more sensitive to stress and accelerates leaf senescence (Waditee-Sirisattha et al. 2011). A transgenic Arabidopsis plant overexpressing *SAG* 29 exhibits accelerated senescence and is hypersensitive to salt stress (Seo et al. 2011). The SAG 29 protein may, thus, act as a molecular link that integrates environmental stress responses into the senescing process. Microarray analysis in petals, shows that dehydration stress accelerates many of the gene expression patterns that would normally occur during developmental senescence (Wagstaff et al. 2010). The acceleration of senescence during a severe stress may be viewed as a positive strategy of the plants to retrieve the nutrient resources as quickly as possible before cell death occurs.

D. The Art of Survival: Adaptive Response of Senescing Chloroplasts to Environmental Stress

The response of senescing chloroplasts to a stress may depend on the intensity of the stress, plant genotype, phase of senescence

and acclimation potential of the chloroplast to previous stress exposure. Senescing leaves of wheat, previously acclimated to highlight stress, show enhanced tolerance to osmotic stress during senescence (Behera et al. 2003). During the early phase of progression of leaf senescence, when the leaf still retains photosynthetic potential, the stress induced damage may be counteracted by enzymatic and non-enzymatic modes of defense. An increase in α-tocopherol, an efficient quencher of singlet oxygen, during the first stages of leaf senescence has been demonstrated in several species during drought stress (Munné-Bosch and Alegre 2004). It is also known that senescence is delayed by the activities of antioxidant enzymes (Piacentini et al. 2001; Causin et al. 2006). However, during the later stages of progression of leaf senescence, a decline in the activities of antioxidant enzymes with a concomitant increase in lipid peroxidation has been widely reported (Munné-Bosch and Alegre 2004; Sairam et al. 2009). Casano et al. (1994) have shown that oxidative stress could be favored by inability of senescing leaves to modulate the level of mRNA of antioxidant enzymes. Nevertheless, the regulation of the whole process of senescence has an adaptive value as it contributes to the survival of the plants under stressful conditions (Ono et al. 2001).

E. Senescence Induced Reprogramming of Sugar Metabolism and Adjustment of the Sugar Status

Photosynthesis and the transport of photosynthetic products to different organs take place efficiently in a mature leaf ensuring optimal synthesis and use of sugars, the source of energy for plant growth. During senescence and stress response, the level of sugar in the cell is altered leading to modulation of gene expression and enzyme activities in both sugar producing and sugar consuming processes.

Senescence and stress responses are known to decrease photosynthetic efficiency and result in sugar-limiting conditions. Plants counter this challenge by inducing the degradation of starch and protein and accelerating lipid catabolism to carry on respiration and metabolic activity and adapt to this limitation by slowing down many biosynthetic activities to conserve energy and to protect cells from sugar depletion (Biswal et al. 2012). While the degradation processes are regulated by *SAGs*, the slowdown of biosynthetic activities is controlled by senescence down-regulating genes (*SDGs*) (Biswal et al. 2012). The induction of *SAGs* during senescence in response to environmental stress factors could trigger the disassembly of chloroplasts. The decrease in the sugar level as a result of the decline in photosynthesis up-regulates *SAGs* and hexokinase is considered to be the enzyme involved in their over or limited expression for the regulation of senescence (Dai et al. 1999). However, the *SAGs* are regulated by many other factors besides sugars (He and Gan 2001; Lopez-Molina et al. 2003). Under cold condition, the accumulation of sugar is accompanied by the accumulation of many other osmolytes (Kaplan et al. 2007; Krasensky and Jonak 2012), which help in adjusting the osmotic pressure within the cell to protect it from disruption of the plasma-membrane and thylakoid membrane (Hare et al. 1998). The involvement of these osmolytes in regulating redox or sugar signaling, hexokinase-dependent signaling, and interaction of trehalose synthesis with sugar and ABA signaling has also been reported (Avonce et al. 2004; Paul et al. 2008; Wingler and Roitsch 2008).

Nitrogen availability is known to influence the cellular sugar status (Biswal et al. 2012). Variation in the levels of nitrogen, sugar or both may act as signal in the complex network of senescence (Buchanan-Wollaston et al. 2005; Biswal et al. 2012). The work of Schildhauer et al. (2008) demonstrates a link between nitrogen status and expression of genes associated with leaf senescence. Although the exact mode of signal transduction underlying the regulatory mechanism of stress induced senescence is not properly understood, the interaction of sugar with phytohormones cannot be ruled out (Wingler and Roitsch 2008).

VI. Conclusions

- Chloroplasts, major sensors of stress, play a key role in plant stress responses. The organelle also significantly modulates the development of plants experiencing stress. Green plants not only survive but also grow in extreme environmental conditions but the mechanisms involving their acclimation specifically at different developmental stages are not fully understood. While both plastid and nuclear genome sequencing of many plants was a giant step forward for understanding of the molecular mechanisms of the adaptational processes during plastid development, the integration of genomics with metabolic and physiological functions may provide a clearer picture of the adaptational mechanisms.
- Almost all environmental stresses ultimately result in oxidative stress and plants respond differentially to adapt to different environmental settings. Moreover, the adaptational mechanisms of plants and their cell compartments including chloroplasts are development dependent. Therefore, the differences in pathways operating in plants with different stress factors at the levels of perception, signal transmission, and final responses are understandable. In this background, an integration of all the modes of defenses and responses to these stresses need to be formulated. In spite of the vast literature available in the area of plant stress biology, the complex nature of stress signaling pathways and cross-talk between the pathways still remain unresolved. The work of Sheen's laboratory (Baena-Gonzalez et al. 2007; Baena-Gonzalez and Sheen 2008) on a central integrator of transcriptional network in plant stress signaling is a commendable attempt in that direction.

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